PAEDIATRIC DRUG DEVELOPMENT - REFORMULATION, *IN VITRO,* GENOMIC AND *IN VIVO* EVALUATION

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Thesis Summary

Angiotensin converting enzyme (ACE) inhibitors lisinopril and ramipril were selected from EMA/480197/2010 and the potassium-sparing diuretic spironolactone was selected from the NHS specials list for November 2011 drug tariff with the view to produce oral liquid formulations providing dosage forms targeting paediatrics. Lisinopril, ramipril and spironolactone were chosen for their interaction with transporter proteins in the small intestine.

Formulation limitations such as poor solubility or pH sensitivity needed consideration. Lisinopril was formulated without extensive development as drug and excipients were water soluble. Ramipril and spironolactone are both insoluble in water and strategies combating this were employed. Ramipril was successfully solubilised using low concentrations of acetic acid in a co-solvent system and also via complexation with hydroxypropyl-β-cyclodextrin. A ramipril suspension was produced to take formulation development in a third direction. Spironolactone dosages were too high for solubilisation techniques to be effective so suspensions were developed. A buffer controlled pH for the sensitive drug whilst a precisely balanced surfactant and suspending agent mix provided excellent physical stability. Characterisation, stability profiling and permeability assessment were performed following formulation development. The formulation process highlighted current shortcomings in techniques for taste assessment of pharmaceutical preparations resulting in early stage research into a novel *in vitro* cell based assay.

The formulations developed in the initial phase of the research were used as model formulations investigating microarray application in an *in vitro-in vivo* correlation for carrier mediated drug absorption. Caco-2 cells were assessed following transport studies for changes in genetic expression of the ATP-binding cassette and solute carrier transporter superfamilies. Findings of which were compared to *in vitro* and *in vivo* permeability findings. It was not possible to ascertain a correlation between *in vivo* drug absorption and the expression of individual genes or even gene families, however there was a correlation (R^2 = 0.9934) between the total number of genes with significantly changed expression levels and the predicted human absorption.

Key Words

Oral Drug Delivery, Drug Formulation Development, Cell Culture, Drug Absorption, Microarray.

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TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviations

ABC – ATP-Binding Cassette

ACE – Angiotensin Converting Enzyme

ADH – Anti-diuretic hormone

ADMET – Absorption, Distribution, Metabolism, Excretion and Toxicology

ADP – Adenosine Diphosphate

API – Active Pharmaceutical Ingredient

ASIC – Acid Sensing Ion Channel

ATP – Adenosine Triphosphate

AUC – Area Under the Curve

BCRP – Breast Cancer Resistance Protein

BNaC1 – Brain-type Na+ Chanel 1

bp – Base Pairs

BTRC – Bovine Taste Receptor Cell

BTSS – Biomimetic Taste Sensing Systems

cAMP – Cyclic Adenosine Monophosphate

CD – Cyclodextrin

cDNA – Copy Deoxyribonucleic Acid

CMAX – Peak Concentration

CMC – Critical Micelle Concentration

cNMP – Cyclic Nucleotide Monophosphate

cRNA – Copy RNA

DNA – Deoxyribonucleic Acid

DPI – Dry Powder Inhaler

DRASIC – Dorsal Root Acid Sensing Ion Channel

dsDNA – Double Stranded Deoxyribonucleic Acid

EMEA – European Medical Agency

ENaC – Epithelial-type Na+ Channels

EVD - Eigenvalue decomposition

FDA – Food and Drug Administration

FDR – False Discovery Rate

GI – Gastro-Intestinal

GRAS – Generally Recognised as Safe

HBSS – Hank’s Balanced Salt Solution

HCA – Hierachical Clustering

HPLC – High Pressure Liquid Chromatography

HP-β-CD – Hydroxypropyl-β-cyclodextrin

HYHV – High Yield High Viability

HYLV – High Yield Low Viability

IA – Intra-arterial

IAM – Immobilised Artificial Membrane

IC – Intracardiac

ICH - International Conference on Harmonisation

ID – Intradermal

IM – Intramuscular

IP3 – Inositol trisphosphate

IT – Intrathecal

IV – Intravenous

IVIVC – *in vitro-in vivo* Correlation

KEGG – Kyoto Encyclopaedia of Genes and Genomes

LOD – Limit of Detection

LOQ – Limit of Quantification

LYHV – Low Yield High Viability

LYLV – Low Yield Low Viability

MDI – Metered Dose Inhaler

MIC – Minimum Inhibitory Concentration

mRNA – Messenger Ribonucleic Acid

MVDA – Multivariate Data Analysis

NBD – Nucleoside Binding Domain

NCAM – Neural Cell Adhesion Molecule

NCE – New Chemical Entity

NHS – National Health Service

NPPB - 5-nitro-2-[3-phenylpropylamino]-benzoic acid

PAMPA – Parallel Artificial Membrane Permeation Assay

Papp – Apparent Permeability

PCA – Principal Component Analysis

PDE – Phosphodiesterase

PEG – polyethylene glycol

QSAR – Quantitative Structure Activity Relationship

RAAS – Renin Angiotensin Aldosterone System

RH – Relative Humidity

RSD – Relative Standard Deviation

SAM – Statistical Analysis of Microarray

SC – Subcutaneous

SLC – Solute Carrier

ssDNA – Single Stranded Deoxyribonucleic Acid

TEER – Trans Epithelial Electrical Resistance

TIFF – Tagged Image File Format

TLC – Thin Layer Chromatography

TMAX – Peak Time

TRC – Taste Receptor Cells

UV - Ultraviolet

1 INTRODUCTION

1.1 THE GLOBAL PROBLEM

The United States identified the problem of the poor availability of paediatric medication in the 1990's and since 1998 there has been a regulation in place (Federal Register 66632, 1998) requiring all medicines produced to be assessed for safety and efficacy in paediatrics. As such since the 4th January 2002 under the 'best pharmaceuticals for children' act (FDA/Pub L No. 107-109, 2002) the FDA require, in instances where drugs are intended for paediatric use, evidence that the medication is both safe and effective. In 2007 the European Medical Agency (EMEA) followed suit in the recognition of the problem and has since devised a priority list of off patent paediatric medications with the goal of directing the research and development in the direction of the medications deemed the most important and the most in need of reformulation and investigation.

1.2 DELIVERY ROUTES AND THEIR ASSOCIATED DOSAGE FORMS

The complex physiology of the human body provides a diverse range of routes which can be utilised as a potential pathway for the administration of a dosage form to a patient. The choice of which route is to be taken is dependent upon a number of things such as the desired site of absorption of the drug, the site of action of the drug and stability considerations. For example there would be little point in administering a drug which was rapidly degraded in acidic conditions via the oral route with the aim of achieving absorption in the intestine as the drug would be degraded in the stomach. By altering either the dosage form or if possible the site of absorption this limitation can be overcome, the drug could be orally administered with the intention of absorption through the buccal mucosa or alternatively an alternative route of administration such as injection or suppository could be implemented. From this example it is clear that the correct selection of both delivery route and dosage form is critical in the production of a

successful medication. The most common delivery routes and their associated dosage forms are detailed in this section (Mohammed and Russell 2012).

1.2.1 Oral Administration

The delivery of active pharmaceutical ingredients (API)'s via the oral route can be achieved in a variety of ways depending upon the limitations of the drug in question. Oral dosage forms may be liquid or solid. Solutions, suspensions, and emulsions account for the liquid dosage forms, while tablets, capsules, and powders make up the solid dosage forms that are used in oral administration. A variety of liquid medicines are designed to be swallowed and delivered enterally:

- Oral solutions are normally an aqueous vehicle into which all of the formulation ingredients are dissolved.
- Elixirs involve the incorporation of an alcohol as a co-solvent which is present to optimise the solubilisation of all of the formulation ingredients. As all of the ingredients are solubilized then elixirs qualify as solutions however there are potential limitations for the use of elixirs in paediatric treatment and also for adult groups which avoid alcohol.
- Oral syrups are solutions which contain concentrations of sugars or sugar substitutes at concentrations of up to 85% dissolved in an aqueous vehicle. As syrups are classed as solutions it is important that the API remains soluble in the syrup vehicle and no precipitation is observed.
- Linctuses are normally produced for the treatment of throat irritations and also most cough medicines fall into this category, their high viscosity resulting from their syrup like formulation aids their soothing effect by increasing contact time with the irritated area.
- Gargles and mouthwashes are orally administered aqueous solutions which are designed to treat ailments in the buccal cavity although these are not designed to swallowed, being classified as oropharyngeal formulations.

Suspensions are only employed as a dosage form for oral administration if the API has a low aqueous solubility. They are often alternatively described as colloidal or coarse dispersions depending upon the size of the particles in the suspension. Unlike solutions, suspensions are inherently unstable and sedimentation of the suspended particles often occurs upon storage. As such it is important that homogeneity can be easily achieved before administration in order to ensure accurate dosage. This is of particular relevance when viscosity increasing excipients are used to slow sedimentation. A balance must be reached where sedimentation is slowed without inhibiting re-suspension. Ideally a suspension should display pseudoplastic flow properties which yields a vehicle with greater viscosity during storage which decreases upon dispensation of the formulation. The aesthetics of a suspension are also important from a consumer point of view (Jones 2008, Mohammed and Russell 2012).

Emulsions, although more often used in external applications they are at times also used in oral liquids but only when the emulsion is in the oil in water form. This refers to the API being contained in a disperse oil phase stabilised within a water external phase. Emulsions are of benefit over suspensions where insoluble drugs are more stable in oil than they are in aqueous environments. They can also at times provide formulations which are more readily absorbed than suspensions. A major drawback of emulsions is that they are often difficult to manufacture (Griffin *et al* 2007, Mohammed and Russell 2012).

Tablets and capsules account for the solid dosage forms which are orally administered. These are widely used as they provide many characteristics which makes them attractive as a dosage form. For example they allow for easy administration and can be modified to provide controlled release both in the rate and location of release. They are also useful in the taste masking of unpleasant tasting drugs at the same time as being easy to manufacture and providing an ‘elegant’ dosage form. There are many sub-classes of tablets including; conventional compressed, multiple compressed, sugar-

coated, film-coated, enteric-coated, chewable, buccal, sublingual and effervescent (Mohammed and Russell 2012). Tablets are normally produced in one of four ways; dry-granulation, wet-granulation, direct compression and roller compression. Other methods such as freeze-drying are also used however carry higher running costs (Bolhuis and Zuurman 1995).

- Conventional compressed tablets provide rapid drug release as they are designed to have rapid disintegration rates upon digestion in the stomach, they are produced via the direct compression of granules or powders containing the drug.
- Multiple compressed tablets involve the use of layering different granules or powders compressing the tablet after each addition to produce a controlled release tablet which is capable of releasing its API at different locations in the gastro-intestinal (GI) tract or at different rates. Layering also allows for increased potential for taste masking and layering also allows for the incorporation of more than one API where in other conditions one API may be incompatible with the other(s). Increasing the number of layers incorporated results in greatly increased complexity of the manufacturing process.
- Sugar coated tablets involve the coating of a conventional compressed tablet with a sugar or sugar substitute to improve the palatability or the aesthetic appeal of a tablet. Although once a popular technique the advent of film-coated tablets has seen a reduction in the incidence of sugar-coated tablets formulated.
- Film-coated tablets are similar to sugar-coated tablets only the sugar solution previously used has been replaced with a polymer or a blend of polymers. They can be designed to target the release of the API where sugar coated tablets cannot. In some cases the drug release can be brought about as a result of diffusion of the API through the insoluble coating, this allows a specific site of the GI tract to be targeted.

- Enteric-coated tablets are designed for targeted release and this is achieved by coating the tabled in a polymer which remains intact through much of the GI tract thanks to its pH dependent solubility and as such reaches the intestine before any breakdown of the tablet will occur. This has advantages in circumstances where the API is sensitive to acid degradation in that the drug is protected until it arrives in the alkaline environment of the small intestine. There are also the benefits of being able to minimise any irritation caused by the API.
- Chewable, buccal and sublingual tablets are designed for break down in the mouth and this can be for a number of reasons. Buccal absorption is one of the most rapid means of delivering an API and also carries the benefit of avoiding first pass metabolism, the main limitation of tablets breaking down in the mouth is bitter tasting drugs. Chewable, buccal and sublingual tablets also have the advantages that they can be produced to larger sizes than tablets which need swallowing and also increase the ease of administration for patients who struggle to swallow solid dosage forms. Buccal and sublingual tables differ in the location in which they should be placed to dissolve, buccal tablets are placed against the inside of either cheek whereas sublingual tablets are placed under the tongue.
- Effervescent tablets are added to water where they rapidly dissolve to produce an aqueous solution or suspension before being drank. The advantage of this is that there are no problems with swallowing solid dosage forms and the absorption of the API will be sped up coming from a pre-dissolved tablet in comparison to a swallowed solid form.

Capsules are less complicated than tablets and normally fall into one of two categories; hard gelatin capsules or soft gelatin capsules. The type of capsule shell used is dependent upon the intended contents of the capsule. Soft gelatin capsules consist of a one piece shell and normally contain non-aqueous liquid in which the API is present. Hard gelatin capsules are formed from two sections which fit together to form the

complete shell and usually contain either a non-aqueous liquid, semisolid or dry preparations such as tablets or powder.

In the case of both tablets and capsules, the instructions for use will indicate when these solid forms should be taken. Some medicines, for example, may need to be taken on an empty stomach or immediately after eating (Mohammed and Russell 2012).

1.2.2 Ocular Administration

Ocular administration involves the treatment of conditions affecting the eyes using either:

- Solutions
- Suspensions
- Ointments

Liquid dosage forms used for ocular administration most commonly include eye-drops. For the most part the conditions treated are external and treated via the application of the solution, suspension or ointment via eye drops. There are also some intraocular ailments which can be treated in the same manor although for most intraocular conditions and more serious conditions intraocular injections are applied. There are many disadvantages of ocular treatment including blurring of vision and local irritation. The main advantages of ocular drops are that they are applied directly to the problem site and with practice they can be administered effectively by the patient (Losa *et al* 1993, Mohammed and Russell 2012).

1.2.3 Nasal Administration

Nasal administration involves delivery of an API via a spray and it is most commonly used in the relief of congestion. The spray is always an aqueous based system as maintaining the action of the cilia in the nasal cavity is considered of upmost importance and non-aqueous systems impair the action of the cilia. The nasal route of

administration provides rapid absorption of an API thanks to the thin membrane good blood supply. Problems can arise in the treatment of congestion if there is any irritation as a result of the presence of preservatives which leads to increased congestion (Ilium *et al* 1998, Mohammed and Russell 2012).

1.2.4 Otic Administration

Otic administration is necessary for the treatment of infection, inflammation and ear ache or more severe pain. Similarly to ocular administration suspensions, solutions and ointments are all used in the treatment of ear conditions. Otic treatment is almost always via the use of solutions and regularly contain more than one API, usually an anaesthetic and an agent to soften the ear wax. The viscosity of otic preparations is important to ensure ease of administration and also the retention of the preparation at the site of action (Koulich *et al* 2010, Mohammed and Russell 2012).

1.2.5 Vaginal Administration

There are many types of dosage form which are suitable for vaginal administration including; semisolid preparations, tablets, capsules, peccaries and implants. The semisolid preparations contain the creams, ointments and gels which are applied via the use of a specially designed applicator. In a similar manner the tablets and capsules which are designed for vaginal application also require the use of an applicator and the patient requires teaching how to correctly use it. Peccaries are similar to rectal suppositories and have a range of usages. Vaginal implants are excellent in the controlled release of the API and the fixed nature of the dosage form ensures the accurate delivery of the API (Ceschel *et al* 2001, Mohammed and Russell 2012).

1.2.6 Rectal Administration

Suppositories are the main mode of rectal administration although ointments, pastes, lotions, liniments, collodions and gels are also used in rectal administration. Suppositories are solid dosage forms which undergo melting, softening or dissolution

and have the advantages that they can be suitably administered to patients who are unconscious or vomiting. Drugs which are liable to degradation in the upper areas of the GI tract can also be administered rectally to avoid this problem. The main disadvantages of rectal administration are the slow absorption, the patient to patient variation in the rate of rectal absorption and also the patient acceptability of the dosage form (Jongjaroenprasert *et al* 2002, Mohammed and Russell 2012).

1.2.7 Pulmonary Administration

The administration of API's via the respiratory system is predominantly for the treatment of respiratory disorders. Due to the physiology of the lungs and their optimisation for exchange of substances drug absorption is understandably efficient and as such has been exploited as a route of drug delivery using dry powder inhalers (DPI), metered dose inhalers (MDI), nebulisers and aerosols, with this route of administration comes the added benefit that the administration in this way avoids direct passage through the liver. DPI and MDI differ in that DPI's administer dry powders containing the API whereas the MDI's administer a suspension or a solution much in the same way (Muller *et al* 2011). Nebulisers involve the administration of solutions to the respiratory tract. The pH of the solution is important as acidic conditions can result in the tightening of the chest and make breathing difficult.

1.2.8 Topical Administration

Topical administration is where gels, pastes, collodions, lotions, ointments or liniments are applied externally to the area in need of treatment. Gels are produced using either dispersed solids or hydrophilic polymers and fall into two categories; Type 1 gels or hydrogels display covalent bonding between the polymer chains and are capable of taking up huge volumes of liquid vehicle whilst still maintaining flexibility and tough mechanical characteristics. Type 2 gels display weaker bonding between the polymer chains than are seen in type 1 gels and as a result display improved flow with

pseudoplastic properties. Pastes and ointments are in essence the same thing with the only real difference the concentration of API present. Pastes usually contain greater than 50% w/w of API where in comparison the concentration present in ointments is much lower. Collodions, lotions and liniments all fall into the categories of either solutions or suspensions and are often applied for the treatment of parasitic, bacterial, fungal and viral infection (Simpson 2010, Mohammed and Russell 2012).

1.2.9 Parenteral Administration

Parenteral administration is used extensively and there are numerous routes of administration; intravenous route (IV), intramuscular route (IM), subcutaneous route (SC), Intradermal (ID), Intra-arterial (IA), Intrathecal (IT), Intradural/extradural and Intracardiac (IC).

- Intravenous (IV) administration is used widely and has many advantages including the administration of large amounts of parenteral preparation up to 500ml. The IV route also allows for the administration of API's directly into a vein which would otherwise result in irritation if administered via another method. The bioavailability of IV administrated drugs cannot be beaten with 100% of the administered dose being available for action. The main drawback of IV administration is the need for training to ensure correct targeting of the injection and that the drug is administered to the vein at the correct rate (Jones 2008).
- Intramuscular (IM) administration is usually delivered to the muscles in the buttocks, the thigh or the upper arm. The maximum volume for administration via IM is lower than IV and the time from delivery to response is slower than the IV method of administration. Response is also slower for suspensions than it is for solutions. Bruising and soreness at the site of injection can often result if the injection technique is poor (Fan *et al* 2010).
- Subcutaneous (SC) administration involves the administration of the preparation to the fat layer directly below the skin. One of the most common uses for SC

administration is in the self-delivery of insulin in the treatment of diabetes. This method of administration is also used when there are problems accessing a vein for IV delivery. The time for the availability of the drug is slower when administered subcutaneously when compared with IV and IM administration. Normally the volume of parenteral preparation administered is low, 1ml or so however in some instances volumes of up to 1000ml can be delivered subcutaneously (Woolums *et al* 2011, Mohammed and Russell 2012).

IV, IM and SC are the three most common routes of administration with the remaining routes of intradermal (ID), intra-arterial (IA), intrathecal (IT), Intradural/extradural and intracardiac (IC) used less frequently but often for more specialist treatments (Jones 2008, Mohammed and Russell 2012).

Solid parenteral implants are also used as a means of drug delivery and these involve the subcutaneous insertion of a sterile solid preparation of a suitable shape and size. This may be placed inside the upper arm to minimize the risk of the implant breaking and includes devices that rely on an osmotic pump to deliver the API. In general, implants have the advantage of being able to deliver an API over a long period of time, which is not possible with other parenteral methods (British Pharmacopeia, 2011).

1.3 Drug and Dosage form selection

Following examination of the drugs listed in the EMA priority list of off patent paediatric medications (EMA/480197/2010) and the NHS specials list for November 2011 tariff (Chaplin 2012), the angiotensin converting enzyme (ACE) inhibitors ramipril and lisinopril in addition to the potassium sparing diuretic spironolactone were selected (Table 1). They were all listed with the specific requirement of the production of an age appropriate dosage form and the drugs all interact with transporters in the intestinal transport network. As an age appropriate dosage form was to be produced and only

tablets and capsules with no liquid alternative existed for the drugs in question it was settled upon that the formulations would be produced as oral liquids.

Table 1 - Lisinopril, ramipril and spironolactone are the drugs selected for formulation. Shown in the table is the information governing the key considerations in their selection. The drugs were required to interact with intestinal transporters and not already exist as oral liquid formulations. Solubility although not an important criteria in drug selection it is one of the most important pre-formulation considerations.

Drugs Selected For Reformulation

Drug	Transporters	Oral Liquid Formulation?	Solubility
Lisinopril	ABCB1 (Inhibitor), SLC15A1 (Substrate).	NO	Soluble
Ramipril	SLC15A1 (Substrate), SLC15A2 (Substrate)	NO	0.0035mg/ml
Spironolactone	ABCC2 (Inducer), ABCB1 (inhibitor), SLCO1A2 (Inhibitor).	NO	0.022mg/ml

1.3.1 ACE Inhibitors

ACE inhibitors act in the renin-angiotensin-aldosterone system (RAAS), a homeostatic mechanism charged with controlling hemodynamic conditions via competitive inhibition of the peptidyl dipeptidase ACE. The ACE inhibitors are an analogue of angiotensin I and occupy the active site on ACE preventing the conversion of angiotensin I to angiotensin II (Dzau *et al* 1981).

There are two ACE isoforms, the first is the somatic isoform, and this is a glycoprotein formed from a single polypeptide chain. The second isoform is known as the testicular isoform, which has a role in reproduction. Somatic ACE has two functionally active domains, N and C, which have distinct physiological roles. The C-domain is predominantly involved in blood pressure regulation while the N-domain plays a role in hematopoietic stem cell differentiation and proliferation. ACE inhibitors bind to and inhibit the activity of both domains, but have much greater affinity for and inhibitory activity against the C-domain.

In normal conditions, a response to a reduction in blood pressure triggers the release of the enzyme renin into the blood stream from the kidneys, here renin acts to convert angiotensinogen to angiotensin I. This is then in turn converted from angiotensin I to angiotensin II by ACE. Angiotensin II is responsible for increasing blood pressure by bringing about multiple responses including increased sodium and water re-absorption in the kidneys and arterial vasoconstriction. The re-absorption of sodium and water in the kidneys is increased indirectly by angiotensin II which acts to bring about the secretion of aldosterone and vasopressin. Aldosterone increases the number of Na^+ channels and Na^+/K^+ATPase pumps present in the distal convoluted tubule and collecting duct of the kidneys. Vasopressin which is more commonly known as the anti-diuretic hormone (ADH) increases the number of aquaporin-2 channels present in the distal convoluted tubule and collecting duct of the kidneys which allows for increased water re-absorption. The increased volume of salts and water re-absorbed into the blood has the effect of increasing the blood volume and therefore the pressure of the system. The increased re-absorption is complemented by vasoconstriction which by decreasing the volume available in the blood vessels increases the blood pressure. It is brought about by the binding of angiotensin II to receptors found in smooth muscle. In addition to stimulating vasoconstriction, vasodilatation is prevented by ACE via the inactivation of bradykinin. Through the use of ACE inhibitors, reductions in blood pressure are achieved as the action of ACE is prevented halting the production of angiotensin II and resulting in both vasoconstriction and salt and water re-absorption being reduced (Dzau *et al* 1981) (Figure 1).

Figure 1 - Renin-Angiotensin-Aldosterone System (RAAS). Under normal conditions the RAAS progresses through the stages in the flow chart displayed in blue and green. When ACE inhibitors are present however the RAAS cannot progress below the dashed line. In this way increases in blood pressure are prevented and coupled with the effect of ACE inhibitors preventing the degradation of bradykinin by ACE, vasodilatation is brought about thereby lowering blood pressure (Dzau et al 1981).

1.3.2 Potassium Sparing Diuretics

Diuretics are a group of medicines which cause the kidneys to remove a higher than normal volume of water from the blood stream. They can be used for the treatment of a variety of symptoms, including hypertension, oedema and ascites in cirrhosis of the liver, malignant ascites, nephrotic syndrome, oedema in congestive heart failure, moderate to severe heart failure (adjunct), resistant hypertension (adjunct) and primary hyperaldosteronism (Paediatric Formulary Committee 2012).

Potassium sparing diuretics are a variety of diuretic which as the name indicates, aid potassium retention whilst still increasing the volume of water removed from the blood. There are two main groups of potassium sparing diuretics, in one group amiloride and triamterene are found and in the other eplerenone and spironolactone exist. The drugs in each group bring about their pharmaceutical action in differing ways.

Amiloride and triamterene interfere with the passage of water and salts across cells in the kidneys by blocking the epithelial sodium channel (ENaC) on the luminal side of the collecting duct. This results in less water being reabsorbed and the blood volume decreasing. This relieves hypertension and allows water to re-enter the blood in sites of oedema for example.

Eplerenone and spironolactone are competitive aldosterone antagonists and achieve a diuretic effect by inhibiting the action of aldosterone. In turn this causes an increase in the net volume of water removed from the bloodstream while maintaining the amount of potassium retained.

Potassium sparing diuretics are relatively weak in their effect so it is not uncommon for them to be used in conjunction with loop diuretics. These have a greater effect on the water volume reduction however do not carry the benefit of potassium retention and so need to be carefully balanced when used to achieve the desired rate of water extraction while maintaining a suitably acceptable potassium level.

1.4 PREFORMULATION CONSIDERATIONS

Prior to any formulation development the physiochemical properties, structure and assay methods for the drug in question need consideration. Information on these are generated during the drug discovery process and referring back to them during reformulation of existing medicines is essential. The various studies that are carried out during drug discovery include:

- The determination of the fundamental physical and chemical properties.
- The analysis of powder characteristics (micrometrics).
- Excipient compatibility studies.
- The development of assays for identification and quantification of the drug.

Preformulation studies consist of two sets of studies and can be broadly classified into drug profiling and analytical characterization. Data generated from analytical studies is also used to help understand the pharmaceutical development of the new chemical entity (NCE). For instance, the development of an assay such as high-pressure liquid chromatography (HPLC) to identify and quantitatively determine the NCE can be used to study the oxidative or photolytic stability, as well as excipient compatibility (Mohammed and Russell 2012).

Assay via ultraviolet (UV) spectroscopy is one of the first stages during preformulation studies. It includes the determination of specific wavelength at which drug exhibits maximum absorption. The development of UV assay is vital as it provides a rapid method to assess quantitative measurements of drug substance. This is achieved by running a broad wave scan in the UV region (approx 200-400nm) and choosing the right absorption wavelength (λ_{max}) for the drug in question. Once a wavelength has been decided upon, the next phase involves the development of a calibration curve to assess unknown drug concentrations for any future experiments. However utmost care should

be taken when developing the calibration curve due to the limitations stated by Beer-Lambert law (Mohammed and Russell 2012).

1.4.1 Solubility

The solubility of drug candidate is one of the critical parameters that have an influence on the amount of drug absorbed into systemic circulation (bioavailability). Drug candidates with solubility values below 1% over the pH range of 1-7 at 37˚C can pose potential problems with drug absorption. Also intrinsic dissolution rate less than 0.1 mg/cm^{2}min can result in dissolution rate limited absorption. Therefore determination of drug solubility and pKa for ionisable drugs helps in choosing the right pH to ensure sufficient drug solubility and decide on the type of counter ion for salt formation. An increase in drug solubility in the acidic media is indicative of basic nature of the moiety and vice versa. However an increase in solubility in both acidic as well as basic media suggests either amphoteric or zwitterionic molecule comprising of two ionisable groups. During preformulation studies, intrinsic solubility (C_o) of the NCE is determined where the drug exists in its unionised form. This is done by measuring solubility in acid medium for acidic drug and in alkaline medium for basic drug and is carried out at two different temperatures of 4 and 37˚C. Solubility measurements at 4˚C are correct as water exhibits anomalous expansion at this temperature and due to its high density results in low solubility. Studies for solubility at 37˚C would be indicative of drug solubility profile in humans (Tong and Wen 2000).

Another important parameter that is studied for ionisable drugs is the measurement of pKa. As a rule of thumb, for acidic drug, two pH units below pKa will result in the formation of unionised form of the drug and two units above will result in complete ionisation. Henderson-Hasselbach equation can be used to calculate the extent of drug ionisation upon changes in pH and in turn predict solubility profiles for ionisable drugs. A solubility value of less than 0.1% presents the need for salt formation if the intended dosage form is a tablet or a capsule. Alternatively novel drug delivery

systems such as liposomes, polymer based nanoparticles and use of co solvents can be implemented (Mohammed and Russell 2012).

1.4.2 Solvents

The solubility of a drug is assessed in various solvents including water and cosolvent mixtures such as ethanol and water. The choice of appropriate solvent or mixtures of solvents is important to facilitate drug extraction and separation in chromatography, improve solubility and formulation development (e.g.-injection for initial pre-clinical evaluation of the drug). When a suitable solvent or mixture of solvents cannot be found due to drug instability or insufficient solubility, oils such as castor oil or liquid paraffin are used.

The choice of the solvent is influenced by drug properties. When the log P of the drug candidate is greater than 2, it results in poor aqueous solubility which necessitates the use of water miscible solvents to improve solubility and stability. The choice of the exact composition of the solvent mixtures can be determined using a phase diagram which measures the effect of increasing/changing the ratio of solvent mixtures on drug solubility. The most suitable solvent/mixtures of solvents is the one where polarity of the solvent matches that of the solute (Millard *et al* 2002). Data from these experiments can be used for both formulation development in the future as well as choosing right combination of solvents for chromatography (HPLC) during assay development (Mohammed and Russell 2012)).

1.4.3 Partition Coefficient

The extent of distribution of drug between water and organic phase (usually octanol) is termed as partition coefficient(K). The solubility parameter of octanol which is around 10 lies mid-way in the range for majority of the drugs. This enables convenient partition of the drug between the polar and the non-polar phases (Leo *et al* 1971). The most commonly used technique is the shake flask method where a known amount of the

drug is dissolved in one of the phases and is shaken for about 30 minutes, allowed to stand for 5 minutes followed by separation of the aqueous phase (lower layer). The separated phase is then centrifuged and the drug content assayed using either UV spectroscopy or HPLC to determine drug concentration (Mohammed and Russell 2012). Partition coefficient is calculated as follows:

$$K = \left(\frac{[solute]_{octanol}}{[solute]_{water}^{un-ionised}} \right)$$

Determination of partition coefficient has a wide range of applications such as;

a) Identification of the drug's solubility which in turn sheds light on hydrophilic/lipophilic nature of the drug.
b) Prediction of the drug permeability *in vivo*.
c) Allows for the choice of mobile phase and stationary phase for chromatography techniques such as HPLC and thin layer chromatography (TLC).

1.4.4 Melting Point

Determination of melting point of a drug provides information on drug purity and polymorphism. A pure compound is characterised by a sharp well defined melting temperature and the existence of polymorphism is detected by multiple melting temperatures (Lipinski *et al* 1997). Understanding of the melting point of an API allows for decisions to be made with regards to the developmental procedure, for example an API with a low melting point may be unsuitable to undergo processes such as high speed rotary pressing which produces significant heat due to friction and as such implementing encapsulation of the API would be a more suitable option for the dosage form production (Mohammed and Russell 2012).

1.4.5 Micromeritics

Micrometrics is the science and technology of small particles and is used during investigations into:

- Particle size and size distribution.
- Identification of particle shape and surface area.
- Pore size.

In pharmaceutical formulations, the size, shape, and surface area of the drug particles play a large role in the physical stability of that formulation and also the rate of release of an API from the formulation. Generally, reducing the particle size increases the rate of dissolution and this enhances absorption. The flow properties of granules and powders are also linked with particle size. This is of particular relevance during the production of tablets where the uniformity of the tablets produced can be compromised if there are large variations in particle sizes and flow properties (Mohammed and Russell 2012).

1.5 SOLUBILISATION OF INSOLUBLE DRUGS

As the best option for paediatric administration is the production of an oral liquid formulation the aqueous solubility of a drug can be a determinate in the dosage form selection, soluble drugs lend themselves easily to the production of solutions. For insoluble drugs however the options are to produce a suspension where the drug remains undissolved or alternatively to utilise techniques to solubilise the insoluble drug, e.g. complexation, co-solvency or micelles.

Cyclodextrins are used to aid the solubilisation of a compound in water via the complexation of hydrophobic molecules to the cavity formed in the cyclodextrin. This in encapsulates the molecule of interest rendering the molecule water soluble. When the

solution becomes more dilute, e.g. in the body, then the much larger volume of aqueous solvent reverses the encapsulation releasing the molecule of interest.

There are three main sub classes of cyclodextrins; α, β and γ. These consist of 6, 7, and 8 glucopyranose units respectively and form cyclic oligosaccharides (Figure 2) (Brewster and Loftsson 2007).

Figure 2 - General Cyclodextrin Structure – The above example is that of β-CD exhibiting 7 glucopyranose units where n=1. Alteration of 'n' to 0 or 2 would then depict the structure of α-CD and γ-CD respectively.

Cavity size is the major determinate as to which sub-group of cyclodextrin is used for complexation. β – cyclodextrins are the most widely used and are well suited to molecules the size of hormones and vitamins. α – cyclodextrins are the smallest of the sub classes and γ – cyclodextrins are the largest (Challa *et al*. 2005).

Co-solvency incorporates the use of two miscible solvents in which the compound of interest is known to be soluble. The reason for using a co-solvent system is that the compound's stability can be increased and as a result, more compound can be dissolved than if either solvent was used on their own. Although co-solvents can consist of more than two solvents, they most commonly consist of only two.

Micelles can be either oil in water or water in oil formations and work by encapsulating the molecule of interest. For oral administration the type of micelle would have to be an oil in water formation. Micelles are formed from surfactants present at a concentration above the critical micelle concentration (CMC) in a system at a temperature above the critical micelle temperature (Krafft Temperature) (Francis *et al*. 2004). Although micelles can be used for the solubilisation of insoluble drugs the formation of micelles can reduce the effectiveness of preservatives and so could be unfavourable for use in a pharmaceutical formulation intended for long term storage and use.

1.6 Excipients for Inclusion in Oral Liquid Formulations

The formulation of solutions and even more so for suspensions requires the use of several excipients. It is important that all ingredients in the formulations are chemically compatible to ensure the stability of a formulation. In addition to the previously mentioned solubilising agents, flavours and sweeteners there is the inclusion of preservatives to protect against microbial contamination and in the majority of cases antioxidants are included to protect the API from oxidising agents.

1.6.1 Preservatives

The preservatives for inclusion in oral liquid formulations should be effective over a broad spectrum in order to prevent contamination as a result of bacterial (Gram negative and Gram positive) and fungal infection. They must also however be harmless to the patients taking the medication and also remain active and stable for the life of the product. There are a multitude of preservatives available for use with some of the most commonly used including benzoic acid, sorbic acid and the Parabens group of preservatives (Bean 1972).

There are many factors in liquid formulations which can have an effect on the efficacy of the preservatives included in the formulation. The first determinant is the

concentration of preservative included in the formulation. Below the minimum inhibitory concentration (MIC) the preservative will not be sufficiently effective to fully protect the formulation from contamination. Other factors which can be of incidence on the efficacy of the preservatives are the pH of the system, the presence of micelles and the presence of hydrophilic polymers (Jones 2008).

The pH of a system determines whether a compound will be present in its ionised or unionised form. In some cases only the unionised form of a preservative displays antimicrobial activity. As a result increased antimicrobial activity will be seen in acidic formulations, an example of such a preservative is benzoic acid. There are other preservatives which are less pH dependent include the parabens which thanks to their high pKa are suitable for use over a pH range of around 4-8.

Micelles can impact preservative activity if the preservative displays hydrophobic properties, where this is the case the preservative can pass into the oil phase of the micelle leaving concentrations of preservative remaining dissolved in solution which may fall below the MIC. If micelles and hydrophobic preservatives are to be used in conjunction it is important that the concentration of preservative initially added to the formulation is greater than indented for use so as that the concentration of preservative present in the solution remains above the MIC (Jones 2008).

Hydrophilic polymers have been shown to interact with the preservative dissolved in solution via chemical reactions which reduces the availability of preservative. If this is the case then as with the presence of micelles the concentration of preservative initially added to the formulation must be greater than is indented for use so as that the concentration of preservative present in the solution remains above the MIC. Electrostatic interactions between the hydrophilic polymers and the preservatives may also render the two incompatible. As this is the case then formulations involving cationic hydrophilic polymers in formulations along with acidic preservatives should be avoided (Jones 2008).

1.6.2 Antioxidants

Antioxidants are included in pharmaceutical preparations to protect the API when there is the possibility of oxidative degradation. Antioxidants are typically either compounds that inhibit free radical induced decomposition or they are redox systems which exhibit higher oxidative potential than the drug they are protecting. They work to protect the drug by being oxidised in preference of the drug itself. Antioxidant concentration decreased markedly between production and the end of the shelf life of a formulation as the antioxidant is used up over time. Antioxidants are used in low concentrations and can be combined with chelating agents which provide added protection (Shah *et al* 2010).

1.6.3 Additional Excipients Required in the Formulation of Suspensions

Suspensions are more complicated formulations than solutions to produce as they are inherently unstable formulations. Due to the fact that the insoluble particles in a suspension will in most cases sediment at the bottom of the container. If the particles sediment in a very compact manner then caking can result where homogenous redistribution of the particles into the suspension is impossible. Controlling the interaction between the particles so that any aggregation is reversible or preventing any interaction between particles makes it possible to prevent caking. In order to do this excipients such as; electrolytes, surface active agents and hydrophilic polymers are often included in suspension formulations. Electrolytes entirely and surface active agents on the whole improve the stability of a suspension by modifying the zeta potential of the suspended particles and before their action can be understood explanation of the interaction between particles in a suspension is necessary (Jones 2008).

1.7 Production of a Palatable Formulation - Current Techniques and Shortcomings of Existing Taste Assessment Methods.

One of the most crucial areas to address in the production of a new medicine which is intended for oral administration is that of taste. This is of particular importance to ensure patient compliance and is of increased importance when formulations are intended for paediatric use as children are less likely to be willing to take unpleasant tasting medications that an adult may tolerate. The consideration of formulation taste is of particular relevance in solutions more so than suspensions as the bitter taste of many API's is only fully prevalent once the API is solubilised whereas when in suspension the bitter taste can be somewhat masked by the suspending agent used in the suspension. Fortunately there are many modes of taste masking and taste modification which are successfully employed on a routine basis by formulation scientists.

The inclusion of taste enhancers such as concentrates, fruit juices and sweetening agents are widely used however are limited to their capacity in taste masking very bitter drugs. Another approach is to coat the drug particles with agents which are not pharmaceutically active for example eudragit, polyethylene glycol (PEG) and ethyl cellulose. These coatings prevent the interaction of the bitter tasting drug particles with the taste receptors in the mouth and thereby mask the bitter taste. It is important that the coating of the drug particles does not limit the bioavailability of the drug. Complexation of the drug molecule with a host molecule is another method by which the bitter taste of a drug can be masked, in this way the whole drug molecule or at least the section of the molecule is contained within a host molecule for example cyclodextrin and in a similar way to the coating procedure prevents the interaction of the drug with the taste receptors. Yet another widely used approach involves the production of solid insoluble poly-electrolytes which have a high molecular weight known as Ion exchange resins. The inclusion of a bitter tasting drug into a solid dispersion, liposomes or emulsions or alternatively the combination of the drug with amino acids are four further methods for

taste masking with the formation of pro-drugs or salts of the drug being two methods of improving palatability via chemical modification (Ayenew *et al* 2009).

The most commonly used sweetening agents included in pharmaceutical preparations are sucrose, liquid glucose, glycerol, sorbitol, saccharin sodium and aspartame. Increasingly artificial sweeteners are being used in formulations and in paediatric formulation the use of sugar is to be avoided. This is also the case for formulations intended for use in patients suffering from diabetes mellitus (Jones 2008).

In the same manner that sweeteners are included to mask the taste of unpleasant tasting formulations, purpose made flavourings can be added. The flavour selected for use must be effective at masking the taste and in order to do this the flavours to choose from depend on the initial taste of the formulation. For example, when a formulation is unpleasant as the result of a salty taste then some of the best flavours to mask this include; butterscotch, apricot, peach, vanilla and mint. In the same way, a bitter taste is best masked with flavours such as; cherry, mint and anise. Formulations which are overly sweet are best improved by including vanilla or fruit and berry flavours while sour tasting formulations are best masked using the citrus flavours and raspberry.

In most cases a combination of more than one flavouring agent coupled with a sweetening agent will produce the most palatable formulation and excipients charged with the roll of enhancing the flavours present are often included. Flavour adjuncts are at times also included and these have the effect of reducing the sensitivity of the taste buds at the same time as adding to the flavour of the formulation.

There is however still a problem in the formulation development; due to the complexity of taste as a sense, the methods available for assessing the taste improvements or taste suitability are limited and are in undoubted need of improvement.

Taste transduction begins with taste receptor cells (TRC's) found in taste bud clusters within structures known as taste papillae. These taste bud containing papillae

exist in three distinct forms which are located in specific areas on the tongue and they are known as fungiform, circumvallate and foliate papillae. Fungiform papillae are located on the anterior portion of the tongue while circumvallate and foliate fungiform are located in posterior areas of the tongue. The taste buds which are contained within the papillae are constructed of four different cell types, the support cells (Type 1 cells, or dark cells), account for 50% of the cells present in a taste bud. The sensory cells (Types 2 &3 or light cells), account for around 25% of the cells present in a taste bud. Basal cells (Type 4 cells) account for the remaining 25% of the cells. Of the sensory cells, type 2 cells are thought to be responsible for the detection of bitter, sweet and umami tastants through transduction pathways involving proteins such as gustducin and phospholipase C (PLC)-β_2, which are used as reliable indicators of type 2 TRC presence in TRC cultures. Likewise, neural cell adhesion molecule (NCAM) is a reliable indicator of type 3 sensory cells. These are thought to be the cells responsible for the detection of acid taste while the cell type responsible for the detection of salty taste is yet to be identified.

Bitter compounds are thought to elicit a taste response through two separate pathways. The first pathway, as activated by denatonium involves the activation of the G-protein coupled receptors T2R/TRB which activates gustducin heterotrimers. Cyclic adenosine monophosphate (cAMP) is hydrolysed by phosphodiesterase (PDE) following stimulation by the α-gustducin subunit of the heterotrimer. The β and γ subunits which are released activate PLC-β_2 leading to the generation of inositol trisphosphate (IP_3) which in turn leads to an increase in intracellular calcium concentrations following the release of calcium from internal stores. The second pathway stimulated by bitter compounds involves the inhibition of apical K^+ channels which leads directly to the depolarisation of the TRC and subsequent increase in intracellular calcium (Gilbertson *et al* 2000).

Sweet compounds again have multiple taste transduction pathways and the cascade mediated is dependent upon whether the compound in question is an artificial

sweetener or a natural sugar. Artificial sweeteners activate ionotrophic receptors which are linked to cation channels and G-protein coupled receptors. This results in elevated levels of IP_3 and a subsequent increase in intracellular calcium following the action of PLC. Natural sweeteners activate G-protein coupled receptors linked with adenylyl cyclase to bring about an increase in intracellular cAMP. This is then thought to bring about phosphorylation by cAMP activate protein kinase A and in turn the inhibition of basolateral K^+ channels. This leads to the depolarisation of the TRC and intracellular calcium increase (Gilbertson *et al* 2000).

Umami taste is elicited by the amino acid L-glutamate (L-Glu). L-Glu causes the activation of the taste form of the G-protein coupled receptor mGluR4. This causes reductions in intracellular cAMP through PDE activation. This reduction in cAMP is thought to reopen cyclic nucleotide monophosphate (cNMP) inhibited channels which leads to an increase in intracellular calcium. Other amino acids activate ionotrophic receptors which leads to the direct depolarisation of the TRC and in turn the increase in intracellular level (Gilbertson *et al* 2000).

Salty taste is elicited by sodium salts. These cause direct depolarisation of the TRC leading to intracellular calcium increases following the permeation of Na^+ ions entering the cell through amiloride sensitive epithelial-type Na^+ channels (ENaC) (Gilbertson *et al* 2000).

Sour taste is a result of protons entering the TRC. There are a variety of proposed transduction mechanisms for sour taste however there is still much work required in this area to reveal the main transduction components involved in the taste cascade following exposure to a sour stimuli. This is due mainly to the pH sensitivity of ion channels, intracellular signalling components and transport proteins. Currently proposed ideas are similar to the transduction of salty taste through ENaC, where following permeation of H^+ ions have been shown to cause depolarisation of TRC's leading to increases in intracellular calcium. H^+ ions also have the effect of activating proton activated cation

channels such as brain type Na^+ channel-1 (BNaC1), acid sensing ion channel (ASIC) and dorsal root acid-sensing ion channel (DRASIC) all of which may play a role in the signalling cascade. Sour stimuli have also been shown to activate an 5-nitro-2-[3-phenylpropylamino]-benzoic acid (NPPB) sensitive conductance similar to that seen through ASIC's, again suggesting that NPPB may play a role in the transduction of sour taste (Gilbertson *et al* 2000).

As it stands, the most common means of assaying taste of new formulations *in vitro* is through the use of *in vitro* drug release studies or *in vitro* assay methods. Drug release studies examine the release of a tastant from a formulation using modified dissolution apparatus or such like, utilising artificial saliva they are used to measure the effectiveness of coatings or complexation within a formulation. This method however is not applicable to the testing of liquid formulations. *In vitro* assay methods are currently reliant upon the measurement of the activation of transducin and/or gustducin meaning that these assays are not applicable to transducin/gustducin independent taste modifiers, of which there are many (Ruiz-Avila *et al* 2000). The most common *in* vivo method for taste assessment is through the use of a taste panel (Anand *et al* 2007). This involves the sensory analysis of tastants using trained healthy human volunteers and although being well established and the standard method for the taste assessment of drugs and drug products there are significant drawbacks. Finding volunteers who will be available on a long term basis is tricky especially when unpleasant tasting samples are being tested. Once recruited the volunteers must undergo extensive training and even after which the testing is still subjective. In addition the process of testing is time consuming and as a result low throughput. Finally potential problems regarding toxicity and additional human ethical issues are also limiting factors. Other *in vivo* test methods involve animal preference testing (Tordoff and Bachmanov 2003) and electrophysiological methods (Dewis *et al* 2013), both of which are far from ideal. The animal preference testing is a low throughput, qualitative means of taste assessment

and the electrophysiological methods involve complicated animal based surgery which brings with it significant cost and also animal ethical issues. In addition the data analysis and interpretation can be problematic.

Most recently advances in biomimetic taste sensing systems (BTSS) have provided the first high-throughput techniques which are of use in formulation development and optimisation. They employ electrochemical sensing methodologies linked to chemometric methodologies to provide both qualitative and quantitative analysis (Baldwin *et al* 2011). There are however still limitations with these systems. It is almost impossible to generate absolute statements relating to the taste of a particular sample analysed using an electronic tongue and data is susceptible to change as a result of changes in analytical conditions and as a result reproducibility of data can be hampered (Woertz *et al* 2011). In addition to the experimental and data analysis limitations, the initial setup cost is considerable.

One area of interest which is seemingly underexplored and exploited is that of *in vitro*, tissue culture based models. Investigations into taste receptor cell (TRC's) function has been done using explant cultures from rodents (Mbiene 1997), semi-intact taste buds in tissue slices (Caicedo 2000) and primary cultures established from freshly isolated TRC's (Ruiz *et al* 2001, Ookura 2002, Qin 2008). The lack of progress in this area is quite likely due to the limitations of primary culture, most notably limited passage number typically below P5, which have been encountered by many of the groups who have attempted primary culture of TRC's. One group in particular has reported more promising results and have successfully established long term cultures of both rat (Ozdener 2006) and human (Ozdener 2011) TRC's. Both of these cell lines are useful in their own right however there are refinements which can be made to significantly reduce both the cost and complexity of establishing and maintaining the cultures while at the same time maintaining and adding to the reported advantages which each of these cultures provide.

1.8 Stability Testing of Liquid Formulations

Once a formulation has been developed it is necessary for it to undergo stability testing to provide information with regards to how the quality of a formulation varies over time when exposed to environmental factors including temperature, humidity, and light. This controlled testing allows for the identification of any degradation in the formulations and determine a re-test time or shelf life for the product. Recommended storage conditions can also be determined following stability testing.

The conditions for storage are set out in the international conference on harmonisation (ICH) guidelines (Q1A (R2)) which states that 'a drug substance should be evaluated under storage conditions that test its thermal stability, and if applicable its sensitivity to moisture'. There are three studies recommended for general use termed; long term, intermediate and accelerated (Table 2);

Table 2 - Storage conditions for long term, intermediate and accelerated storage conditions as detailed by the ICH guidelines

Study	Storage Conditions	Minimum Time Period
Long Term	25°C ± 2°C at 60% Relative Humidity ± 5% or 30°C ± 2°C at 65% Relative Humidity ± 5%	12 Months
Intermediate	30°C ± 2°C at 65% Relative Humidity ± 5%	6 Months
Accelerated	40°C ± 2°C at 75% Relative Humidity ± 5%	6 Months

It is necessary to test the formulations at regular time points for the duration of the testing period in order to establish the full stability profile of the formulation. For long term studies it is recommended that testing should be carried out at least every three months over the first twelve months and then every six months thereafter for the duration of the study. For the accelerated conditions it is recommended that testing should be carried out at least for day zero, the end time point and one other time point during the duration. The intermediate study follows the same pattern as the accelerated study however requires at least one extra time point between day zero and the end time point.

1.9 Intestinal Drug Absorption Modelling – Current trends *in vitro*, *in vivo* and *in silico*.

The extent of a drug's ability to successfully traverse from the intestinal lumen into the blood is the greatest limiting factor in the absorption of orally administered drugs. Physiological components such as tight junctions between intestinal enterocytes and the hydrophobicity of cell membranes are combined with biochemical components such as enzymes and transporter proteins to provide a barrier function.

The passage of drugs across the intestinal membrane can occur in a variety of ways (Figure 3) including:-

1.) Passive diffusion via the paracellular route.
2.) Transcellular carrier mediated transport across the cell membrane.
3.) Efflux transporters at the apical membrane may actively drive compounds back into the intestinal lumen.
4.) Apical efflux transporters may facilitate the intestinal clearance of compounds that are already present in the blood.
5.) Intracellular metabolising enzymes may modify compounds before they enter the blood.
6.) Intracellular metabolising enzymes and apical efflux transporters may co-ordinately metabolise and excrete compounds forming an effective barrier against intestinal absorption.

With this in mind, a model for the prediction of drug absorption across the intestinal membrane should be capable of generating information relating not only to the extent to which a drug is absorbed but also the transport pathway by which the drug passes across the intestinal membrane.

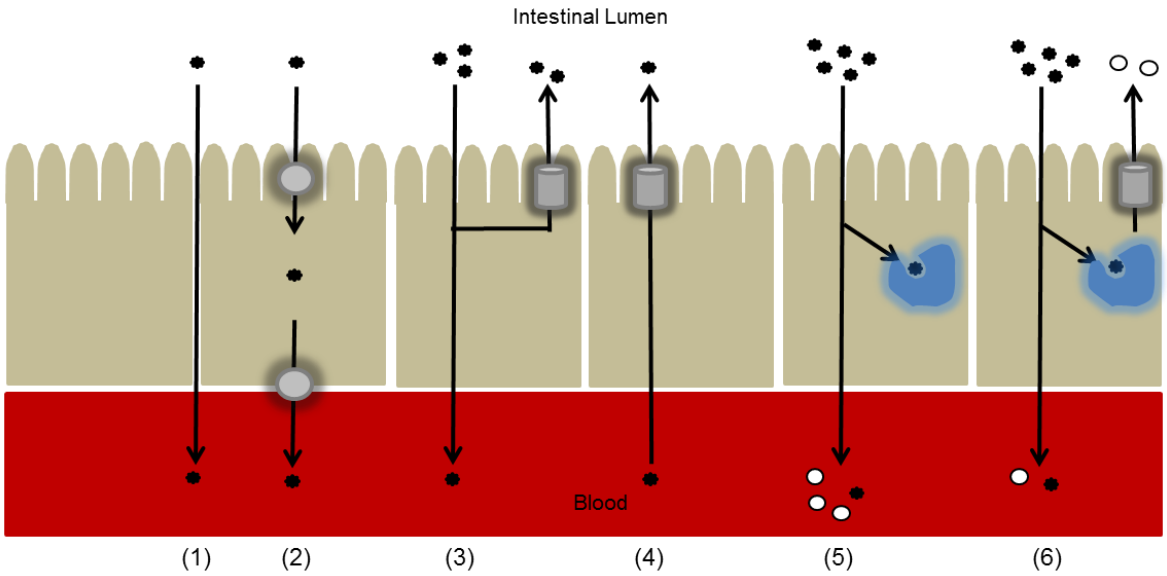


Figure 3 - Potential transport pathways a drug molecule may take in the intestinal membrane. (1) Passive diffusion via the paracellular route, (2) Transcellular carrier mediated transport across the cell membrane, (3) Efflux transporters at the apical membrane may actively drive compounds back into the intestinal lumen, (4) Apical efflux transporters may also facilitate the intestinal clearance of compounds that are already present in the blood, (5) Intracellular metabolising enzymes may modify compounds before they enter the blood, (6) Intracellular metabolising enzymes and apical efflux transporters may co-ordinately metabolise and excrete compounds forming an effective barrier against intestinal absorption.

There are many methods which have been developed for the modelling and prediction of intestinal absorption and these are categorised broadly into *in vitro, in vivo* and *in silico*. *In vivo* investigations involves the use of living humans or animals in order to identify the permeability of a drug. *In vitro* investigations are models of the *in vivo* conditions and are used to produce estimates of the drug permeability. These often do not reproduce all of the conditions seen in the intestine and as such the identification of correlations between *in vivo* and *in vitro* analysis is important to ensure the validity of an *in vitro* analysis. *In silico* modelling and prediction is computer based models and is becoming an increasingly commonly use tool in drug discovery and design.

1.9.1 *In Vitro* Permeability Models

In vitro analysis of drug permeability can be done through a multitude of techniques which are broadly classified into, excised tissue assays, cell based assays, membrane based assays and physiochemical based analysis. Each class of technique has its advantages however they all come with limitations also. Selection of the model to use is at times determined by the specific parameters relating to the drug permeability which are to be investigated.

Excised tissue assays involve the use of; isolated intestinal segments, everted sacs, intestinal rings and stripped and unstripped mucosal sheets mounted in ussing chambers. The main advantage of excised tissue is that there is a full complement of the physiological structures present and this provides a very accurate representation of the intestinal membrane. The major disadvantage however is that experiments are limited in their duration by deterioration of the tissue as cell viability is limited (Table 3).

Cell based assays involve the use of cell cultures such as Caco-2, HT-29, T-84 and primary cultures of isolated epithelial cells. Cell based assays provide many of the major features of the intestinal membrane and Caco-2 cells are well established as a technique for modelling intestinal permeability. The main limitations of cell based assays are that there is often only one cell type present in the culture, mucus production is not achieved in well-established models and enzyme levels may be lower than seen in the *in vivo* environment. Primary cultures also carry with them increased complications including being technically difficult to culture and having limited viability (Table 4).

Membrane based assays involve immobilised artificial membrane (IAM), parallel artificial membrane permeation assay (PAMPA), mucosal cell membrane vesicles and isolated mucosal cells. The cell free membrane based assays tend to be rapid and high throughput techniques and correlation with cell based assays have been shown. There is the disadvantage however that in most models, transporter proteins as well as

enzymes are not present, also the membrane based assays tend not to provide any insight into paracellular absorption (Table 5).

Physiochemical methods look at a range of parameters which include, molecular weight, atomic make up of drug compounds, partition coefficient and distribution coefficient. The logarithm of the partition coefficient (LogP) is the hydrophobicity descriptor and it is used in a number of *in vitro* models and is the logarithm of the octanol/water partition coefficient. Much of this *in vitro* analysis provides the data used in producing *in silico* models. Lipinski's rule of five is one example where physiochemical properties are used to predict drug absorption. The main limitation of physiochemical methods is that they tend to form a basic check rather than an all encapsulating predictive model. Many modern API's do not fit the patterns of the physiochemical prediction methods, examples include drugs which are substrates for transporter proteins as well as many naturally occurring compounds.

Table 3 – Advantages and limitations of the most common in vitro permeability models using excised tissue samples.

***In Vitro* Permeability Models - Excised Tissue**		
Permeability Model	Advantages	Limitations
Isolated Intestinal Segments	Full Physiological Profile is present. Allows for the study of specific functions at an organ level.	The effect of Cell alterations limits the length of experiments.
Everted Sacs	All factors involved in the Intestinal Barrier are present (Cells and Mucus Layer). Rapid and Low Cost Method. Can be useful in identifying the pathway of absorption. Allows for the investigation of permeability in different sections of the Intestine.	Not a perfused model. Low viability of the tissue. Drug must cross the whole intestinal barrier.
Ussing Chambers	Can be used for both human and animal tissue. Allows for the investigation of permeability at in different sections of the intestine. Both absorption and efflux can be investigated. Allows for metabolic studies. Allows investigation of electrophysiological behaviour.	Limited cell viability. Human material is limited. Not suitable for screening.
Intestinal Rings	All factors involved in the intestinal barrier are present i.e. both cells and mucus layer. Rapid and low cost method. Can be useful in identifying the pathway of absorption. Allows for the investigation of uptake in different sections of the Intestine.	Not a perfused model. Low viability of the tissue.

Table 4 - Advantages and limitations of the most common in vitro cell based permeability models.

***In Vitro* Permeability Models - Cell Models**		
Permeability Model	Advantages	Limitations
Caco-2	Displays the major features of the intestine. Suitably rapid and simple. Suitable for a range of studies. Allows for identification of absorption mechanism. Models uptake and efflux. Allows for Screening. Established in drug discovery. Accurate as uses human cells.	Some physiological factors are not present. Only one cell type present. Low levels of CYP3A4.
HT-29-MTX/Caco-2 co-cultures **MDCK**	Cell combination models mucus-secreting cells. Rapid and simple method. Can be used for screening. Allows for the measurement of passive diffusion.	Not a well-established method. Not an intestinal model. Not a human cell line. Physiological factors not present.
TC-47	Derived from Caco-2 cells. Express high Levels of CYP3A4.	Physiological factors not present. Only one cell type present.
Primary Isolated Epithelial Cells	Displays the major features of the site from which cells are isolated.	Difficult to culture. Limited cell viability. Loss of *in vivo* features.

Table 5 - Advantages and limitations of the most common in vitro membrane based permeability models.

***In Vitro* Permeability Models - Membrane Based Assays**		
Permeability Model	Advantages	Limitations
Immobilised Artificial Membrane (IAM)	Cell Free Assay. Lipids in columns mimic the lipid environment of the cell membrane. Allows high throughput. Has been confirmed to show correlation with Caco-2 permeability study findings.	Uses capacity factor (K) for prediction which results in polar surface area being the stronger predictor of absorption as opposed to binding capacity and molecular size. Ignores enzymes, influx and efflux transporters and the paracellular pathway.
Parallel Artificial Membrane Permeation Assay (PAMPA)	Cell free assay. 96 Well format allows high throughput. Small amounts of compound needed. Can be manipulated to investigate the effect of pH with ease.	The presence of co-solvents in the system can mislead permeability estimations. Requires UV absorbance which many compounds do not exhibit. May require long experimental times. Ignores enzymes, influx and efflux transporters and the paracellular pathway.
Mucosal Cell Membrane Vesicles	Useful in the study of specific membrane processes. Rapid experiments. Small amounts of compound needed. Vesicles produced easily.	Lack of cellular metabolism makes ATP dependent investigation difficult. Provide no insight into paracellular Transport.
Isolated Mucosal Cells		Difficult to culture. Limited cell viability. Loss of *in vivo* features.

1.9.2 *In Vivo* Permeability Models

In vivo permeability analysis is the most 'true to life' means of modelling permeability and for the most part involves the use of animal models, rats are the preferred species used to model the human intestine. On occasions *in vivo* permeability studies are carried out with the use of human volunteers, this is however not an option in cases where there is little toxicological data available for the drug being investigated. The main benefit of *in vivo* testing over *in vitro* and *in silico* methods is that the whole animal/human is involved in the testing, this means that all of the biological parameters such as membrane structure, mucus, transporters, enzymes and drug metabolism are

all accounted for. Additionally there is no need to perform experiments to show correlations. Added benefits of *in vivo* permeability investigations using animal models is that the animals used in the permeability testing can also be used in pharmacological and toxicological evaluations. *In vivo* experiments are however not without limitation, the analytical procedures are usually much more complicated than the equivalents for *in vitro* and *in silico*, also the use of living organisms brings with it significant costs and ethical considerations (Table 6).

Table 6 - Advantages and limitations of the most common in vivo permeability models.

***In Vivo* Permeability Models**		
Permeability Model	Advantages	Limitations
Animal Model **Rat Model**	Brings together all of the factors that can influence the uptake of a drug. Suitably represents the conditions in humans. Allows for the measurement of bioavailability.	Animal model. Metabolism differences between animals and humans. Hard to identify variables in the mechanism of absorption.
Human Model **Administration of specially designed capsules to Human Volunteers**	Human Model. Regulatory authorities are recommending the importance of this technique in developing sustained release products. Permits regional absorption studies.	

1.9.3 *In Silico* Permeability Models

Recent advances in computing technology has seen the rise of *in silico* analysis which has been limited previously by processing power and data storage constraints. *In silico* models take into consideration the relationship between the structure and activity of an API through the Quantitative Structure-Activity Relationships (QSAR). QSAR's are complex mathematical models which find correlation between the biological activity of an API and the physiochemical properties of that API.

In order to produce an *in silico* method, existing literature is trawled to collect the data used in the construction of the model. Before statistical analysis of the data,

methods used in the data gathering are examined to determine if there is the need to factor in corrections to counteract any differences in the collection methodology which could skew the overall predictions generated. The corrected the data is processed by statistical programmes in order to reveal the significant descriptors for the permeability of the drug. A mathematical equation is ultimately generated and this is used in the prediction of permeability. The predictions made by *in silico* techniques are able to provide guided predictions on a compound's behaviour but there are multiple reasons why the predictions are not exact.

The major benefit of *in silico* modelling techniques is that the results are generated rapidly and being computer based there is no need to use the actual drug in the investigations thus reducing cost and in some instances the investigations can be performed before the API has even been synthesised. The major drawback of *in silico* analysis is that the predicted permeability is only based on the passive transcellular pathway. Although it is understood that this is the most active pathway there are also transport networks involving the active transport and efflux mechanisms that may affect the *in vivo* permeability of an API. *In silico* methods for permeability estimation are promising techniques for the future, as they have only more recently began to expand into a feasible technique they currently only play a relatively limited role in API and formulation development (Table 7).

Table 7 - Advantages and limitations of the most common in silico permeability models.

***In Silico* Permeability Models**		
Permeability Model	Advantages	Limitations
Rule of Five	Describes molecular properties important for a drug's pharmacokinetics in the human body. This includes absorption, distribution, metabolism, excretion and toxicity (ADMET).	The rule does not predict if a compound is pharmacologically active.
Solubility Parameters	Predicts the duration of absorption in humans by determining the solubility parameters for each fragment of a drug's structure.	
Multiple Calculated Molecular Descriptors	Investigates a number of strongly inter-correlated physiochemical properties, including lipophilicity, molecular size, hydrogen bonding capability and the degree of ionisation at the pH level of interest.	Experimental data can be collected to provide more reliable data than calculated result.
Dynamic Surface Properties	Allows for the prediction of oral absorption, dynamic polar surface areas of less than 60 A^2 suggest good oral absorption whereas dynamic polar surface areas greater than 140 A^2 suggest poor oral absorption.	Model does not take into account that the compounds also need sufficient water solubility in order to permeate the membrane.
MolSurf/PLS	Used to model permeability across Caco-2 monolayers and differentiate between drug's with poor permeability and those with good permeability.	Permeability is modelled for an *in vitro* test and so *in vivo* performance may differ.

1.9.4 Importance of *In vitro - In vivo* Correlations (IVIVC) and *In Silico* – IVIVC's

Establishing clear correlations with the observed *in vivo* behaviour is a vitally important part of establishing any method which has been developed for the modelling of permeability. In identifying that a correlation exists in the form of a mathematical relationship between the predicted results and the expected *in vivo* observations it is possible to use the model as an alternative to human or animal testing.

In the case of IVIVC's, the correlation between the *in vitro* findings and the *in vivo* observations should produce a linear relationship with an R^2 value close to 1.00. In general the findings from the *in vitro* method are required to be within ± 10% of the findings *in vivo* at each time point for the duration of an investigation. For models where this is achieved, the *in vitro* method is acceptable and becomes a useful tool in both the prediction of *in vivo* permeability and also the quality control of a drug product should it progress through the drug development pipeline and make it into production. It is important to mention however that any IVIVC, is restricted in its use to the modelling of the permeability for the certain drug product. In the same fashion that finding IVIVC's are essential in the production of useable *in* vitro permeability models, *in silico* – IVIVC's are necessary to show that permeability predictions produced by any *in silico* technique are representative of the observed *in vitro* and *in vivo* findings (Mohammed *et al* 2011).

1.10 Genomics as a Novel Contributor in Revealing an IVIVC for Intestinal Absorption Prediction.

Genes are at the hub of so many of the biological events and focusing studies on human genetics allows for advances in drug discovery through a multitude of avenues including the understanding of disease onset and progression and providing a means to the identification of drug targets and even therapeutic agents by observing genes and their transcription products. Application of genomics to areas including, biomarkers, tissue expression profiling, side effect profiling, pharmacogenomics and genome wide epigenetics are some of the main areas through which genomic techniques are already contributing to the drug discovery and development processes. Investigation into fluctuations in the *in vitro* gene expression levels of intestinal transporters with the aim of producing an IVIVC is one of the ways in which genomic profiling can further contribute to drug discovery and development.

1.10.1 Membrane Transporters

There are two main types of membrane transporters, the first type are membrane channels, the second is carrier mediated transporters. Carrier mediated transporters are then sub divided based upon the energy needs for the transport process. Carrier mediated transporters include transporters active in facilitated diffusion, primary active transporters and secondary active transporters (Stenberg *et al* 2000). Primary active transporters transport substrates out of the cell against concentration gradients and are driven by the energy released following the conversion of ATP into ADP, the ATP-binding cassette (ABC) transporters fall into this group of transporters. Secondary active transporters or co-transporters use the energy liberated during the transport of Na^+ and Ca^{2+} ions across the membrane by ion pumps. Solute carrier (SLC) transporters fall into the secondary active transporter group.

ABC Transporters are responsible for the transport of a large number and wide variety of substrates. They were initially identified as a result of their role in drug resistance when intestinal efflux transporters were recognised as limiting drug absorption (Gottesman *et al* 2002 and Huang *et al* 2007). The sub classes of ABC transporters are arranged into four groups according to their predicted two-dimensional structure in which the number of transmembrane regions and the extent of N terminus glycosylation varies (Juliano *et al* 1976).

The first group have two symmetrical sub units constructed of 6 transmembrane segments and 2 nucleoside-binding domains (NBD) which extend in to the cytoplasm and it is to the NBD's that ATP binds and is hydrolysed. The first extracellular loop of the protein is highly glycosylated. The second group is identical in structure to that of the first with the only difference being that the glycosylation is present on the fourth extracellular loop. The third group exists with an additional transmembrane segment and there is additional glycosylation on this region. The final group is the most recently discovered and contains breast cancer resistance protein (BCRP), this protein exhibits

only one 6 transmembrane segment and glycosylation on the last extracellular loop. Having only one sub unit it is referred to as a half-transporter and is believed to be involved more in the stabilisation of the cell membrane than in an actual transport role (Mohammed *et al 2011*).

The SLC transporters are another superfamily of transporters found in the intestinal membrane. Somewhere in the region of 300 genes for SLC transporters have been cloned and these have been clustered in to 43 sub families. The SLC transporters mediate the uptake of a variety of hydrophilic substrates including sugars, organic cations, anions, phosphates, metals, monocarboxylic acids, water soluble vitamins and drugs with structures which resemble natural substrates (Huang *et al* 2006).

ABC and SLC transporters are involved in the absorption of many of the drugs listed in the EMA/480197/2010 and the NHS specials list for November 2011 tariff including the drugs intended for reformulation - lisinopril, ramipril and spironolactone (Table 8 and Table 9).

Table 8 - Drugs listed in the EMA Revised Priority List for Studies into off-patent paediatric medicinal products and the transporters with which they interact (Transporter information collected from sources listed in the bibliography).

EMEA Revised Priority List for Studies into off-patent paediatric medicinal products	
Drug	**Transporters**
Amiodarone	ABCB1 (Inhibitor, Inducer)
Dobutamine	None
Milrinone	None
Propanolol	SLC22A2 (Inhibitor), ABCB1 (Substrate, Inhibitor)
Fluoxetine	ABCB1 (Inhibitor)
Androstanolone gel	ABCB1 (Substrate(Skin))
Cholestyramine	None
Glibenclamide	ABCC3 (Inhibitor), ABCB11 (Inhibitor), ABCB1 (Inhibitor), ABCC1 (Inhibitor), SLC15A1 (Inhibitor), SLCO1A2 (Inhibitor), SLC15A2 (Inhibitor), hROAT1 (Inhibitor), ABCC2 (Inhibitor), ABCG2 (Inhibitor), SLC22A7 (Inhibitor), SLCO2B1 (Substrate).
Hydorcortisone	ABCB1 (Substrate, Inhibitor), ABCG2 (Inhibitor), SLCO1A2 (Inhibitor).
Metformin	SLC22A2 (Substrate, Inhibitor), SLC22A1 (Substrate, Inhibitor), SLC47A1 (Inhibitor)

Carbimazole	None
Bisacodyl	Not Absorbed
Macrogol	Not Absorbed
Unfractionated Heparin	None
Azathioprine	None
Etopophos	ABCC3 (Substrate, Inhibitor), ABCC6 (Substrate Inhibitor), ABCB1 (Substrate, Inhibitor), ABCC1 (Substrate, Inhibitor), ABCC10 (Substrate, Inhibitor), ABCC2 (Substrate, Inhibitor), ABCG2 (Substrate)
Fludarabine	SLC29A1 (Substrate)
Melphalan	SLC22A3 (Inhibitor)
Methotrexate	ABCC3 (Substrate, Inhibitor), ABCC4 (Substrate, Inhibitor), ABCC1 (Substrate, Inhibitor), hROAT1 (Substrate, Inhibitor), ABCC10 (Inhibitor), SLC22A8 (Substrate, Inhibitor), ABCC2 (Substrate, Inhibitor), ABCB1 (Substrate), SLCO1A2 (Substrate), SLC16A1 (Substrate), ABCC11 (Substrate), SLCO1B3 (Substrate), SLC22A11 (Substrate), SLCO1C1 (Substrate), SLCO3A1 (Substrate), ABCG2 (Substrate), SLC22A7 (Substrate), SLCO1B1 (Substrate), SLC46A1 (Substrate, Inhibitor).
Mycophenolate Mofetil	None
Amphotericin B	None
Clindamycin	None
Ganciclovir	SLC22A1 (Inhibitor), hROAT1 (Inhibitor), SLC22A8 (Inhibitor), SLC22A7 (Inhibitor)
Itraconazole	ABCB1 (Inhibitor)
Clonidine	SLC22A1 (Inhibitor), SLC22A3 (Inhibitor), SLC22A5 (Inhibitor), SLC22A4 (Inhibitor), ABCB1 (Substrate)
Diclofenac	ABCC4 (Inhibitor), ABCB1 (Inhibitor), ABCC1 (Inhibitor), hROAT1 (Inhibitor), SLC22A8 (Inhibitor), SLCO1C1 (Inhibitor), SLC22A11 (Inhibitor).
Propofol	None
Spironolactone	ABCC2 (Inducer), ABCB1 (inhibitor), SLCO1A2 (Inhibitor).
Amiloride	SLC22A2 (Inhibitor), SLC22A1 (Inhibitor), SLC22A4 (Inhibitor)
Ciclosporin	ABCB1 (Substrate, Inhibitor, Inducer), ABCC3 (Inhibitor), ABCB11 (Inhibitor), ABCC1 (Inhibitor), SLCO1A2 (Inhibitor), SLC10A2 (Inhibitor), SLC10A1 (Inhibitor), hROAT1 (Inhibitor), ABCC10 (Inhibitor), ABCC2 (Inhibitor), ABCG2 (Inhibitor), SLCO1B1 (Inhibitor)
Deflazacort	None
Captopril	ABCB1 (Inhibitor), SLC15A1 (Inhibitor), hROAT1 (Inhibitor)
Enalapril	ABCB1 (Inhibitor), SLC15A1 (Substrate, Inhibitor), hROAT1 (Inhibitor), SLC22A8 (Inhibitor), SLC22A7 (Inhibitor), SLCO1A2 (Substrate).
Lisinopril	ABCB1 (Inhibitor), SLC15A1 (Substrate).

Ramipril	SLC15A1 (Substrate), SLC15A2 (Substrate)
Allopurinol	SLC22A8 (Substrate), SLC22A7 (Substrate).
Ethosuximide	None
Lidocaine	SCL22A5 (Inhibitor), ABCB1 (Inhibitor)
Topiramate	None
Cyclophosphamide	ABCB1 (Inhibitor)
Daunorubicin	ABCB1 (Substrate, Inhibitor, Inducer), ABCC1 (Substrate, Inhibitor), ABCC2 (Inhibitor), SLC22A2 (Substrate), ABCC6 (Substrate), ABCB11 (Substrate), ABCC10 (Substrate), ABCG2 (Substrate).
Topotecan	ABCG2 (Substrate, Inhibitor), ABCB1 (Substrate).
Granisetron	None
Tropisetron	None
Ondansetron	None
Carbamazepine	ABCB1 (Substrate, Inhibitor, Inducer)
Gabapentin	None
Ibuprofen	SLCO2B1 (Substrate), ABCB1 (Substrate), ABCC4 (Inhibitor), ABCC1 (Inhibitor), SLCO1A2 (Inhibitor), hROAT1 (Inhibitor), SLC22A8 (Inhibitor), SLC22A11 (Inhibitor)
Azithromycin	ABCB1 (Inhibitor)
Dornase Alfa	None

Table 9 - Drugs listed in the NHS Specials list for November 2011 Drug Tariff and the transporters with which they interact (Transporter information collected from sources listed in the bibliography).

NHS Specials list for November 2011 Drug Tariff	
Drug	**Transporters**
Amisulpride	None
Amitriptyline	ABCB1 (Substrate, Inhibitor)
Amlodipine	ABCB1 (Inhibitor)
Azathioprine	None
Bendroflumethiazide	None
Captopril	ABCB1 (Inhibitor), SLC15A1 (Inhibitor), hROAT1 (Inhibitor)
Chloral hydrate	None
Clobazam	None
Clonazepam	None
Co-Dydramol	None
Dipyridamole	ABCC4 (Inhibitor), ABCC5 (Inhibitor), ABCB1 (Inhibitor)
Ferrous sulphate	None
Gabapentin	None
Gliclazide	None
Glycopyrronium Bromide	None
Haloperidol	ABCB1 (Substrate, Inhibitor)
Lorazepam	None
Midazolam	ABCB1 (Substrate, Inhibitor, Inducer), SLC22A1 (Inhibitor)
Primidone	None
Ramipril	SLC15A1 (Substrate), SLC15A2 (Substrate)
Spironolactone	ABCC2 (Inducer), ABCB1 (inhibitor), SLCO1A2 (Inhibitor).
Tarolimus	ABCB1 (Substrate, Inhibitor, Inducer), ABCA5 (Substrate)
Topiramate	None
Zopiclone	None
Glyceryl Trinitrate	N/A
Hypromellose	N/A
Sodium Chloride	N/A

1.10.2 Microarrays

The development of DNA arrays in the early 1980's (Augenlicht *et al* 1982) was one of the most important advances in the field of genome profiling. Within 15 years of conception, by 1995 (Schena *et al* 1995) DNA arrays had been miniaturised giving the world the first DNA microarrays which were quickly utilised and in 1997 the first full eukaryotic genome microarray for *Saccharomyces cerevisiae* was reported (Lashkari *et al* 1997). In modern day genomics they find routine application in a broad

spectrum of disciplines including advancing areas of drug discovery and development through comparative assessment of normal and diseased state tissues, transcription and/or expression profiling, side effect profiling, pharmacogenomics and the identification of biomarkers (Russell *et al* 2013).

In the production of DNA microarray slides probes are immobilized onto a flat, solid support in an organized grid like fashion producing rows and columns. Each probe is designed to match and identify its corresponding molecule in an unambiguous fashion following hybridization (Karakach *et al* 2010). Single stranded (ssDNA) and double stranded (dsDNA) DNA oligomers as either short length (25 base pairs (bp)) oligonucleotide probes (Li *et al* 2009) or long (50-80bp) oligonucleotide probes (Zhao *et al* 2005 and Barrett *et al* 2003) can be used. cDNA can also be used in the production of microarray probes which are of longer and highly variable cDNA probes (Grover *et al* 2010). Although the microarray production procedure varies, the end point of slide scanning and fluorescence quantification prior to data analysis are common components for all microarrays (Figure 4).

In microarray experiments, mRNA isolated from tissue or cell samples is fluorescently labelled to produce samples for analysis referred to as targets. These targets are then hybridized to their corresponding probes on the microarray slide followed by slide scanning allowing for the simultaneous quantification of expression levels of thousands of transcripts from each sample facilitating analysis on genome wide level. It is this ability which makes microarray such a powerful resource in the high throughput assessment of gene expression. Each slide scan generates huge amounts of data and it is through the detailed statistical processing of the datasheets that trends in the gene expression levels can be revealed. In addition to the specific investigation being performed, the data gathered also contributes to the production of genomic databases and genetic maps such as the human genome project (Schena *et al* 1995)

and the Kyoto encyclopaedia of genes and genomes (KEGG) (Russell *et al* 2013, Kanehisa and Goto 2000).

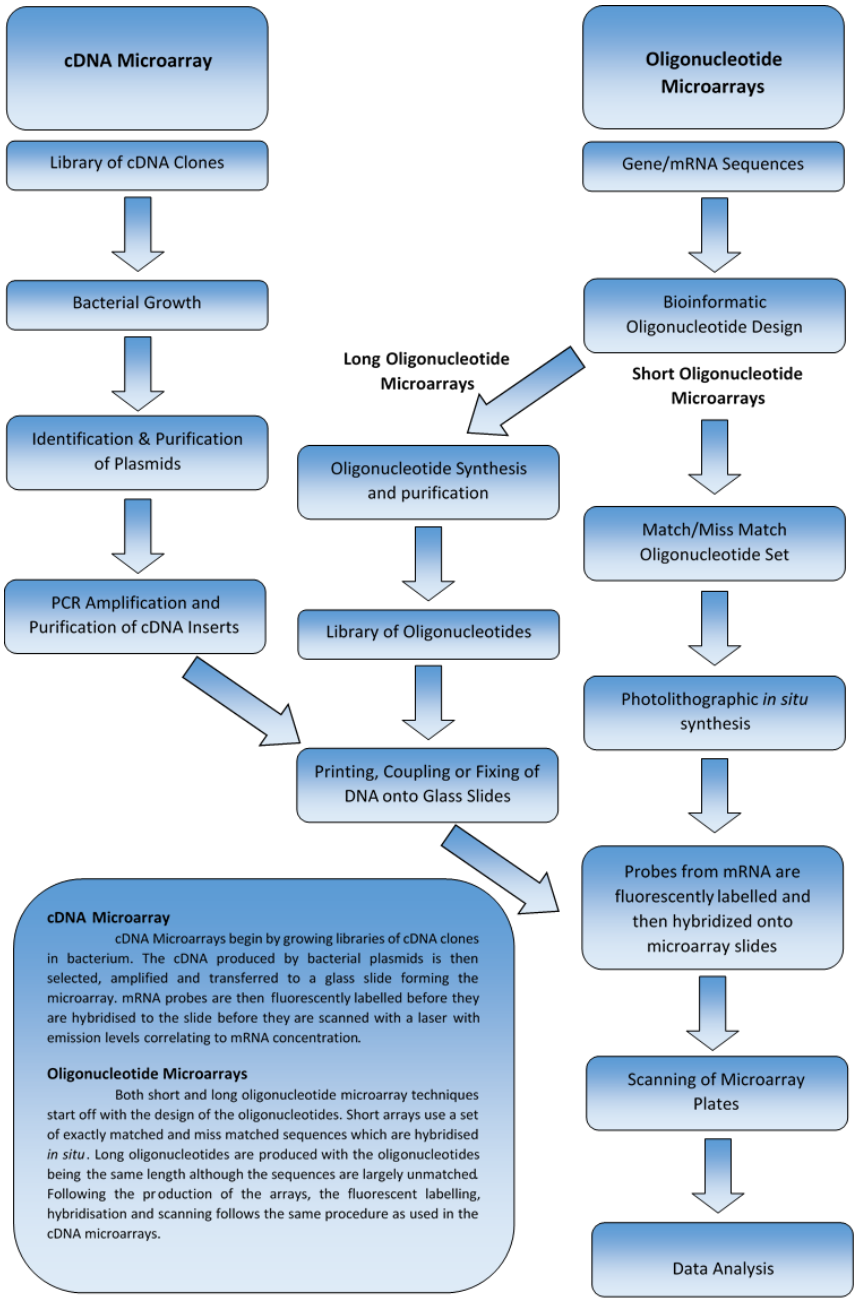


Figure 4 – Flow chart illustrating the different processes in the use of cDNA microarrays, long oligonucleotide microarrays and short oligonucleotide microarrays. cDNA microarrays begin by growing libraries of cDNA clones in bacterium, this cDNA is then transferred to glass slides to form the array. In contrast, oligonucleotide arrays start with bioinformatic oligonucleotide design to produce the array slides. Once microarray slides are produced, the fluorescent labelling, hybridisation and data analysis processes are the same for both types of array.

Various software programs such as TMEV have been developed perform hierarchical clustering (HCA), principal component analysis (PCA) and statistical analysis of microarray (SAM) to facilitate the comparison of gene expression leading to the identification of potentially important genes.

HCA clusters data from samples or investigations in order of closeness of the gene expression profiles. That is samples showing the largest difference to controls are clustered furthest from the controls and likewise samples showing the least difference are clustered closest. HCA allows for the visual identification of samples or genes which are providing similar degrees of expression in the experimental conditions (Heyer et al., 1999; Tefferi et al., 2002). The main limitation of this technique is the data set is not reduced in any way therefore there are still tens of thousands of data points for consideration and thus identifying specific correlations in the data is complex and time consuming.

Data reduction techniques such as PCA overcome the limitations of HCA by separating the data set into multiple principal components, PCA is multivariate data analysis (MVDA) tool, the likes of which have been used extensively in the past, it is only within the last decade or so however that they have found application in cellular biology analysis (Orr & Scherf, 2002; Michaud et al., 2003).

PCA is a tool used to simplify the data analysis procedure making large scale data easier to handle, and crucially without the loss of information. This is achieved by assessing principal components in a three dimensional space. The term principal component describes how we choose to describe our data in a more concise or convenient way, redefining variables by combining factors showing correlation (Orr & Scherf, 2002; van der Werf et al., 2005). Eigenvalue decomposition (EVD) is one of the most popular ways to define principal components. Principal components are required to be mathematically orthogonal to each other, this means that there is no linear correlation between components. This is useful as variables in raw data are not

independent and any variables which display correlation to one another result in redundancy. It is in this way PCA minimises variables without the loss of information. Principal components are numbered in order of the magnitude of variance meaning that component 1 has the largest degree of variance in the data followed by component 2 then 3 and so on and it is the separation along a component between data sets that illustrates the degree of variation between them. It is common that there are large numbers of components however so to simplify the data analysis further components which contribute to low levels of variance can be discarded without impacting the findings.

SAM algorithms are also a useful tool in the analysis of genomic data as they are capable of identifying individual genes as significant based on the differential expression between sample sets. The statistical analysis can be tuned to the data set being investigated to maximise the data mining process. An additional benefit of SAM which is not provided by other techniques is the identification of the false discovery rate (FDR), this reveals what percentage of the significant genes have been identified by chance. The incorporation of SAM into microarray data analysis means that gene lists can be produced for significantly different genes and used in pathway analysis investigations with the confidence that the findings are statistically relevant.

KEGG is one of the many online resources in existence alongside databases the human genome project and METLIN amongst others which are utilised across a range of genomic, proteomic and metabolomic applications where it is used to build genome wide models of pathways and processes. The key to its usefulness is in the integration of information recorded regarding molecular pathways, genome projects and compound databases which is complimented by the logging of information referring to cellular pathways, both metabolic and signalling and also information on human disease and drug development in a largely open access manner (Russell *et al* 2013).

1.11 REFORMULATION

Reformulation of existing medicinal products is performed for a variety of reasons. Pharmaceutical companies 'tweak' their own products so as to allow for re-patenting and in doing so maintain their market exclusivity in that area, to expanding the scope of an existing drug to better target other patient segments (Yoshitani and Cooper 2007). Also there are many factors of existing formulations which can be improved in an effort to maximise their potential for targeting specific patient groups. Where paediatric reformulations are concerned some of the most common areas for improvement are; increasing the understanding of a drug's pharmacokinetics, improving a drug's safety and developing an age appropriate formulation.

Reformulation of off-patent medications in an attempt to improve the availability of medication to children is a much more attractive proposition when compared to developing a completely NCE as using an existing API which is recognised by the FDA means that all of data collection via clinical trials associated with the production of a NCE will already have to have been carried out in order for the FDA advisory committee to approve the product (Bhattaram *et al* 2005). This will remove much of the cost associated with the formulation of a new drug. Reformulation is generally categorised into three sub categories including; the reformulation of a molecular entity, new deliveries and new indications (Yoshitani and Cooper 2007).

The reformulation of molecular entities involves the modification of an existing API just enough so as that the changed molecule can be patented however not enough to alter how it behaves in the body. In this way the FDA views the modified API as the same drug under the guidelines regarding bioequivalence. In the area of molecular entity reformulation there are three further subsections which involve metabolites, polymorphs and chiral switching.

Metabolites are the molecules produced following a chemical reaction in the body, the modification implemented with regards to metabolites is the creation of prodrugs which enter the body in an inactive form and following metabolism the active form of the drug is produced (Yoshitani and Cooper 2007).

Polymorphs which exist with different crystalline structures, waters of hydration, solvents and amorphous forms come into play where reformulation is concerned as the FDA recognise a modified API so long as there is no change in its physical form and that it is also bioequivalent to the original drug (Yoshitani and Cooper 2007). It is often difficult to produce reformulations which display characteristics which are bioequivalent to the original product as changes in the polymorphism often bring about changes in the solubility of the drug which in turn can result in changes in the drugs dissolution properties (Wiliams *et al* 2008).

Chiral switching is implemented in reformulation as the ratio of each chiral version can be changed resulting in a more pure S- enantiomer or a more pure R- enantiomer and as each chiral version bring about very different responses in the body (Fleming and Ma 2002). A more pure mix would produce a more effective response in favour of one or other enantiomer depending on which was present in the highest concentration. In this way the efficacy of a drug is improved without altering the molecule itself (Yoshitani and Cooper 2007).

Reformulation to produce new delivery methods is fairly self-explanatory with factors such as the alteration of the dosage form say from an oral liquid such as a suspension to an oral solid such as a tablet or a capsule. Alternatively reformulating to change the route of entry into the body for example changing from a nasal spray to an oral tablet or a capsule. In both cases it is essential that the behaviour of the drug once inside the body is identical to the original formulation (Yoshitani and Cooper 2007).

New indications involves the production of an existing drug however using the existing medication to treat a disease or condition not covered in an existing drug patent (Ashburn and Thor 2004). If this is achieved then there are no changes to the existing API and it is just a new method of use for an existing medication. This is a relatively uncommon occurrence as drug companies go to great lengths to protect their products in order to maintain market exclusivity (Yoshitani and Cooper 2007).

1.12 Aims and Objectives of Thesis

As discussed above it is recognized globally that the drug products currently available for treating the paediatric population, which suitably meet the needs of the patient segment are lacking in their coverage. In order to improve on this situation bodies such as the FDA and the EMA have devised legislation to guide the research and development of paediatric medicines through priority listings. By selecting and reformulating off patent medications as oral liquids in cases where oral liquids are not already available the paediatric population can be more appropriately targeted.

Additionally, previous research has shown how the gene expression of intestinal membrane transporters changes in response to drug transport. It is therefore considered worthwhile investigating if there is a correlation between the expressional changes seen and the extent of drug absorption. The development of an *in vitro – in vivo* correlation would be of benefit to a number of fields, potentially aiding in the areas of drug discovery and development as well as potentially reducing the need for animal based drug absorption studies.

The objectives of the work can be categorized into three main areas of research;

1. Reformulation of the angiotensin converting enzyme (ACE) inhibitors lisinopril and ramipril and potassium-sparing diuretic spironolactone to produce oral liquid formulations better targeting the paediatric patient segment.

2. Using the model formulations produced in the first stage of the project, an *in vitro – in vivo* correlation between changes in gene expression following *in vitro* transport studies and *in vivo* absorption will be investigated using microarray technology.
3. During the production of any oral formulation targeting any patient segment the palatability of the formulation is an important consideration. It became apparent during the formulation development stages of the project that the available analytical techniques for taste assessment are inadequate. As such initial stages into the development of an *in vitro* cell based taste assessment method merited investigation.

2 Reformulation of Lisinopril to Produce an Age Appropriate Oral Liquid Dosage Form Targeting the Paediatric Patient Segment

2.1 Chapter Aims and Objectives

- Develop a HPLC method for the detection of Lisinopril and validate according to ICH guidelines.
- Formulate Lisinopril as an oral liquid dosage form with a particular focus on effectively targeting the paediatric patient segment.
- Characterise formulations including stability testing in long term and accelerated conditions as specified by ICH guidelines.

2.2 Introduction

According to the BNF for children September 2013 edition, the use of Lisinopril is limited to treatment, under specialist supervision, of hypertension and proteinuria in nephritis, diabetic nephropathy and heart failure. The recommended dosage varies significantly depending upon the desired application. For the treatment of hypertension and proteinuria in nephritis in children aged 6-12 years the recommended initial dose is 70µg/kg (Max 5mg) being administered once daily increasing in intervals of 1-2 weeks to 600µg/kg (Max 40mg) given once daily. In children aged 12-18 years the recommended dose is initially 5mg given once daily with a usual maintenance dose of 10-20mg given once daily with a maximum of 80mg given once daily. In the case of diabetic nephropathy, the recommendation is only for the treatment of children aged 12-18 years and again the dosage is initially 5mg given once daily with a usual maintenance dose of 10-20mg given once daily with a maximum of 80mg given once daily. For the treatment of heart failure the recommendation is only for children aged 12-18 and the dosage is initially 2.5mg given once daily which is increased by no greater than 10mg at a time and at intervals of at least two weeks up to a maximum of 35mg given once daily if tolerated by the patient (Paediatric Formulary Committee 2012).

Lisinopril (Table 10), along with Captopril is one of the few ACE inhibitors which in administered in its active form and not as a pro-drug. Lisinopril is not currently available as a liquid dosage form (Table 11) and the existing formulations are not currently licensed for use in pediatrics.

Table 10 - Lisinopril Monograph listing important physiochemical properties which should be considered before formulation.

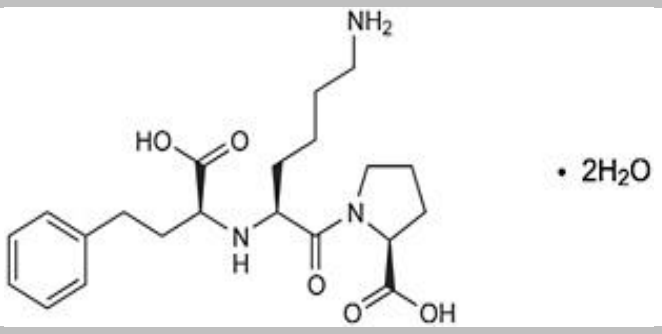
Lisinopril Monograph	
Chemical Name	(2S)-1-[(2S)-6-Amino-2-[[(1S)-1-carboxy-3-phenylpropyl]amino]hexanoyl]pyrrolidine-2-carboxylic acid dehydrate
Other Names	Prinivil, Zestril
Molecular Formula	$C_{21}H_{31}N_3O_5.2H_2O$
Molecular Weight	441.5
CAS Number	83915-83-7
Lisinopril Structure	
Appearance	White Crystalline Powder
Solubility	Lisinopril is soluble in water (100mg/ml), soluble in methanol (14.3mg/ml). Lisinopril is practically insoluble in alcohol, acetone, chloroform and ether.
Sensitivities	Lisinopril is sensitive to Oxidation and Alkaline conditions.

Table 11 - Available Lisinopril dosage forms detailing product name, drug dosage and dosage form.

Available Lisinopril Dosage Forms	
Prinivil 90 20 mg tablet Bottle	Prinzide 10-12.5 mg tablet
Prinzide 30 20-12.5 mg tablet Bottle	Zestril 20 mg tablet
Prinivil 30 2.5 mg tablet Bottle	Zestril 10 mg tablet
Lisinopril 100% powder	Zestoretic 20-25 tablet
Zestril 40 mg tablet	Zestril 5 mg tablet
Zestril 30 mg tablet	Zestoretic 20-12.5 tablet
Prinivil 40 mg tablet	Lisinopril 20 mg tablet
Zestoretic 20-25 mg tablet	Prinivil 20 mg tablet
Zestoretic 20-12.5 mg tablet	Zestril 2.5 mg tablet
Prinzide 20-25 mg tablet	Lisinopril 10 mg tablet
Zestoretic 10-12.5 mg tablet	Prinivil 10 mg tablet
Zestoretic 10-12.5 tablet	Lisinopril 5 mg tablet
Lisinopril 30 mg tablet	Prinivil 5 mg tablet
Lisinopril 40 mg tablet	Apo-Lisinopril 20 mg Tablet
Prinzide 20-12.5 mg tablet	Mylan-Lisinopril 20 mg Tablet

Given the recommendation for the production of an age appropriate formulation, coupled with the need for dose flexibility and the lack of an oral liquid formulation, the production of an oral liquid formulation created with a focus on paediatric use would be highly advantageous.

Detailed herein is the formulation of, and characterization of an oral Lisinopril solution, including details of the production of a suitable HPLC method, excipient selection and stability assessment of the formulations in accordance with ICH guidelines.

2.3 MATERIALS AND METHODS

2.3.1 HPLC Method Development for the Detection of Lisinopril

The Lisinopril HPLC method was developed using a Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler. The stationary phase is a Phenomenex Gemini 5µ C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size). The detection wavelength was set at 228nm. The injection volume is 20µl with a run time of 10 minutes. Preparation of Calibration Standards involves the production of six standards produced via serial dilution in mobile phase. Standards at 2.0, 1.0, 0.5, 0.25, 0.125 and 0.0625mg/ml and samples are prepared for analysis by dilution so as to fall within the calibration range and are diluted with Lisinopril mobile phase. Method validation was carried out following ICH Guidelines (Q2(R1)).

2.3.2 Formulation Development

The Lisinopril for the formulation was purchased from Discovery fine chemicals while citric acid, Propyl Paraben, Butyl Paraben and Xylitol were all supplied by Sigma. The flavour concentrates used in the formulation development were free samples provided by S. Black's (Azelis). Formulations were produced using volumetric glassware. Ultrapure water was first added into which excipients were dissolved with the aid of a magnetic stir bar before it was removed and the solutions made up to volume. Upon production formulations were transferred to amber glass bottles.

2.3.3 Stability Analysis

The conditions for storage are set out in the ICH Harmonisation Guidelines (Q1A(R2)) 2003 (Table 12) which states that 'a drug substance should be evaluated under storage conditions that test its thermal stability, and if applicable its sensitivity to moisture'. The conditions are listed below;

Table 12 - Storage conditions for stability testing as outlined in the ICH guidelines (ICH Guidelines Q1A(R2) 2003). (R.H = Relative Humidity)

Study	Storage Conditions	Minimum Time Period
Long Term	25°C ± 2°C at 60% R.H ± 5% or 30°C ± 2°C at 65% R.H ± 5%	12 Months
Accelerated	40°C ± 2°C at 75% R.H ± 5%	6 Months

Samples were stored in Firlabo SP-BVEHF stability cabinets and analysed on a monthly basis, quantitatively for drug recovery via HPLC and pH and qualitatively for colour and odor.

2.4 Results and Discussion

The content of this chapter comprises of two key areas of investigation, firstly there is the development stages and secondly there is the assessment stages.

2.4.1 HPLC Method Development for the Detection of Lisinopril

The development of a HPLC method for the detection of Lisinopril was initially carried out following the method described by Nahata and Morosco (2004). This involved the use of a reverse phase Phenomenex Gemini C_{18} column (150 × 4.6 mm) 5µm particle size at 27°C, using a mobile phase of 0.8% diethylamine in water and acetonitrile (43:57, v/v). The flow rate was 0.4 mL/min. UV detection was carried out at 220nm. The Lisinopril stock solution was prepared in methanol at 2mg/ml and further calibration standards were produced from this via serial dilution. Six standards were produced in total and these were ran using the above conditions.

The trace (Figure 5) produced by following the method described by Nahata and Morosco (2004) was very noisy and a large solvent front was present close to the Lisinopril peak with the Lisinopril peak appearing ahead of the solvent front.

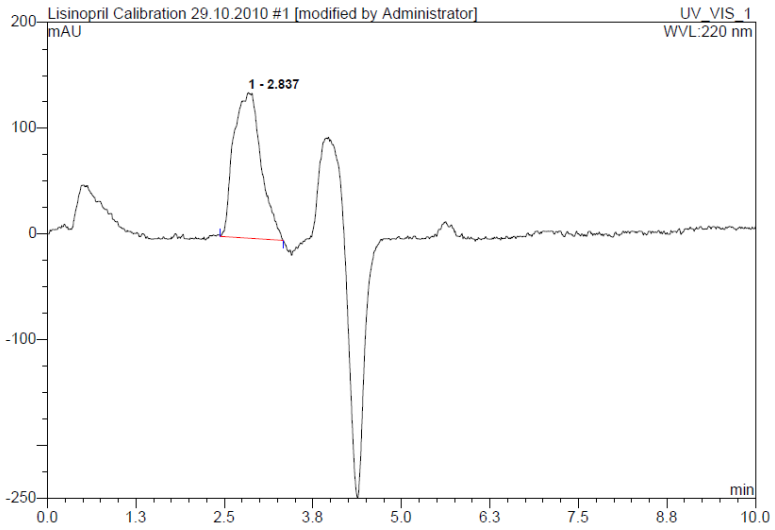


Figure 5 - HPLC trace for 2mg/ml Lisinopril calibration standard ran using the method described by Nahata and Morosco (2004).

In order to reduce the noise on the trace the stock solution and subsequent serial dilutions were produced using mobile phase in place of the methanol. The resulting trace (Figure 6) displayed a much smoother readout as desired however the large solvent front was still present with the Lisinopril peak appearing ahead of the solvent front.

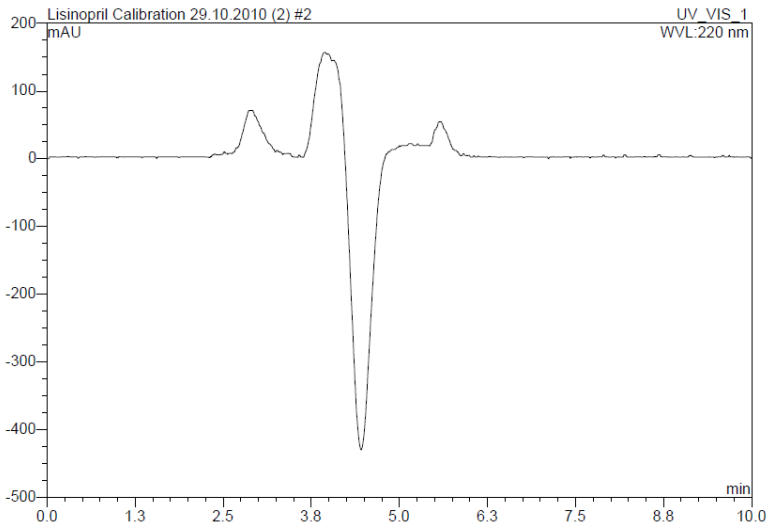


Figure 6 -HPLC trace for 2mg/ml Lisinopril calibration standard ran following modification to the method described by Nahata and Morosco (2004). Calibration standards produced in mobile phase rather than in methanol.

To correct this problem the mobile phase used was adjusted from 0.8% diethylamine in water and acetonitrile (43:57, v/v) to 0.8% diethylamine in water and acetonitrile (20:80, v/v). This was designed to increase the polarity of the mobile phase which would lead to an increase in the retention time of the Lisinopril. Running the Lisinopril standards using the modified mobile phase resolved the problem of the solvent front and improved the detection of the Lisinopril (Figure 7).

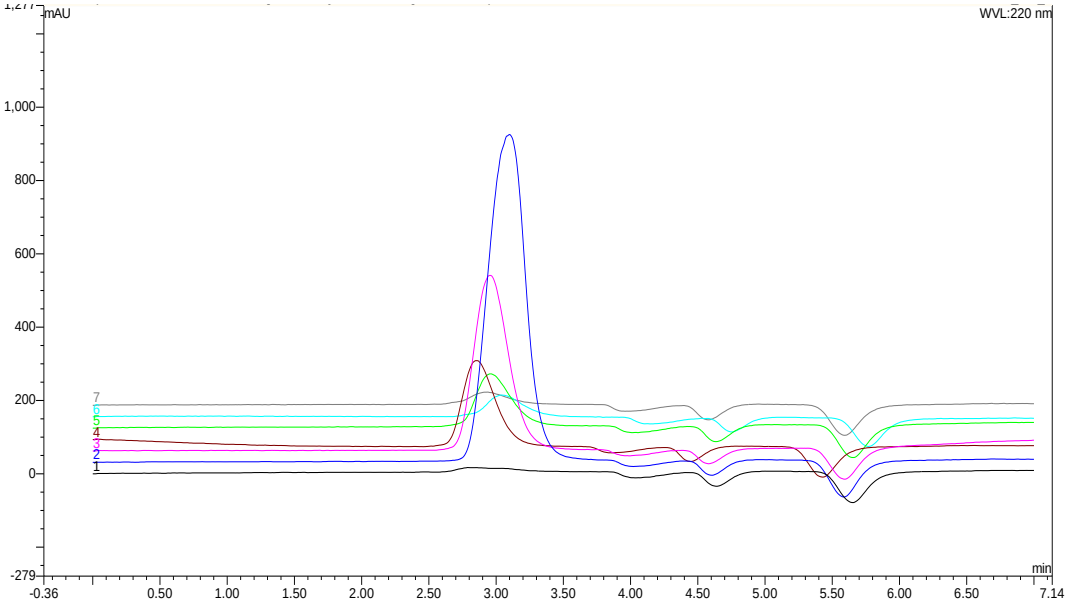


Figure 7 - HPLC trace for Lisinopril calibration standards ran following modification to the method described by Nahata and Morosco (2004). Mobile phase altered from 0.8% diethylamine in water and acetonitrile (43:57, v/v) to 0.8% diethylamine in water and acetonitrile (20:80, v/v). Calibration standards produced in mobile phase rather than in methanol.

Although the trace displayed vast improvement there still remained some variation in the retention time and the peaks produced were larger than desired. In order to maximize the detection of Lisinopril and reduce the variation in retention time a Lisinopril solution produced using the modified mobile phase was analysed using UV spectroscopy to identify λ_{max}. This was found to be 228nm. The injection volume was reduced to decrease the peak size and following adjustment of the detection wavelength the calibration standards were re-ran and the resulting retention times were found to be more consistent (Figure 8)

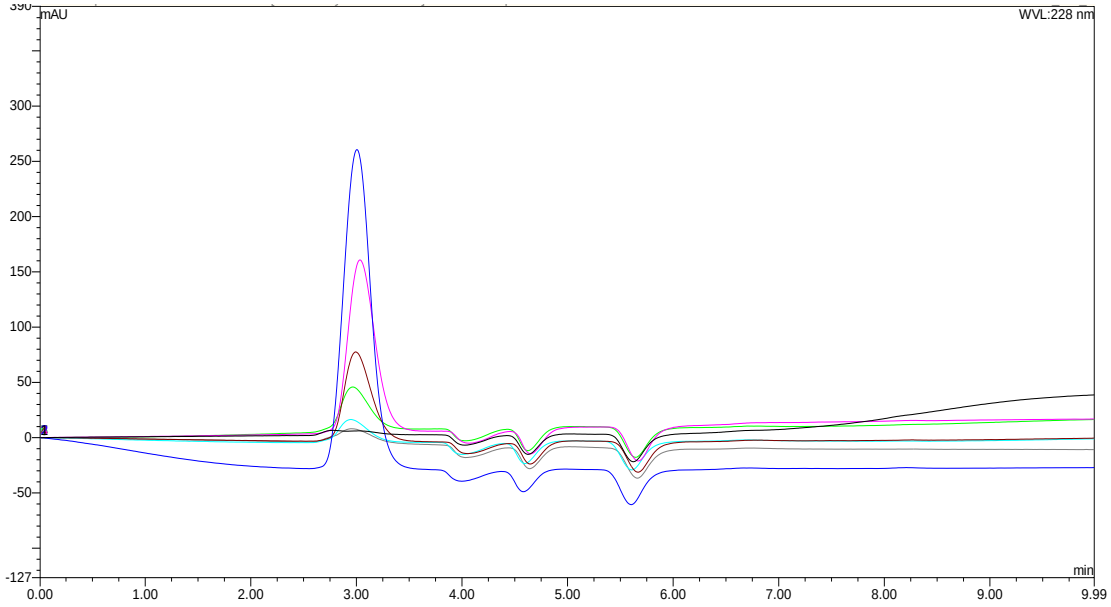


Figure 8 - HPLC trace for calibration standards ran following modification to the method described by Nahata and Morosco (2004). Mobile phase altered from 0.8% diethylamine in water and acetonitrile (43:57, v/v) to 0.8% diethylamine in water and acetonitrile (20:80, v/v). Calibration standards produced in mobile phase rather than in methanol. Reduction of the injection volume to 20µl and adjustment of the detection wavelength to 228nm.

With the detection optimized the method was investigated for its ability to resolve Lisinopril from a preliminary formulation. The first stage in this was to produce a calibration curve for the method using the calibration standards produced from the stock solution. The calibration curve (Figure 9) displayed good linearity with an R^2 value of 0.9979.

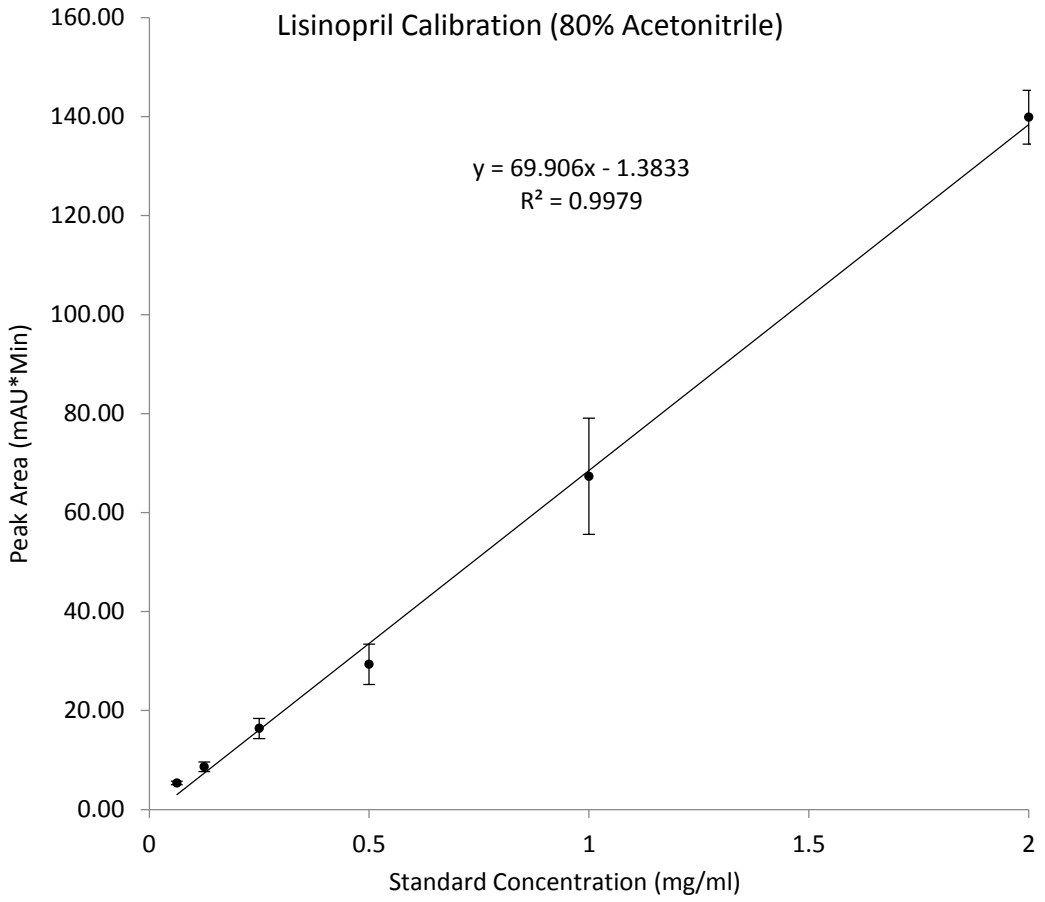
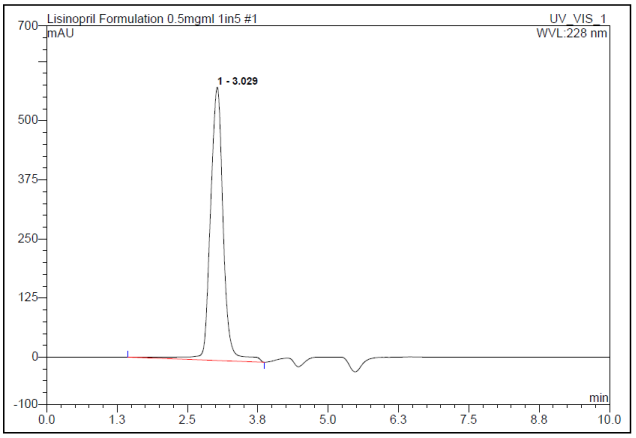


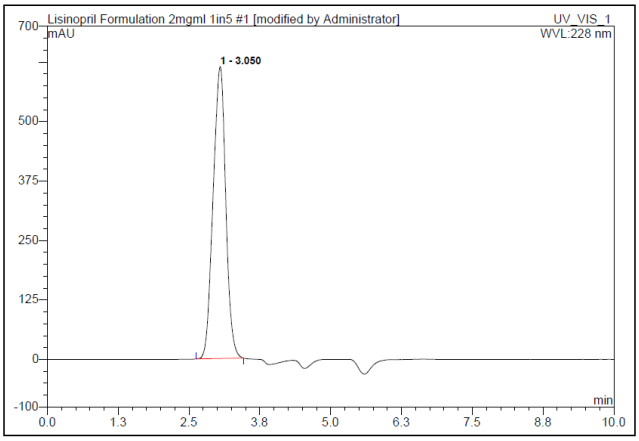
Figure 9 - HPLC calibration curve for Lisinopril following modification to the method described by Nahata and Morosco (2004). Mobile phase altered from 0.8% diethylamine in water and acetonitrile (43:57, v/v) to 0.8% diethylamine in water and acetonitrile (20:80, v/v). Calibration standards produced in mobile phase rather than in methanol. Reduction of the injection volume to 20µl and adjustment of the detection wavelength to 228nm.

Following the production of a suitable calibration curve, two preliminary formulations were produced and contained 2mg/ml and 0.5mg/ml Lisinopril, 1mg/ml Ascorbic acid and 1mg/ml Benzoic acid as a common antioxidant and preservative. These formulations were then ran following the modified method with the sample formulation being diluted 1:5 in mobile phase (Figure 10 and Figure 11).



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3.03	n.a.	577.972	153.540	100.00	n.a.	BMB
Total:			577.972	153.540	100.00	0.000	

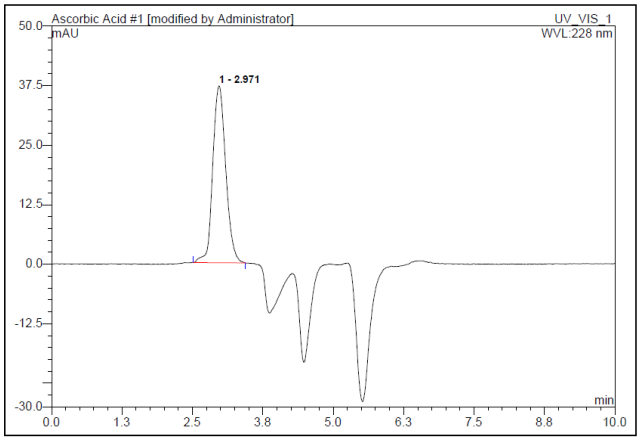
Figure 10 - HPLC trace for a preliminary Lisinopril formulation containing 0.5mg/ml Lisinopril, 1mg/ml Ascorbic acid and 1mg/ml Benzoic acid.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3.05	n.a.	613.441	158.679	100.00	n.a.	BMB*
Total:			613.441	158.679	100.00	0.000	

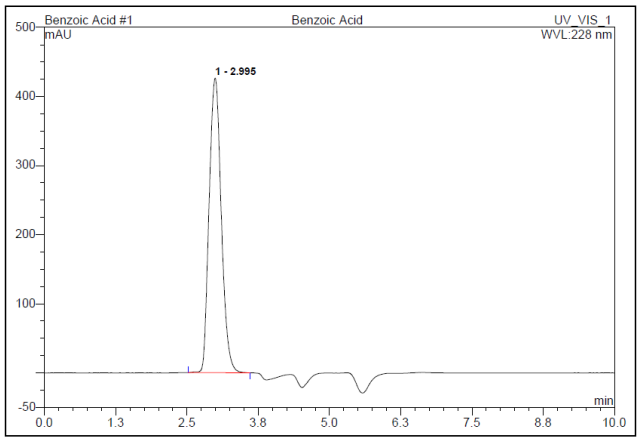
Figure 11 - HPLC trace for a preliminary Lisinopril formulation containing 2.0mg/ml Lisinopril, 1mg/ml Ascorbic acid and 1mg/ml Benzoic acid.

Although the HPLC method produced a good calibration curve the Lisinopril recovery from the preliminary formulations was found to be >500% of the expected. This indicates that the excipients in the formulation are interfering with the Lisinopril peak. To confirm this an ascorbic acid (Figure 12) and a benzoic acid (Figure 13) solution were both ran using the Lisinopril HPLC method.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.97	n.a.	37.179	9.922	100.00	n.a.	BMB*
Total:			37.179	9.922	100.00	0.000	

Figure 12 - HPLC trace for the detection of ascorbic acid using the Lisinopril HPLC method.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.99	n.a.	426.407	105.390	100.00	n.a.	BMB
Total:			426.407	105.390	100.00	0.000	

Figure 13 - HPLC trace for the detection of benzoic acid using the Lisinopril HPLC method

As the two traces confirm the retention time of ascorbic acid and benzoic acid (2.9 minutes) are at the same time as the lisinopril retention time, the only way to avoid this problem was to again modify the mobile phase composition. To do this the ratio of 0.8% diethylamine to acetonitrile was reversed and increased from (20:80) to (90:10).

Although it was expected that the solvent front previously present would re-appear, increasing the polarity of the mobile phase sufficiently may separate the sample peak suitably from the solvent front. Repeating the HPLC for the preliminary formulation with the modified mobile phase produced the following trace (Figure 14).

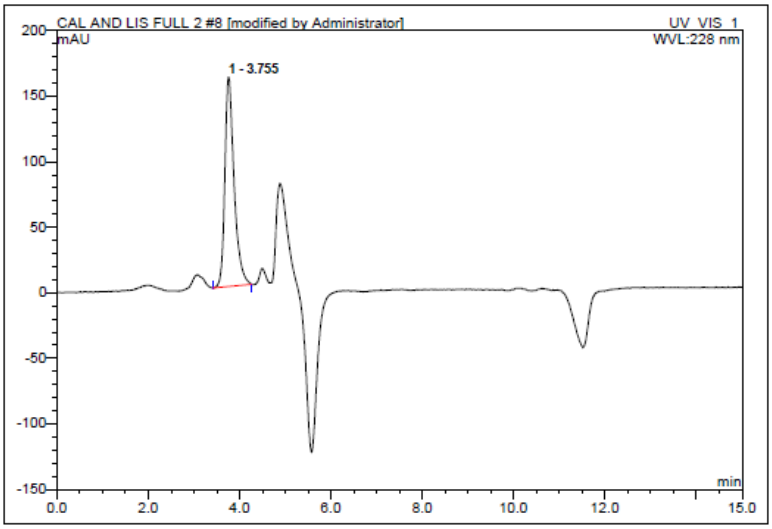


Figure 14 - HPLC trace for preliminary formulation using modified mobile phase of 0.8% diethylamine and acetonitrile (90:10).

This produced a clear peak and following the production of a calibration curve (Figure 15) the recovery was found to be 102.71%. With a suitable method developed the next stage was the validation of the method. This was performed as detailed in the ICH Guidelines Q2(R1).

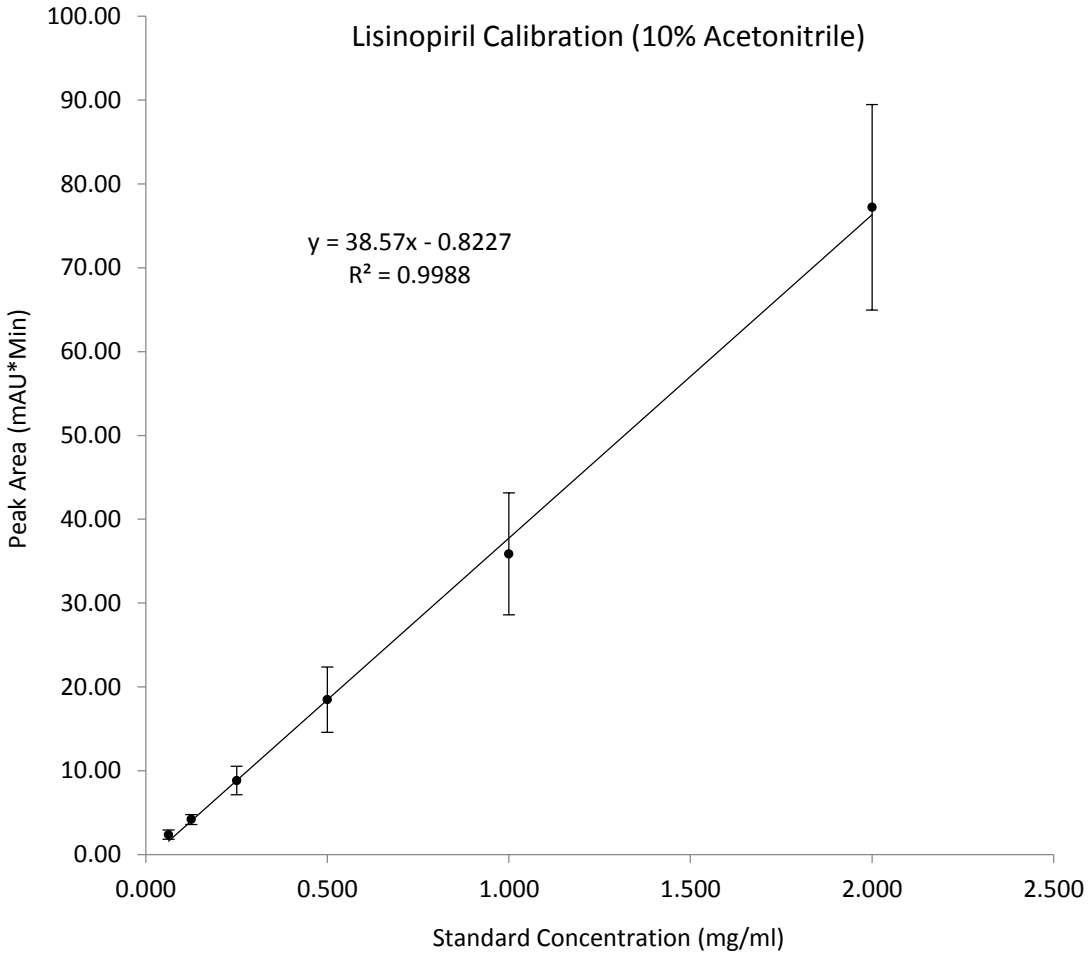


Figure 15 - HPLC calibration curve for Lisinopril using modified mobile phase of 0.8% diethylamine and acetonitrile (90:10). Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

To provide indication of the method's selectivity the Mobile Phase (0.8% diethylamine and acetonitrile (90:10)) was used as a blank (Figure 16) in order to show that there are no placebo peaks eluting at the same retention time as Lisinopril (Figure 17).

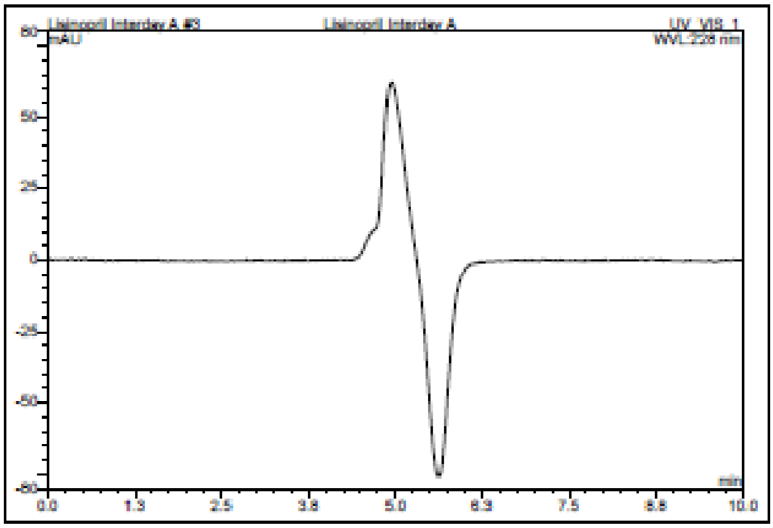


Figure 16 - Mobile Phase Blank

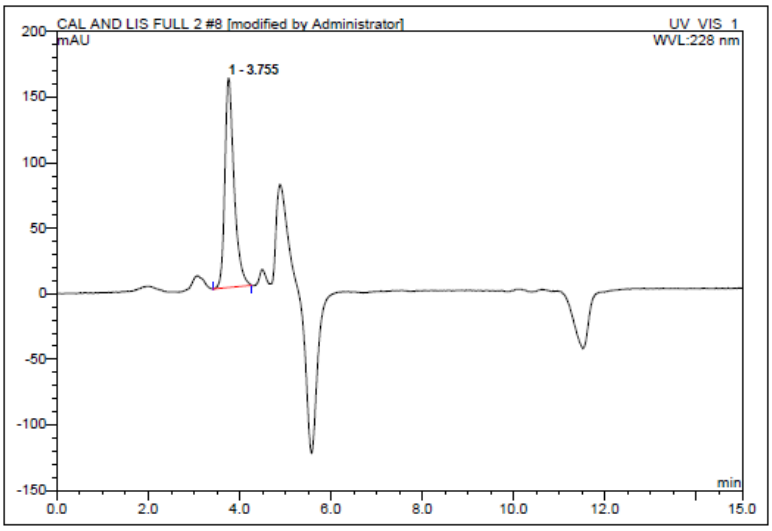


Figure 17 - Lisinopril Spiked Mobile Phase (2mg/ml)

The Accuracy of the method was determined by spiking mobile phase solution with known amounts of Lisinopril (2mg/ml, 1mg/ml and 0.5mg/ml). Three different concentrations within the calibration range were ran in triplicate. The recoveries ranged from 97.14% to 106.86% with a relative standard deviation (RSD) of 4.13%. Response for the detector was determined to be linear over a range of 2mg/ml to 65.5µg/ml. Correlation coefficients and slopes were obtained by plotting standard concentration (mg/ml) against peak area (mAU*min) (Figure 18).

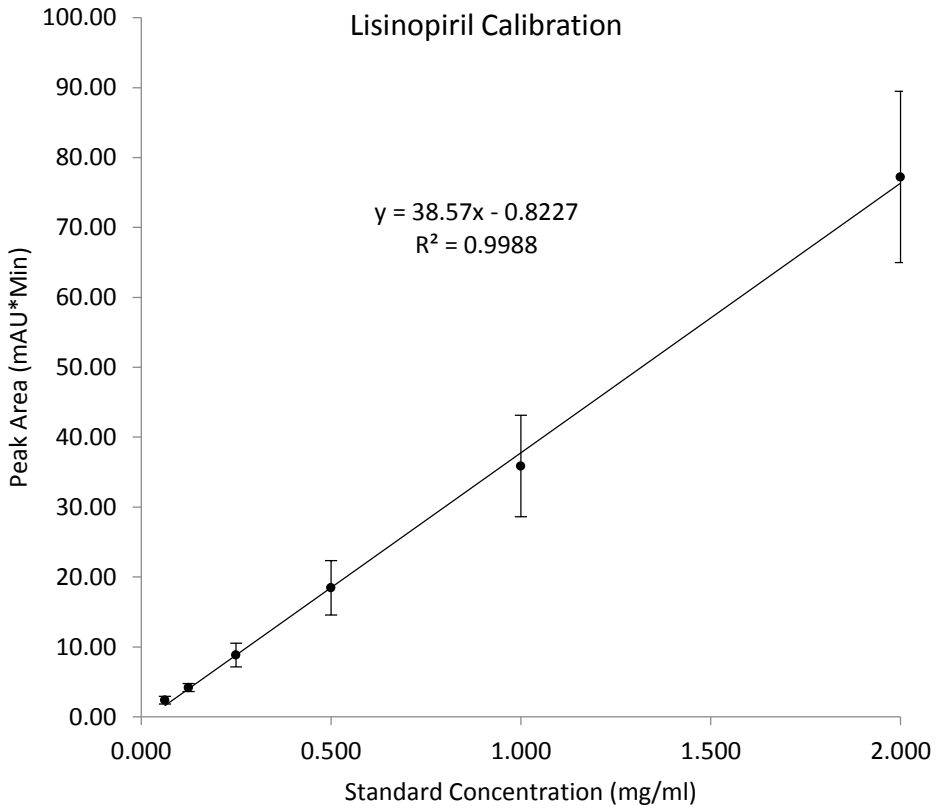


Figure 18 - Lisinopril Calibration chart illustrating good linearity with an R^2 value of 0.9988 and an equation (y = 38.57x – 0.8227) to be used in the calculation of sample concentrations. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The method precision is determined via 3 intraday HPLC runs, running six standards each in triplicate (Figure 19, Figure 20 and Figure 21). The intermediate precision for the method is determined via 3 interday HPLC runs, running six standards each in triplicate (Figure 22, Figure 23 and Figure 24).

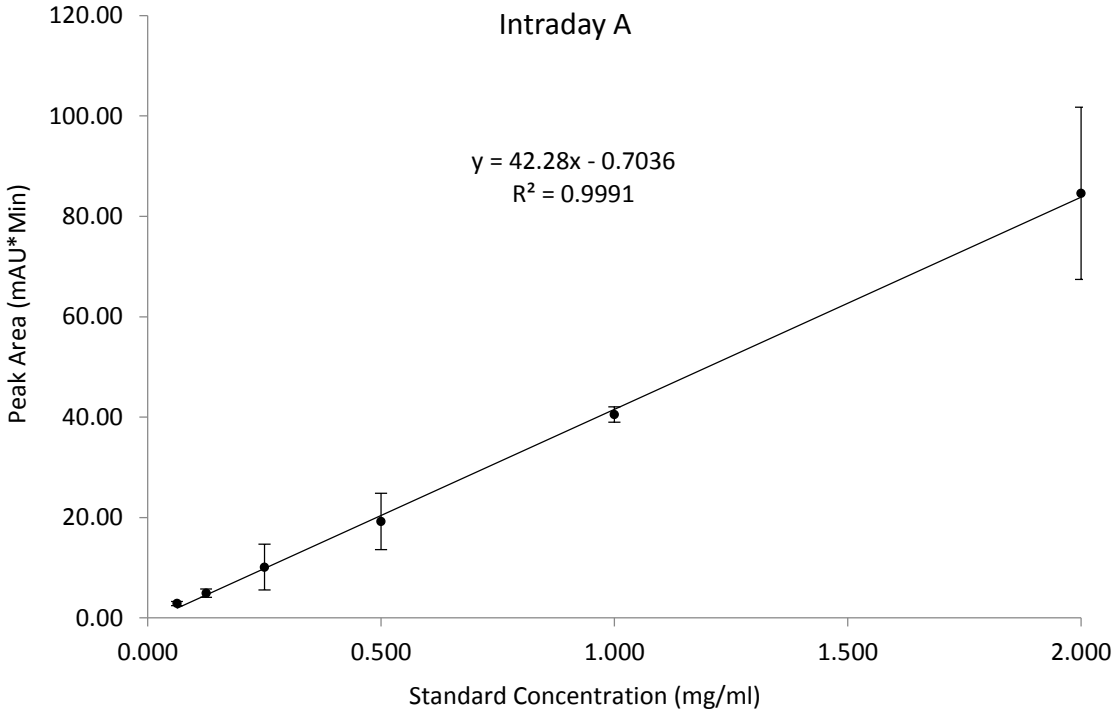


Figure 19 - Lisinopril Validation - Intraday Run A. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

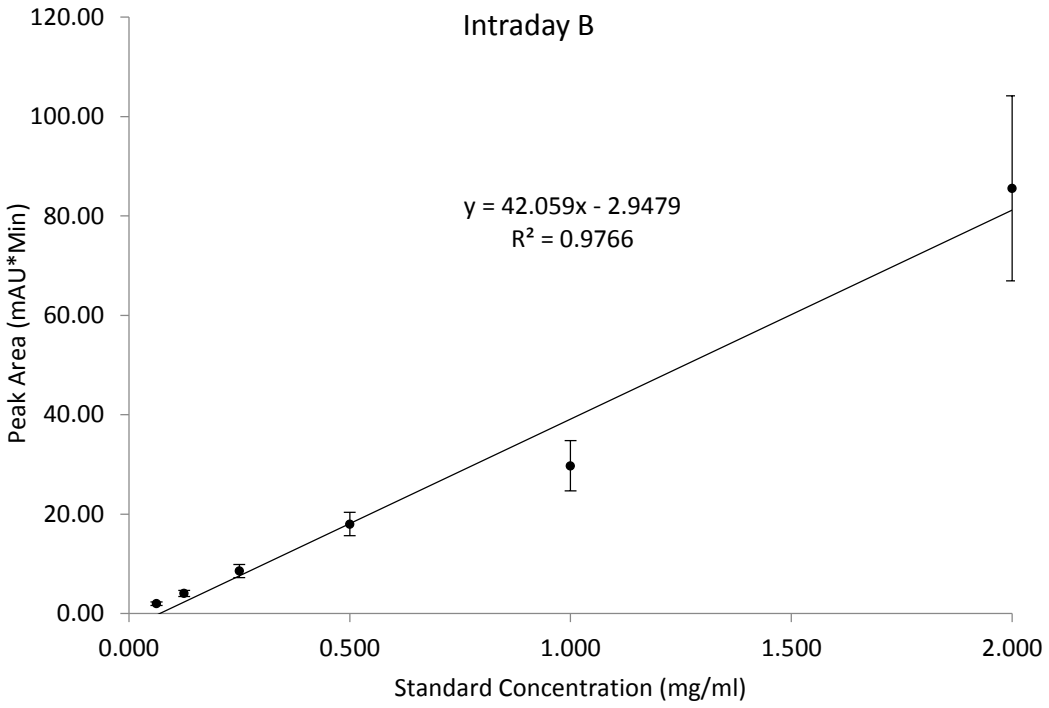


Figure 20 - Lisinopril Validation - Intraday Run B. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

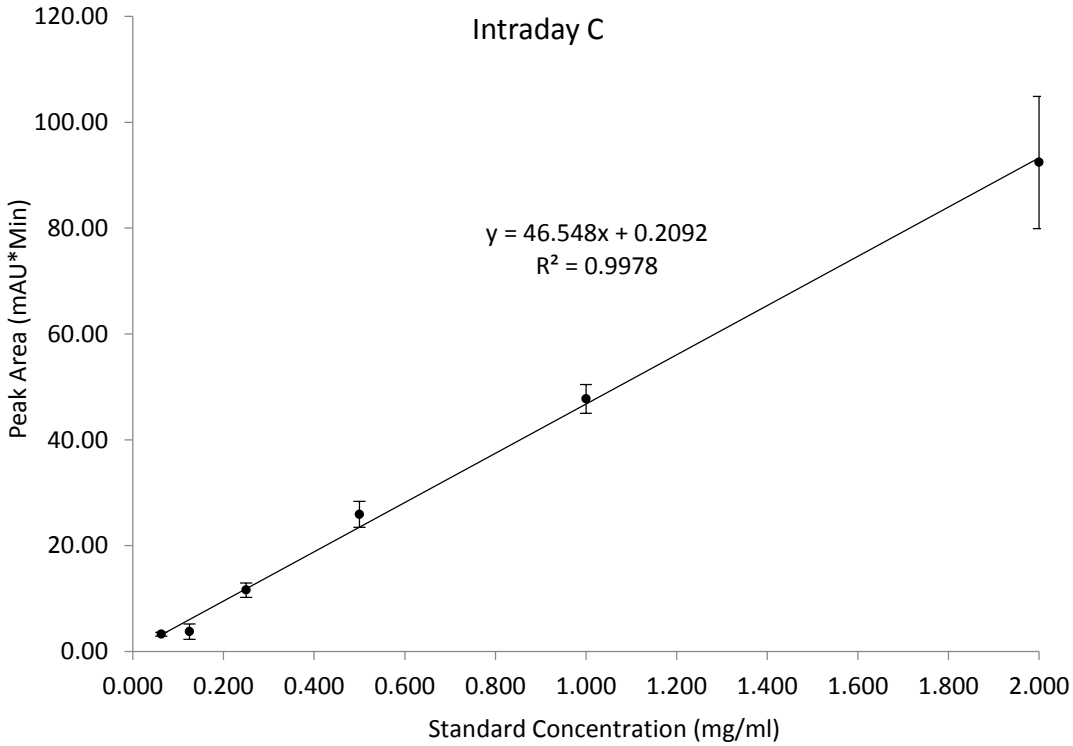


Figure 21 - Lisinopril Validation - Intraday Run C. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

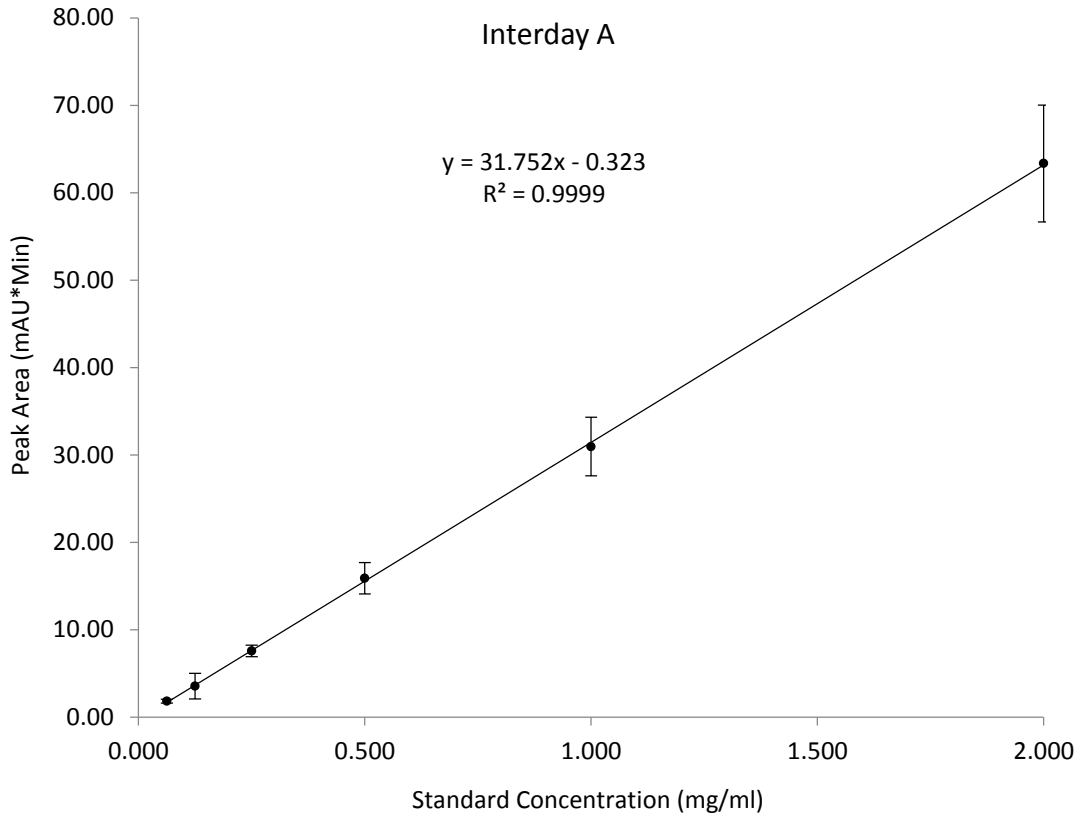


Figure 22 - Lisinopril Validation - Interday Run A. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

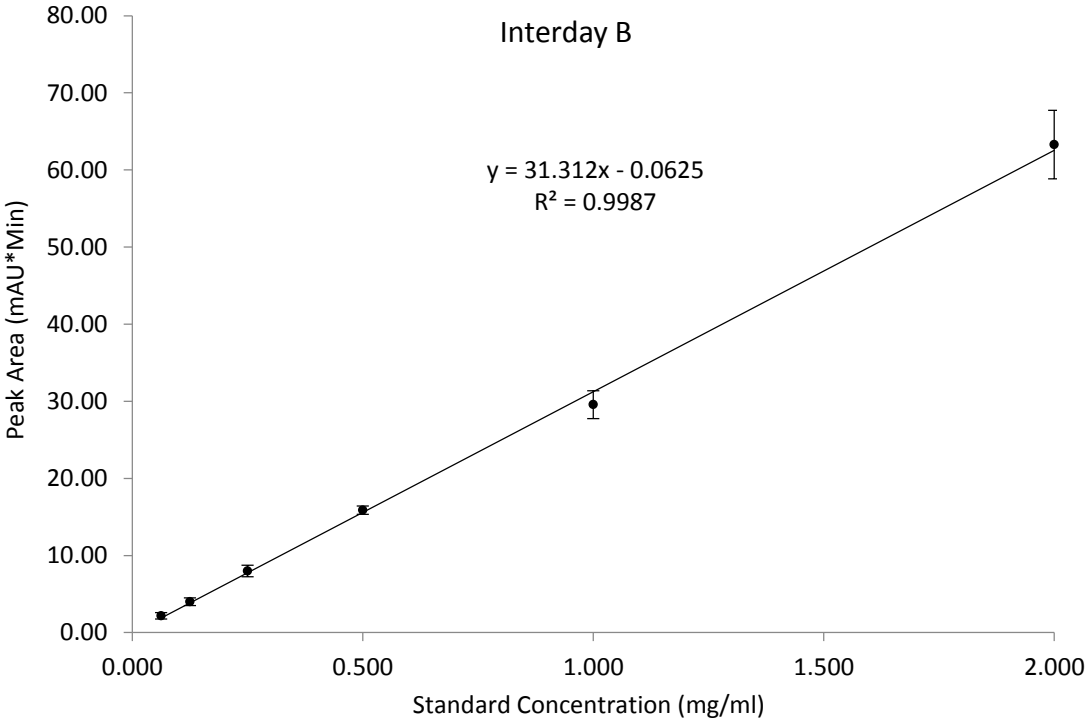


Figure 23 - Lisinopril Validation - Interday Run B. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

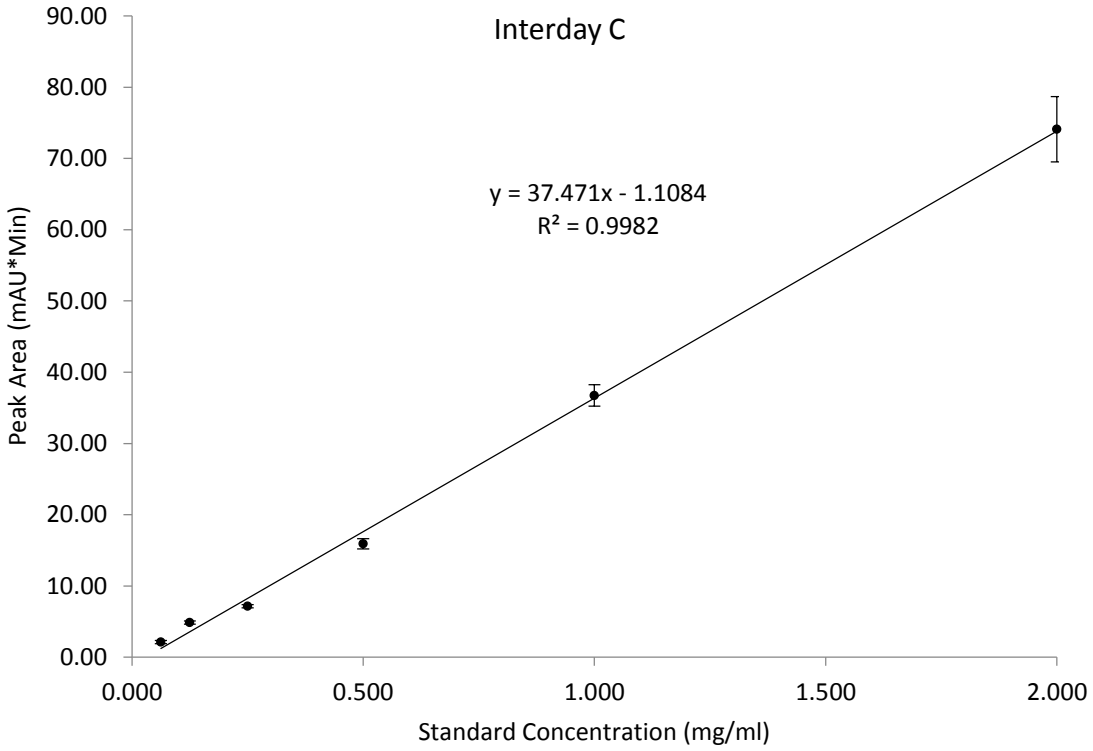


Figure 24 - Lisinopril Validation - Interday Run C. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The Limit of Detection (LOD) for the method is the lowest amount of analyte in a sample which can not necessarily be used in order to calculate an exact value but the lowest amount which can be detected by the detector. The LOD can be expressed as;

$$LOD = \frac{3.3\sigma}{S}$$

σ = is standard deviation of the response.

S = the slope of the calibration curve.

For the Lisinopril method the LOD was 0.12mg/ml.

The Limit of Quantification (LOQ) for the method is the lowest amount of analyte in a sample which can be used in order to calculate a suitably accurate and precise value for the amount of analyte in the sample. The LOQ can be expressed as;

$$LOQ = \frac{10\sigma}{S}$$

σ = is standard deviation of the response.

S = the slope of the calibration curve.

For the Lisinopril method the LOQ was 0.38mg/ml.

Having preformed the validation for the Lisinopril HPLC method it was determined that the method was not sensitive enough for the purpose required. In order to overcome this problem a second HPLC method for the detection of Lisinopril was investigated.

The new method was adapted from a method described by Beasley *et al* (2005). The new mobile phase consisted of a Potassium Phosphate Buffer mixed 80:20 with acetonitrile. Standards were produced using the Potassium Phosphate mobile phase ranging from 2mg/ml – 62.5µg/ml and these were ran at a wavelength of 228nm using a flow rate of 1.0ml/min and an injection volume of 20µl through a Phenomenex Gemini C_{18} column (150 × 4.6 mm) 5µm particle size at 27°C.

Figure 25 - HPLC trace for Beasley et al (2005) Lisinopril HPLC method.

The Beasley *et al* (2005) Lisinopril HPLC method produced a clean peak at around 2 minutes retention time (Figure 25). The peak produced was very narrow and the difference in peak area between standards was relatively small. In an effort to broaden the peak to increase the peak area and also potentially increase the separation of peaks produced by excipients the flow rate was reduced from 1ml/min to 0.5ml/min. The standards were re-ran with the flow rate reduced and this produced the following trace (Figure 26).

Figure 26 - HPLC trace for Beasley et al (2005) Lisinopril HPLC method implementing reduction to the flow rate from 1ml/min to 0.5ml/min.

The modification to the flow rate increased the peak area by around 100% and increased the retention time to approximately 4 minutes. As a result the separation of any excipient peaks will also be doubled. The calibration curve for this method produced good linearity with an R^2 value of 0.9996 (Figure 27). The gradient of the calibration curve was also increased which indicates improved LOD and LOQ values.

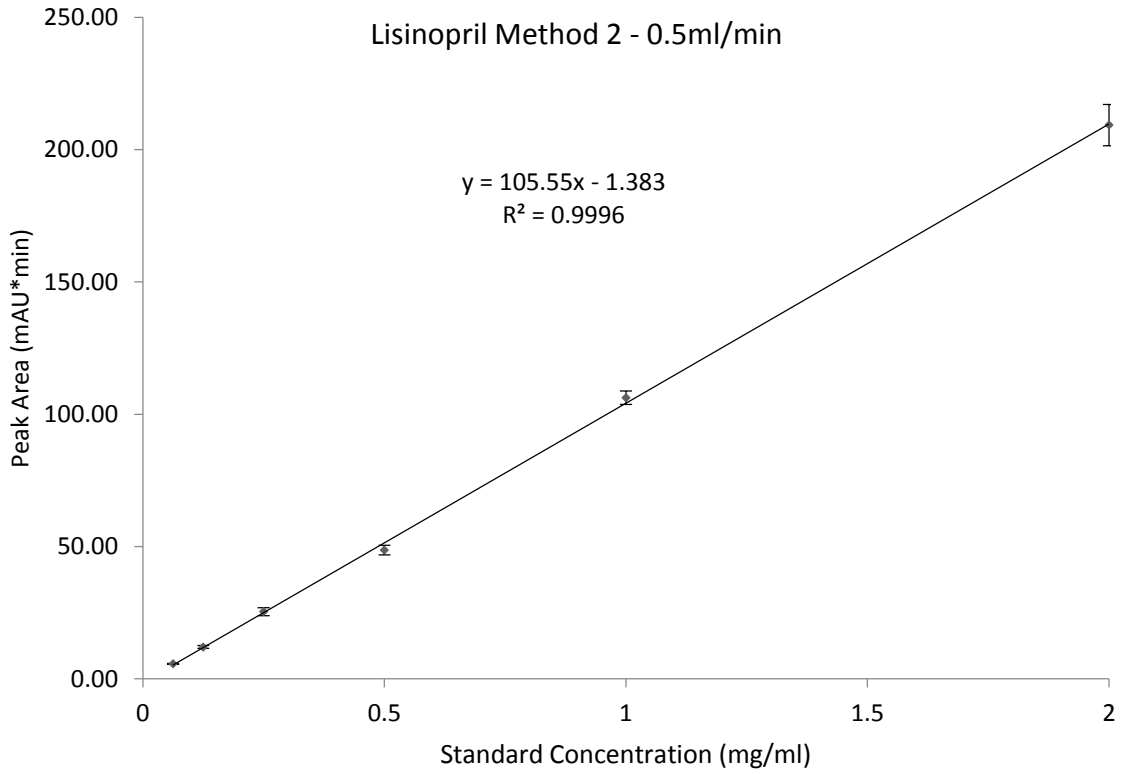


Figure 27 - Calibration curve for second Lisinopril HPLC method illustrating improved linearity with an R^2 value of 0.9996 and an equation with a steeper gradient (y = 105.55x – 1.383) to be used in the calculation of sample concentrations. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The Lisinopril preliminary formulation was ran on this method in order to ensure there was no interference from the excipients and also to ensure that the recoveries were 100%. This was proven to be the case (Figure 28).

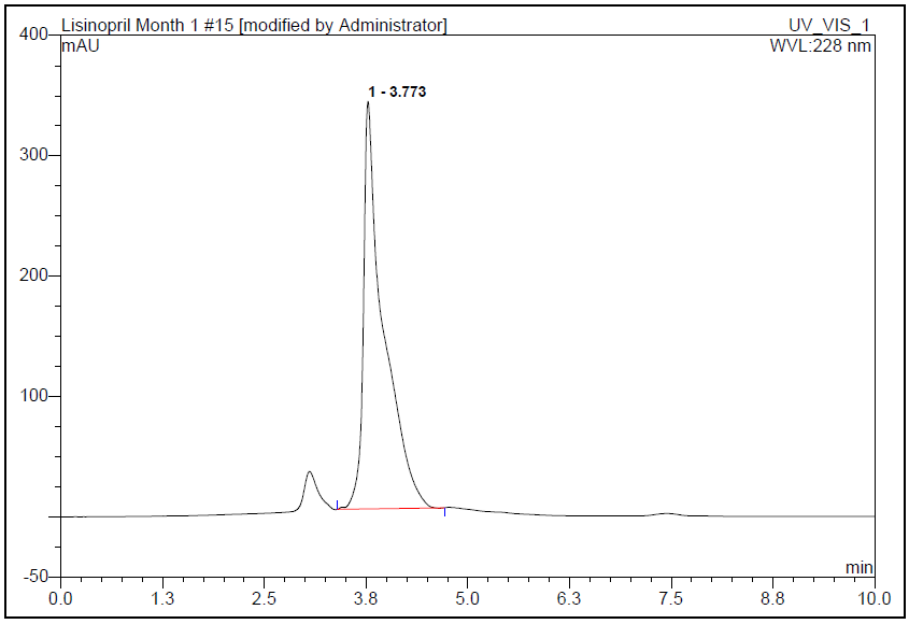


Figure 28 - HPLC trace for preliminary Lisinopril HPLC formulation.

Validation of this method was performed as previously described and as per the ICH guidelines. A summary of the validation is shown below (Table 13);

Table 13 - Validation Summary for improved Lisinopril HPLC method

Lisinopril Validation	
Selectivity	The Method was deemed to be selective for Lisinopril by running 'blank' samples and Lisinopril spiked samples
Accuracy	Determined by spiking mobile phase with known amounts of Lisinopril (2.0mg/ml, 1.0mg/ml and 0.5mg/ml). The recoveries ranged from 98.34% to 103.15% with an average standard deviation of 2.43%.
Linearity	Response for the detector was determined to be linear over a range of 2mg/ml to 62.5µg/ml. Correlation coefficients and slopes were obtained by plotting standard concentration (mg/ml) against peak area (mAU*min). R^2=0.9996 m=105.55
Precision	Precision and intermediate precision were tested via the comparison of three intraday and interday calibration curves. These showed suitable repeatability.
Limit Of Detection	LOD is 13.5µg/ml.
Limit Of Quantification	LOQ is 40.92µg/ml

The improved HPLC method produced a greatly improved standard deviation of 2.43%, the Linearity was also improved with an R^2 value of 0.9996. The gradient of the line was also increased which lead to improved LOD and LOQ values of 13.55µg/ml and 40.925µg/ml respectively. Following the development of a suitable method of detection the next stage in the production of the Lisinopril oral liquid formulation was the excipient selection.

2.4.2 Formulation Development

As Lisinopril is sensitive to oxidation then it was necessary to include antioxidants to protect the drug from degradation as a result of oxidation over the life of the formulation. Similarly it was necessary to include preservatives which protect the formulations from microbial infection over the shelf life of a product. Also to be included were sweeteners and flavours to improve the palatability of the formulation owing to the bitter taste of the drug. The key criteria for the excipients selected were that; they must be generally recognised as safe (GRAS) listed by the FDA and also compatible with the drug, the other excipients and also the formulation conditions the drug requires. Through investigation into existing literature detailing information on potential excipients, summary tables were produced to allow directed selection of excipients for inclusion in formulations.

The inclusion of preservatives in pharmaceutical preparations has long been known as a necessary means of protecting a product from microbial contamination. Preservatives are not universal and some are better suited to one dosage form more so than another (Table 14);

Table 14 - Preservative Options

Preservative	Advantages	Disadvantages	Application
***Acids (organic)*: benzoic acid, parabens, sorbic acid**	Active against bacteria and fungi	Highly pH dependent	Oral and topical formulations; gums and syrups
***Alcohols*: ethyl or isopropyl, chlorbutol, bronopol**	Broad spectrum, including that against acid-fast bacteria	Volatile; poor penetration of organic matter	Solvent; eye drops and injections; synergistic properties
***Aldehydes*: formaldehyde, gluteraldehyde**	Broad spectrum antibacterial, antifungal, and sporicidal activity	Acid solutions inactivated with temperature; toxic and carcinogenic	Chemical sterilization and storage of surgical instruments (e.g., endoscopes)
***Biguanides*: chlorhexidine, polyhexamethylene biguanide**	Mainly active in cationic form against gram-positive bacteria	Water insoluble; inactivated by organic matter; limited antifungal activity	Solution for hard contact lenses and other opthalmic products
***Halogens*: hypochlorite, povidone-iodine, chloroform**	Broad spectrum of antibacterial, fungal, and viral activity	Unstable; corrosive; inactivated by organic matter	Limited use nowadays
***Organic mercurials*: mercury, silver, thiomersal, phenylmercuric acetate**	Broad spectrum of antibacterial activity	Low capacity to organic matter, ionic and some non-ionic surfactants; toxicity	Eye drops; contact lens solutions
***Phenolics*: cresol, chlorocresol, bisphenol**	Cheap, rapid activity against gram-positive bacteria and fungi	Low water solubility; adsorbed by rubber; volatile, irritant, pH dependent	Creams
***Quaternary ammonium compounds*: cetrimide, benzalkonium chloride**	Narrow spectrum (gram-positive bacteria) of activity; surfactant properties	Low capacity to organic matter; low activity at acidic pH; incompatible with soaps, ionic and non-ionic surfactants	Eye drops; surgical creams; ointments

For the Lisinopril formulations the selected preservative/s needed to be suitable for use in an oral preparation. As such and using the information provided in the table above (Table 14), Benzoic Acid, Sorbic Acid and the Paraben group of preservatives were further investigated (Table 15, Table 16 and Table 17) (Gilbert and Allison, 2006).

Table 15 - Summary Table for Parabens intended for use as preservatives.

Parabens				
	Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben
Solubility in Water (%w/v)	0.25	0.08	0.02	0.02
pH Range	3-6	4-8	4-8	4-8
Stability at 20°C	4 Years	4 Years	4 Years	4 Years
RDA	10mg/kg Bodyweight	10mg/kg Bodyweight	10mg/kg Bodyweight	10mg/kg Bodyweight
Buffer Required	No	No	No	No
Antioxidants Required	No	No	No	No
Limiting Factors	Activity Reduced by use of micelles			
Advantages	Non-toxic, Acid when dissolved in water, Effective at Low Concentrations			
GRAS Listed	Yes	Yes	Yes	Yes
Amount for Use (mg/ml)	4	4	4	4

Table 16 - Summary Table for Benzoic Acid intended for use as a preservative.

Benzoic Acid	
Solubility in Water (%w/v)	0.33
pH Range	2.5-4.5
Stability at 20°C	8 Weeks
RDA	5mg/Kg Body Weight
Buffer Required	No
Antioxidants Required	Yes
Limiting Factors	Inactive above pH5
Advantages	GRAS listed. Accepted as a food additive in Europe. Included in the FDA Inactive Ingredients Database.
GRAS Listed	Yes
Amount for Use (mg/ml)	0.1

Table 17 - Summary Table for Sorbic Acid intended for use as a preservative

Sorbic Acid	
Solubility in Water (%w/v)	0.25
pH Range	Optimum 4.5
Stability at 20°c	Sensitive to light, Oxidising agents, Some loss of antimicrobial activity occurs in the presence of non-ionic surfactants and plastics.
RDA	25mg/Kg Body Weight
Buffer Required	No
Antioxidants Required	Yes
Limiting Factors	Sensitive to oxidation, Particularly in the presence of light. Oxidation occurs more readily in an aqueous solution than in the solid form. When stored in glass containers, solutions become very ph sensitive, Aqueous Solutions of sorbic acid without the addition of antioxidants are rapidly decomposed when stored in polypropylene, polyvinylchloride and polyethylene containers. No activity above pH 6.
Advantages	Optimum pH in suitable range for lisinopril preparation, GRAS Listed
GRAS Listed	Yes
Amount for Use (mg/ml)	2

Investigation into the properties of benzoic acid, sorbic acid and the parabens allowed for sorbic acid to be discounted as an option due to more complicated conditions for storage and the maintenance of stability than benzoic acid and the parabens with no extra benefit as a preservative. At this parabens are the preservative of choice as they fit all of the necessary criteria and have no limitations which will be of consequence in this application. It should be noted however that parabens should be used together to improve their activity, e.g. methyl and propyl parabens used in conjunction.

As discussed, antioxidants are necessary for inclusion in the Lisinopril formulation as lisinopril is sensitive to oxidation. Excipients included in the formulations may also be sensitive to oxidation and therefore the presence of antioxidants may be necessary to maintain the effect of preservatives or flavours for example. In the same was as for the selection of a preservative, the antioxidant chosen must be compatible with the active ingredient and also feature in the GRAS database. Investigation into

possible antioxidants for use in an oral liquid preparation lead to the short listing of Citric Acid, Ascorbic Acid, Sodium Metabisulphate and α-Tocopherol. Investigation into the individual properties of these antioxidants (Table 18, Table 19 and Table 20) allowed for the elimination of α-Tocopherol as it has a low solubility in water.

Table 18 - Summary Table for Citric Acid intended for use as an antioxidant.

	Citric Acid
Solubility in Water (%w/v)	61.8
pH Range	2-6
Stability at 20°c	Citric Acid Anhydrous is stable if stored under cool and dry conditions
RDA	None Specified
Buffer Required	No
Limiting Factors	On storage, sucrose may crystallize from syrups in the presence of citric acid.
Advantages	Correct pH range for use. Has some taste masking properties
GRAS Listed	Yes
Amount for Use (mg/ml)	3

Table 19 - Summary Table for Ascorbic Acid intended for use as an antioxidant.

	Ascorbic Acid
Solubility in Water (%w/v)	28.57
pH Range	Acid
Stability at 20°c	Powder Form = Stable. Unstable in solution, especially alkaline solution, made worse by light and heat. In the absence of oxygen and other oxidizing agents it is also heat stable
RDA	15mg/kg Bodyweight
Buffer Required	No
Limiting Factors	Can be unstable in solution
Advantages	Active in correct pH range
GRAS Listed	Yes
Amount for Use (mg/ml)	1

Table 20 - Summary Table for sodium metabisulphate intended for use as an antioxidant.

Sodium Metabisulphate	
Solubility in Water (%w/v)	52.63
pH Range	Acid
Stability at 20°c	Powder Form = Slowly oxidised in air. Aqueous sodium metabisulphate solutions decompose in air, especially on heating. Photosensitive.
RDA	3.5mg/kg Bodyweight
Buffer Required	No
Limiting Factors	Links with allergic reactions, Potential irritant.
Advantages	Correct pH, GRAS listed, Has some antimicrobial properties, Used in acidic preparations.
GRAS Listed	Yes
Amount for Use (mg/ml)	10

All of the summarised antioxidants would be suitable for use however Citric acid was selected as it is already used extensively as an antioxidant in pharmaceutical preparations and has no limiting factors that will be of consequence in this investigation. It also has the added advantage that citric acid can improve the taste of a formulation.

For the selection of flavours and sweeteners, flavours were provided by S. Black's and included at concentrations based upon manufacturer recommendations. Where sweeteners are concerned, artificial sweeteners were targeted to avoid using sugar, both for the age appropriate dosage form and also to produce a formulation which would be suitable for diabetics. Numerous sweeteners were investigated, all of which would have been suitable however Xylitol was selected for its multiple advantages which are discussed below.

Xylitol is a non-cariogenic sweetening agent in a variety of pharmaceutical dosage forms, including tablets, syrups, and coatings. It is also widely used as an alternative to sucrose in foods and as a base for medicated confectionery. Unlike sucrose, Xylitol is not fermented into cariogenic acid end products and does not contribute to the formation of dental caries. As Xylitol has an equal sweetness intensity to sucrose, combined with a distinct cooling effect upon dissolution of the crystal, it is

highly effective in enhancing the flavour of tablets and syrups and masking the unpleasant or bitter flavours associated with some pharmaceutical actives and excipients. In liquid preparations, Xylitol is used as a sweetening agent and vehicle for sugar-free formulations. In syrups, it has a reduced tendency to 'cap-lock' by effectively preventing crystallization around the closures of bottles. Xylitol also has a lower water activity and a higher osmotic pressure than sucrose, therefore enhancing product stability and freshness. In addition, Xylitol has also been demonstrated to exert certain specific bacteriostatic and bactericidal effects, particularly against common spoilage organisms.

As the API and all excipients are soluble in water the production of the formulation is simple. Following excipient selection the constituent parts for Lisinopril solutions are as follows;

- Lisinopril (2mg/ml)
- Citric Acid (3mg/ml)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (30% w/v in sweetened formulation) (10% w/v in flavoured formulation)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

2.4.3 Stability Analysis

With the formulation produced the next stage in the investigation was to proceed into stability testing. This is done by simulating an environment which a drug product would be likely to experience during its production, distribution and storage. Stability testing is important as it not only ensures that the drug content of the product remains constant over the duration of the products life in storage, but also that the organoleptic properties of the product remain unchanged. Something as simple as a colour change

with may be harmless can be detrimental as a patient may be reluctant to take the medication.

2.4.3.1 Accelerated Conditions

Over the six month course of the stability testing in accelerated conditions with 40°C and 75% relative humidity, the drug content of the formulations dropped below 95% of the starting dose by the third month of testing falling to a low of approximately 80% of the starting dose after six month (Figure 29). The pH remained constant for the duration (Figure 30).

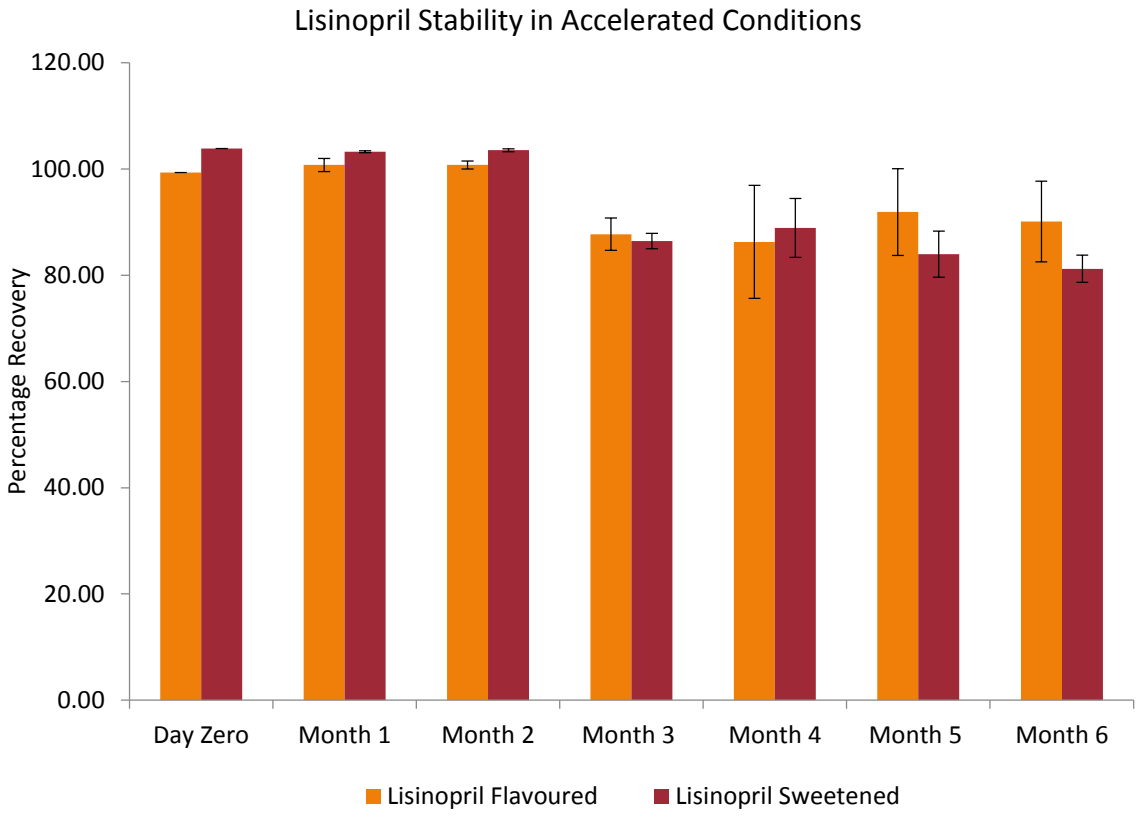


Figure 29 - Lisinopril recovery following HPLC for formulations in accelerated storage conditions. Before the third month of testing the formulations proved stable with little variation in drug content and consistent drug content between samples of the same batch. From the third month onwards a reduction of up to approximately 20% of the drug content was seen when compared with the starting concentration and in addition the variation between samples also increased. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

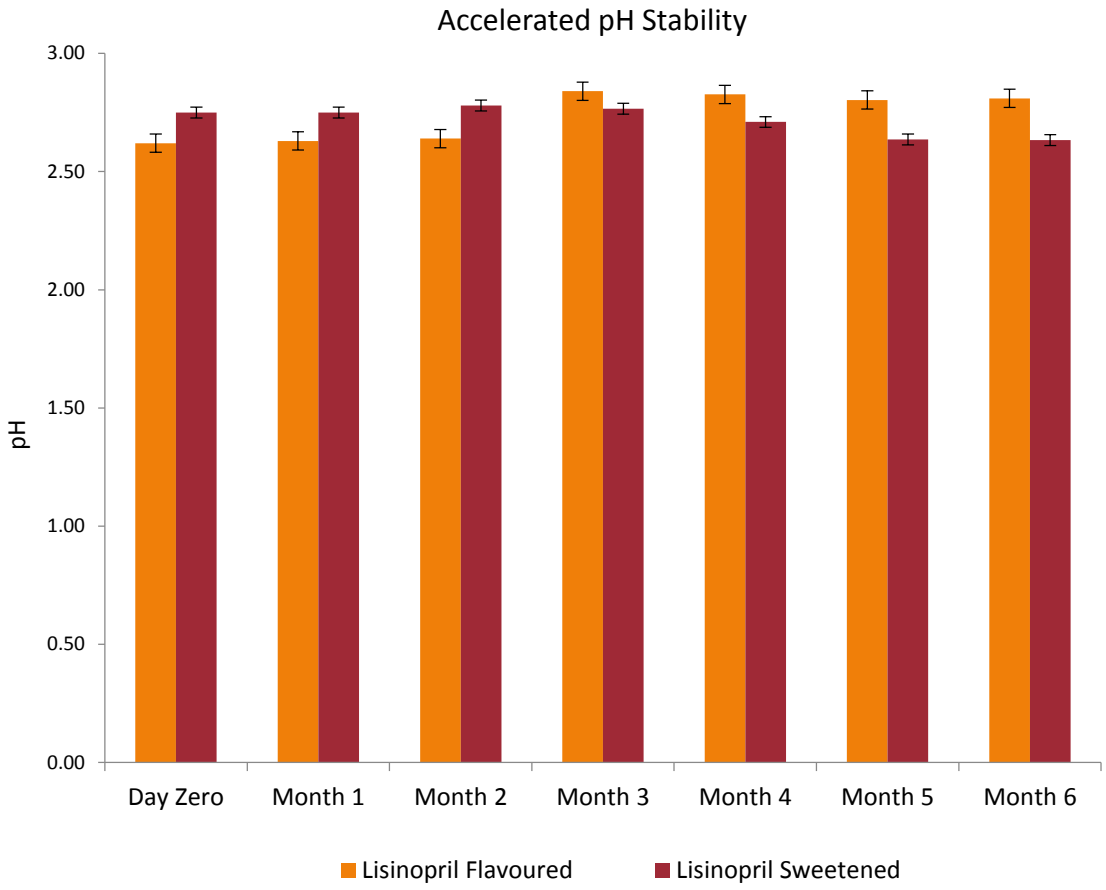


Figure 30 - Lisinopril pH stability for formulations in Accelerated storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

2.4.3.2 Long Term Conditions

Over the twelve month course of the stability testing in long term conditions with 25°C and 60% relative humidity, the drug content of all of the formulations remained >95% of the starting dose (Figure 31) and the pH remained constant for the duration (Figure 32).

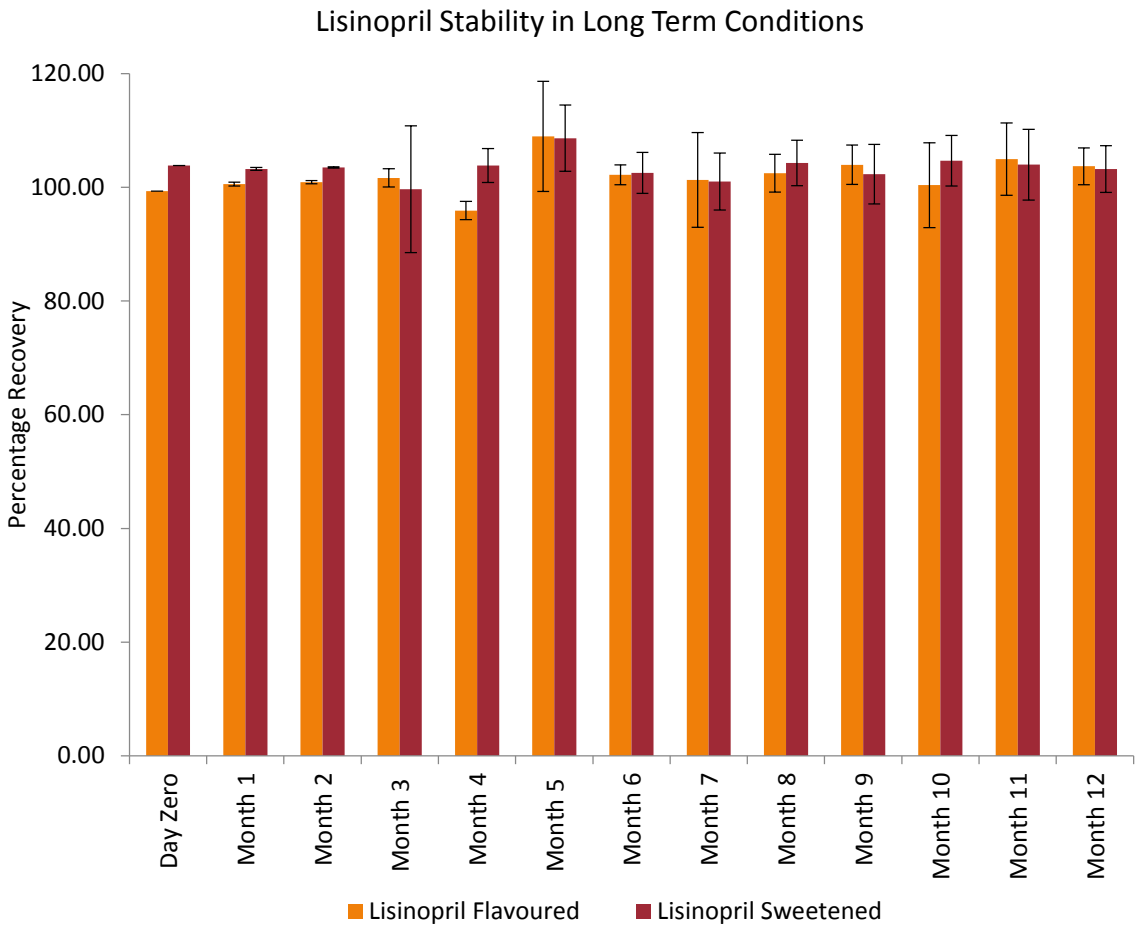


Figure 31 - Lisinopril recovery following HPLC for formulations in Long Term storage conditions. For the duration of testing the formulations proved stable with little variation in drug content and consistent drug content between samples of the same batch. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

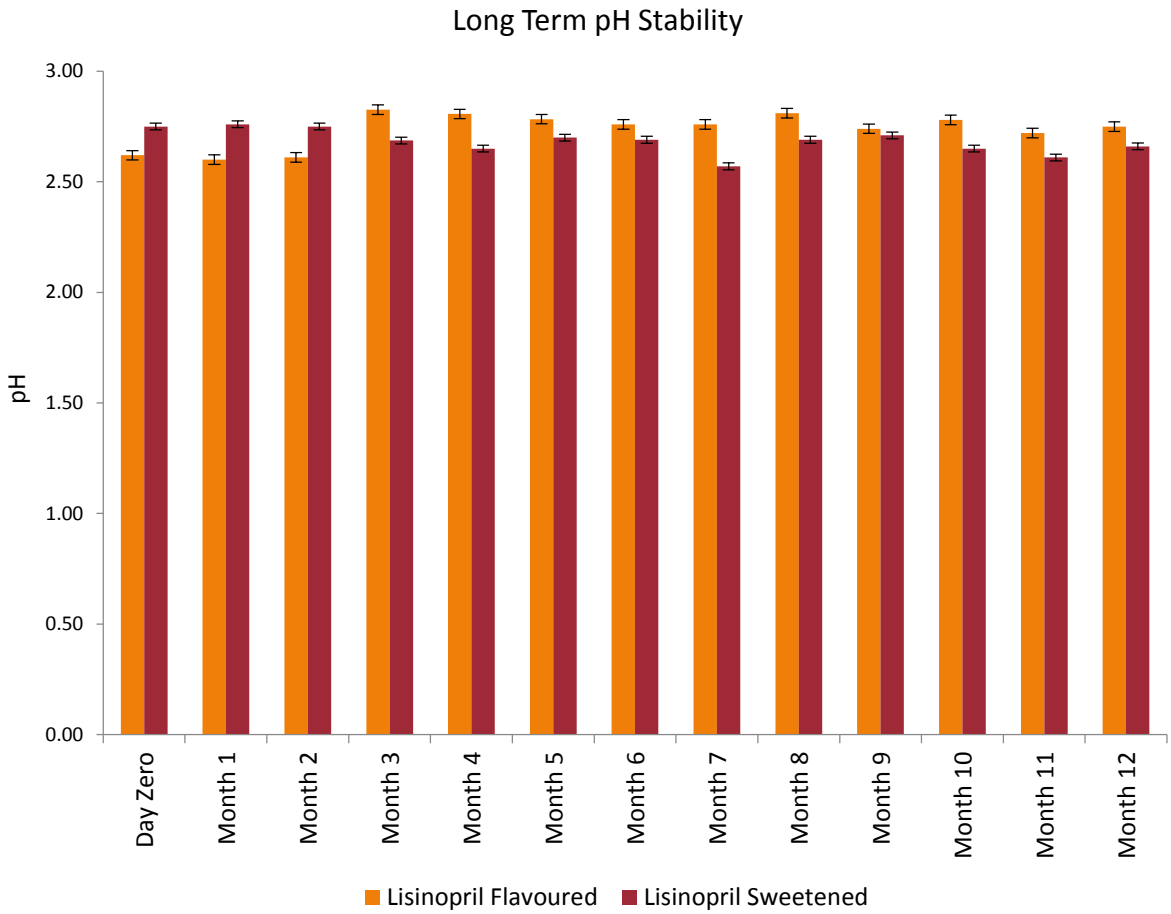


Figure 32 - Lisinopril pH stability for formulations in Long Term storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

According to the parameters outlined by the ICH harmonised tripartite guideline stability testing of new drug substances and products Q1A(R2**)**, "significant change" for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation product's exceeding its acceptance criterion;
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., colour, phase separation, resuspendibility, caking,

hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH; or
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

The Lisinopril formulations tested under accelerated conditions showed significant decrease in the drug assayed in comparison to the day zero analysis (Figure 29). The formulations in long term storage conditions (Figure 31) did not show any significant decrease in the drug assayed even after 12 months in storage. This suggests that the degradation seen under accelerated conditions is a result of the elevated temperature. It is known that Lisinopril diketopiperazine (DKP) degradate is the primary degradation compound for Lisinopril. Degradation occurs through intramolecular condensation and sees the formation of a second amide bond (Figure 33).

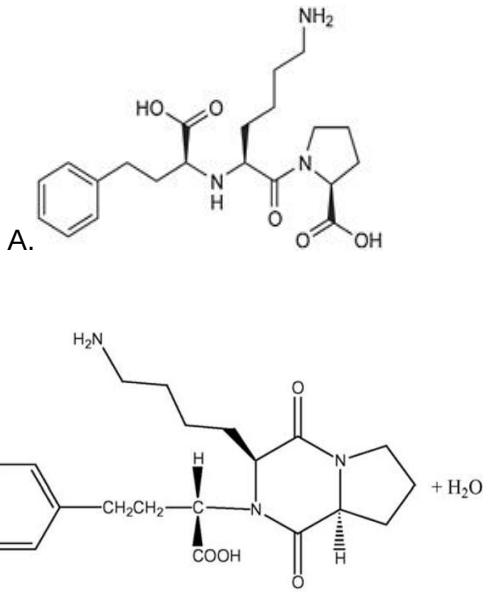


Figure 33 – Lisinopril DKP Structure (B) - primary degradation product of Lisinopril (A).

This reaction is known to occur when Lisinopril experiences conditions of elevated temperature (Demeter *et al* 1998), as such the advice for storage of the medication would have to emphasise storage in a cool location (<25°C). The humidity of the storage conditions is not a large issue as the medication will be contained within bottles impervious to moisture. With regards to excursions outside of the storage conditions such as transport during distribution, degradation due to temperature should not be a concern so long as excursions are short in duration and infrequent. This is as degradation was only seen to be significant after >1 month and the temperature at which degradation was seen is higher than most environmental temperatures the medication would experience.

3 Reformulation of Ramipril to Produce an Age Appropriate Oral Liquid Dosage Form Targeting the Paediatric Patient Segment

3.1 Chapter Aims and Objectives

- Develop a HPLC method for the detection of Ramipril and validate according to ICH guidelines.
- Formulate Ramipril as an oral liquid dosage form with a particular focus on effectively targeting the paediatric patient segment.
- Characterise formulations including stability testing in long term and accelerated conditions as specified by ICH guidelines.

3.2 Introduction

Ramipril is one of the many ACE Inhibitors which is produced as a pro-drug (Figure 34). Similar in nature to other pro-drug ACE Inhibitors such as Quinapril, Fosinapril and Benazepril, Ramipril is converted into its active form Ramiprilat (Figure 35) following metabolism by esterase enzymes in the liver. This is the main site of activation although there is a small degree of activation which occurs in the kidneys.

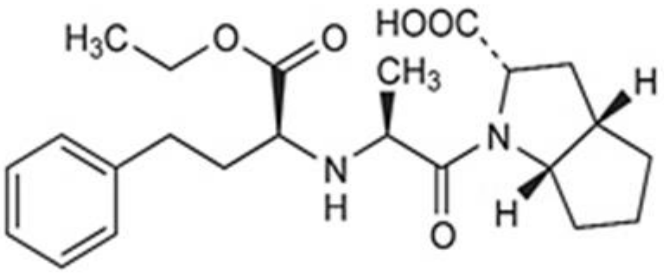


Figure 34 - Ramipril Structure

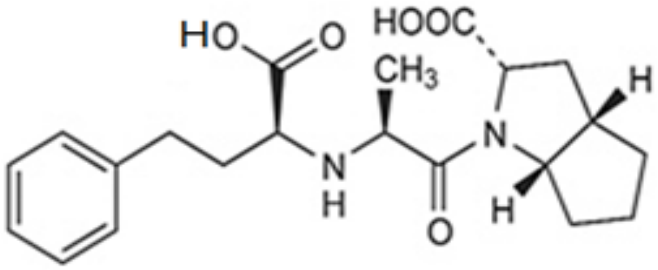


Figure 35 - Ramiprilat Structure

Ramiprilat competes with angiotensin I for binding with ACE and in doing so prevents the production of angiotensin II. In this way the conversion of Angiotensin I to Angiotensin II is prevented which in turn leads to the suppression of Angiotensin II activity resulting in the reduction of blood pressure. This effect is complemented via the prevention of bradykinin inactivation allowing for vasodilatation resulting in increased blood pressure reduction (Betzing *et al* 1999).

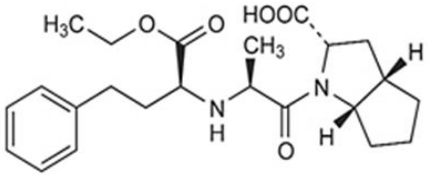
Ramipril is used mainly in the treatment of mild to severe hypertension but is also used to reduce the incidence of fatality following heart attacks in patients who develop indications of congestive heart failure. Also Ramipril is used to reduce the risk of heart attack and strokes in patients who are at high risk. As with all ACE Inhibitors Ramipril may be used to slow the progression of kidney disease in patients who suffer from high blood pressure whilst at the same time suffering from conditions such as diabetes (Ravid *et al* 1998, Lewis *et al* 2001, Molitch *et al* 2001), microalbuminuria (albumin in the urine) (Lewis *et al* 2001) or nephropathy (Molitch *et al* 2001). Once administered maximum absorption tends to be approximately 50-60% (Senthilkumar *et al* 2011) and is through the PEPT1 transporters in the small intestine (Knutter *el al* 2008). In the paediatric population Ramipril is used most commonly for the treatment of heart failure, hypertension in children with type 1 diabetes with nephropathy and also in the treatment of diabetic nephropathy (Paediatric Formulary Committee 2012). At the time of this research there was no liquid Ramipril formulation available (Table 21).

Table 21 - Ramipril Dosage Forms

Ramipril Dosage Forms	
Altace 1.25 mg Tablet	Novo-Ramipril 10 mg Tablet
Altace 10 mg Tablet	Novo-Ramipril 2.5 mg Tablet
Altace 2.5 mg Tablet	Novo-Ramipril 5 mg Tablet
Altace 5 mg Tablet	Pms-Ramipril 1.25 mg Tablet
Altace 1.25 mg capsule	Pms-Ramipril 10 mg Tablet
Altace 1.25 mg tablet	Pms-Ramipril 2.5 mg Tablet
Altace 10 mg capsule	Pms-Ramipril 5 mg Tablet
Altace 10 mg tablet	Ramipril 1.25 mg Tablet
Altace 2.5 mg capsule	Ramipril 10 mg Tablet
Altace 2.5 mg tablet	Ramipril 2.5 mg Tablet
Altace 5 mg capsule	Ramipril 5 mg Tablet
Altace 5 mg tablet	Ramipril 1.25 mg capsule
Apo-Ramipril 1.25 mg Tablet	Ramipril 10 mg capsule
Apo-Ramipril 10 mg Tablet	Ramipril 2.5 mg capsule
Apo-Ramipril 2.5 mg Tablet	Ramipril 5 mg capsule
Apo-Ramipril 5 mg Tablet	Ran-Ramipril 1.25 mg Tablet
Co Ramipril 1.25 mg Tablet	Ran-Ramipril 10 mg Tablet
Co Ramipril 10 mg Tablet	Ran-Ramipril 2.5 mg Tablet
Co Ramipril 2.5 mg Tablet	Ran-Ramipril 5 mg Tablet
Co Ramipril 5 mg Tablet	Ratio-Ramipril 1.25 mg Tablet
Jamp-Ramipril 1.25 mg Tablet	Ratio-Ramipril 10 mg Tablet
Jamp-Ramipril 10 mg Tablet	Ratio-Ramipril 2.5 mg Tablet
Jamp-Ramipril 2.5 mg Tablet	Ratio-Ramipril 5 mg Tablet
Jamp-Ramipril 5 mg Tablet	Sandoz Ramipril 1.25 mg Tablet
Mylan-Ramipril 1.25 mg Tablet	Sandoz Ramipril 10 mg Tablet
Mylan-Ramipril 10 mg Tablet	Sandoz Ramipril 2.5 mg Tablet
Mylan-Ramipril 2.5 mg Tablet	Sandoz Ramipril 5 mg Tablet
Mylan-Ramipril 5 mg Tablet	

The aim of this investigation was to reformulate Ramipril as an Oral liquid dosage form overcoming the various hurdles, such as poor solubility and drug stability, which are present in between the selection of the drug to the production of the final formulation. The main limitation to the formulation of an oral solution is the poor aqueous solubility of Ramipril. The physiochemical properties of Ramipril (Table 22) as with any drug guide the formulation development.

Table 22 - Ramipril Monograph listing important physiochemical properties which should be considered before formulation.

Ramipril Monograph	
Chemical Name	[2*S*-[1[*R**(*R**)],2α,3aβ,6aβ]]-1-[2-[[1-(Ethoxycarbonyl)-3-phenylpropyl]amino]-1-oxopropyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid
Other Names	Altace
Molecular Formula	$C_{23}H_{32}N_2O_5$
Molecular Weight	416.5
CAS Number	87333-19-5
Ramipril Structure	
Appearance	White Crystalline Powder
Solubility	Ramipril is soluble in polar organic solvents and in buffered aqueous solutions. Ramipril is practically insoluble in water.
Sensitivities	Ramipril is sensitive to photodegradation, oxidation and alkaline conditions.

There are numerous methods for solubilising drugs with poor aqueous solubility such as; salt formation (Serajuddin 2007), micelle formation (Sezgin *et al* 2006), co-solvency (Li *et al* 1999) and cyclodextrin complexation (Lockwood *et al* 2003) to name a few. For the purpose of the Ramipril formulation it was decided that the implementation of cyclodextrin complexation and also the use of co-solvency were the two best suited avenues of investigation. This was based on the desire to develop simple formulations containing the minimum amounts of excipients in order to best target paediatrics.

Detailed herein is the production of three oral liquid Ramipril formulations. A solution produced implementing cyclodextrins, a solution produced implementing a co-solvency approach and also the production of a suspension in which excipient selection is optimised to overcome the limitations of a suspensions incipient instability.

3.3 Materials and Methods

3.3.1 HPLC Method for the Detection of Ramipril

The HPLC method for Ramipril was developed from a combination of the stability indicating HPLC methods described by Allen et al (1995) and Patil et al (2008). The method developed used a Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler. Perchlorate Buffer and Acetonitrile (55:45) formed the mobile phase and the stationary phase was a C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size) with a flow rate of 1mg/min and a detection wavelength of 216nm. The injection Volume was 50µl and the run time was 10 minutes. Six calibration standards produced via serial dilution in methanol and acetonitrile (Diluent A) (50:50). The standards are then diluted 1:5 in Diluent B (Water and Acetonitrile, 45:55). Samples were diluted to be within calibration standards using Diluent B. The Mobile Phase (Perchlorate Buffer and Acetonitrile (55:45)) was used as a blank in order to show that there are no placebo peaks eluting at the same retention time as Ramipril. A suitably linear calibration was produced (Figure 36) and the method was validated (Table 23) following ICH guidelines (ICH Topic Q 2 (R1) 1995).

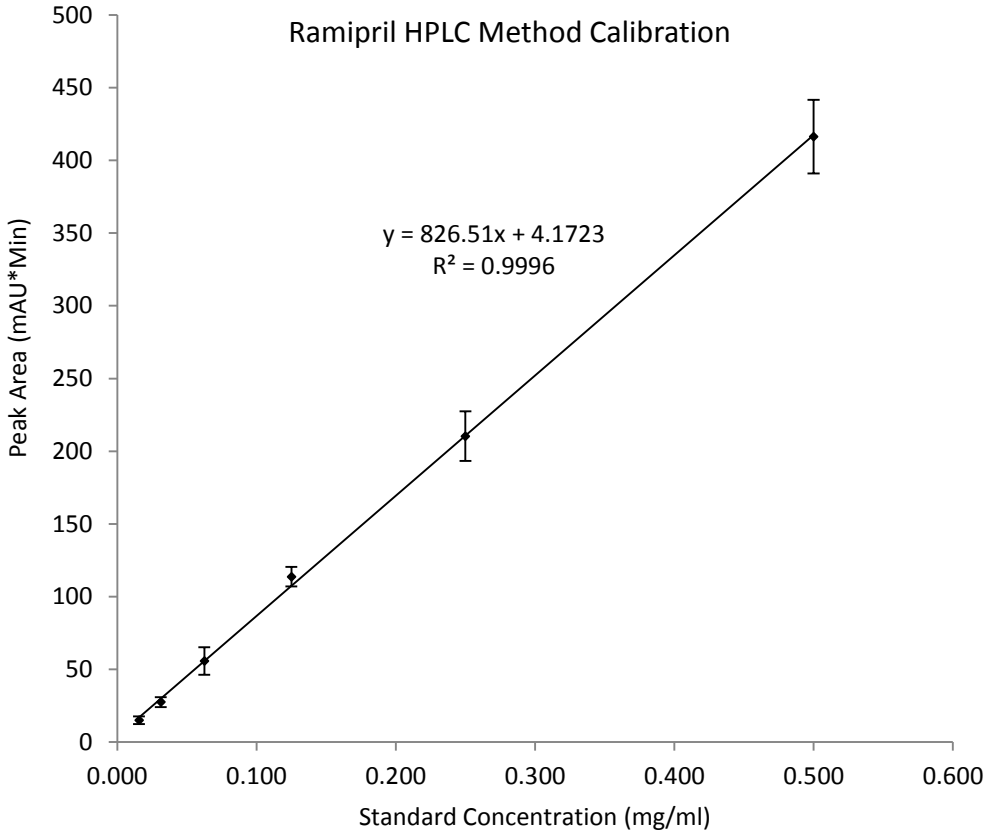


Figure 36 - Ramipril HPLC calibration. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Table 23 - Ramipril HPLC Validation

Ramipril Validation	
Selectivity	The Method was deemed to be selective for Ramipril by running 'blank' samples and Ramipril spiked samples
Accuracy	Determined by spiking mobile phase solution with known amounts of Ramipril (0.5mg/ml, 0.25mg/ml and 0.125mg/ml). Three different concentrations within the calibration range were ran in triplicate. The recoveries ranged from 96.22% to 106.33% with an R.S.D of 4.68%.
Linearity	Response for the detector was determined to be linear over a range of 1mg/ml to 31.25µg/ml. Correlation coefficients and slopes were obtained by plotting standard concentration (mg/ml) against peak area (mAU*min). R^2 = 0.9996 m = 826.51.
Precision	Precision and intermediate precision were tested via the comparison of three intraday and interday calibration curves. These showed suitable repeatability.
Limit Of Detection	LOD is 4.0µg/ml.
Limit Of Quantification	LOQ is 13.4µg/ml

3.3.2 Co-solvent Approach to Dissolve Ramipril

Ramipril (Discovery fine Chemicals, UK) was added (1mg/ml) to acetic acid (Fisher Scientific) solutions at concentrations of 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, 0.04%, 0.03%, 0.02% and 0.01%. The solutions were stirred using magnetic stirrers for 1 hour. The speed at which the solubilisation of Ramipril occurred was observed during this period. Samples were then taken and filtered before analysis by HPLC to confirm the complete solubilisation of Ramipril.

3.3.3 Cyclodextrin Approach to Dissolve Ramipril

3.3.3.1 Phase Solubility

To investigate the phase solubility of Ramipril and HP-β-CD (Sigma) the method implemented was described by Higuchi and Connors (1965). Here, increasing concentrations of HP-β-CD were added to water. To each solution excess Ramipril was added and the system was stirred for 24 hours. At this point samples were taken from each solution, passed through a 0.45µm filter and then analysed via HPLC.

3.3.3.2 Effect of Temperature and pH on Solubility following complexation.

Effect of pH and Temperature on the ability of HP-β-CD to dissolve Ramipril - In order to investigate the effect of pH and Temperature on the ability of HP-β-CD to solubilise Ramipril the phase solubility method described by Higuchi and Connors (1965) was adapted. To assay the effect of temperature, six concentrations of cyclodextrin were produced in distilled water from 1.2mM down to 3.75µM using serial dilution. These solutions were cooled to 15°C using ice and to each solution excess Ramipril was added. The solutions were then maintained at this temperature for 1 hour at which point a sample was taken from each solution, filtered to remove undissolved Ramipril and then diluted 1:10 in water with acetonitrile (45:55). These were then transferred to HPLC vials. The solutions were moved to a water bath set at 30°C and incubated for a further hour

at which point samples were again taken and transferred to HPLC vials as before. This process was repeated at temperatures of 45°C, 60°C and 75°C.

To assay the effect of pH six concentrations of Cyclodextrin were produced in from 1.2mM down to 3.75µM using serial dilution. These were produced in solutions of pH 3, 5 and 7 and to these excess Ramipril was added. This pH range was selected on the grounds that formulations will not be produced with a pH lower than 3 to ensure the compatibility of excipients and will not be produced with a pH greater than 7 as Ramipril is known to degrade in alkaline conditions. Solutions were left stirring for 1 hour before samples were filtered to remove undissolved Ramipril and prepared for HPLC analysis.

3.3.3.3 Effect of Complexation on Ramipril Stability

Accelerated degradation studies were developed from the methods described by Belal *et al* (2001) and Patil *et al* (2008) to test the effect of complexation of Ramipril with HP-β-CD on the drug stability. Solutions of 0.1M NaOH and 1.0M HCL were prepared. For each of these one batch of solution containing Ramipril (0.24mM) only, and one batch of solution containing Ramipril (0.24mM) and HP-β-CD (0.24mM) were produced. These were stirred for two hours and samples were taken at time 0 and then every 20 minutes thereafter. Samples were analysed via HPLC. Photostability was assayed with exposure of test solutions to UV light at 254nm for 24 hours. Once solution contained complexed drug (Ramipril and HP-β-CD) and the other solution contained Ramipril only. Samples were taken at the six hour time point and after 24 hours. Samples were analysed via HPLC.

3.3.3.4 Determination of Ramipril – HP-β-CD Binding Constant and Stoichiometry

The binding constant for the complexation of Ramipril and HP-β-CD was determined. This involved production of a 4.8×10^{-3}M solution of HP-β-CD in water. The conductivity of this cyclodextrin solution was measured to determine a base line. Then, known amounts of Ramipril were added at increasing increments of 1.0×10^{-3}M up to

1.0x10^{-2}M. Temperature was maintained at 25°C and pH was recorded throughout to ensure no variation occurred as varying pH affects the conductance measured. Conductometry readings were taken after each addition of drug. In this way a graph of the conductance readings was produced. Plotting two lines for each section of the graph and calculating their point of convergence allows for the determination of the concentration of cyclodextrin at which complete complexation occurs. In this way the stoichiometry can be determined allowing for the calculation of the binding constant.

3.3.4 Suspension Approach to Ramipril Formulation

3.3.4.1 Xantham Gum and Xanatural 180 Comparison

Xantham Gum (Sigma) and Xanatural 180 (Azelis) were analysed for viscosity using a Brookfield DV – I + Viscometer at a shear rate of 1.7s-1. Measurements were taken from solutions of Xantham Gum and Xanatural at concentrations of 0.1-0.5% for each polymer and compared.

3.3.4.2 Investigation into the Rate and Volume of Sedimentation

The theoretical rate and volume of sedimentation was calculated using Stokes equation;

$$\frac{\delta V}{\delta T} = \frac{d^2(\rho_s - \rho_t)g}{9n_1}$$

Where:

$\frac{\delta V}{\delta T}$ - Rate of Sedimentation (Velocity/Time)

d^2 - Average Particle Diameter

ρ_s - Solid Particle Density ρ_t - Vehicle Density

n_1 - Viscosity of the Vehicle g – Gravity

The average particle diameter was determined using Sympatec HELIOS particle size analysis. This returned an average particle size of around 70µm. The vehicle viscosity was taken to be viscosity recorded at the lowest shear rate investigated in the

rheology experiment to simulate storage conditions and the vehicle density was calculated via measuring the weight of 1ml of vehicle. The solid particle density was calculated by weighing out a known amount of powder (Wp) followed by weighing out a known volume (Vl) of distilled water (Wl). The ratio of Vl/Wl provides the density (Dl w/v g/dm^3) of the liquid. By adding sufficient water to make the powder and water combination the same volume as Vl and measuring the amount of unused water allows for the calculation of how much volume is take up by the powder. As the weight of the powder was recorded at the start and the volume is now known, the density of the powder was calculated. Gravity was taken to be 980.7cm/s.

For investigation into the actual rate and volume of sedimentation Ramipril suspensions in concentrations of Xanatural from 0.1-0.5%w/v were prepared and were allowed to stand undisturbed for the four weeks. Assessment of the actual sedimentation is obtained by measuring the sedimentation volume (F). This is the ratio of the sediment volume (Vs) to the initial volume of the suspension (Vi);

$$F = \frac{V_s}{V_i}$$

Where:

V_s = Sediment volume V_i = The initial volume of the suspension

3.3.4.3 Particle Size and Investigation into Ostwald Ripening

Ramipril suspensions were produced using concentrations of Xanatural 180 at concentrations of 0.1-0.5% w/v and these were measured for particle size using a Sympatec HELIOS/BF particle size analyser. These suspensions were then be heated and cooled repeatedly with particle size being measured after each cycle. Microscope images were also recorded to document any crystal growth that may have been evident. Each cycle involves heating to 40°C of 24 hours then cooling to 4°C for 24 hours. Three cycles were completed.

3.3.4.4 Analysis of Zeta Potential

The Zeta potential of Ramipril suspensions produced using 0.1-0.5% Xanatural 180 were analysed using a Brookhaven instruments corporation zeta plus zeta potential analyser in conjunction with a BI-ZEL electrode assembly (Brookhaven Instruments).

3.3.4.5 Wettability and Investigation into Surfactant Use

The wettability of Ramipril was investigated using a Camtel Ltd CT-100, CCA-100 contact angle tensiometer. The liquid phases for the Ramipril coated slide to be immersed into consisted of water as a control and then Tween 20, 40, 60 and 80 (Sigma) solutions at concentrations of 0.1, 1.5 and 3.0% were produced and also Span 20, 40, 60 and 80 (Sigma) at concentrations of 0.1, 1.5 and 3.0% were produced in order to determine the effect of surfactant presence on contact angle.

3.3.4.6 Suspension Rheology

Suspension Rheology was investigated to provide information on flow properties by measuring suspension viscosity at different shear rates using a Brookfield DV – I + Viscometer at shear rates of 0.08, 0.16, 0.32, 0.83 and 1.67 reciprocal seconds.

3.3.5 Stability Analysis

The conditions for storage are set out in the ICH Harmonisation Guidelines (Q1A(R2)) (2003) (Table 24) which states that 'a drug substance should be evaluated under storage conditions that test its thermal stability, and if applicable its sensitivity to moisture'. The conditions are listed below;

Table 24 - Storage conditions for stability testing as outlined in the ICH guidelines (ICH Guidelines Q1A(R2) 2003). (R.H = Relative Humidity)

Study	Storage Conditions	Minimum Time Period
Long Term	25°C ± 2°C at 60% R.H ± 5% or 30°C ± 2°C at 65% R.H ± 5%	12 Months
Accelerated	40°C ± 2°C at 75% R.H ± 5%	6 Months

The formulations were tested on a monthly basis and assessed quantitatively by HPLC and for pH. The formulations were also assessed qualitatively for colour and smell.

3.4 Results and Discussion

3.4.1 Co-solvent Approach to the Solubilisation of Ramipril

Following the Solubilisation of 1mg/ml of Ramipril in Acetic acid solutions, the solutions were characterised (Table 25) in order to identify the optimum co-solvent concentration. The colour, smell, pH and the solubilising potential of the solutions was compared. All of the Acetic acid solutions achieved the complete solubilisation of Ramipril to form colourless solutions. It was noted however that the 0.01% (v/v) took considerably longer to achieve solubilisation. As expected the pH of the solutions also became less acidic as the Acetic acid concentration decreased. For all of the solutions, the smell of the acetic acid was negligible.

Table 25 - Co-solvent solution characterisation

Acetic Acid			
Concentration	**Colour**	**pH**	**1mg/ml Ramipril Solubilised**
4.00%	Colourless	2.4	Yes
3.00%	Colourless	2.5	Yes
2.00%	Colourless	2.6	Yes
1.00%	Colourless	2.7	Yes
0.50%	Colourless	2.9	Yes
0.10%	Colourless	3.2	Yes
0.05%	Colourless	3.4	Yes
0.04%	Colourless	3.5	Yes
0.03%	Colourless	3.6	Yes
0.02%	Colourless	3.7	Yes
0.01%	Colourless	3.8	Yes (Slowly)

Acetic acid was selected for use as a co-solvent in the solubilisation of Ramipril as it dissolves Ramipril freely, is GRAS listed and is inexpensive. Also in the production of age appropriate formulations the use of harsh co-solvents and alcohols is to be avoided. Ramipril was successfully solubilised (Table 25) using acetic acid as a co-solvent even at concentrations as low as 0.01% (v/v). This lends itself to use in the production of an oral liquid dosage form as low levels of co-solvent are preferred. This is particularly the case with acetic acid as the unpleasant taste and smell are significant factors. Low concentrations of acetic acid mean that in turn less taste and odour masking agents need be used. Also the use of a low concentration of acetic acid results in a less acidic pH than if high concentrations were required (Table 25). This is beneficial in that it increases the choice of excipients such as antioxidants, preservatives and flavours. The optimum concentration of acetic acid solution for use in disolving Ramipril is 0.02% (v/v) (Table 25). This is the lowest concentration at which rapid solubilisation is achieved at the same time as yielding a suitable pH, colour and odour.

3.4.1.1 Final Formulation for Ramipril Solution Implementing a Co-solvency Approach to Achieve Solubilisation.

Following development of a Ramipril solution using acetic acid as a co-solvent the final formulation is as follows;

- Ramipril (1mg/ml)
- Acetic Acid (0.02% v/v)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (20%)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

For the production of the formulation, a 0.02% v/v acetic acid solution was produced. Into this Ramipril was added and under stirring allowed to fully dissolve before any further excipients were added. With Ramipril dissolved in the acetic acid solution the other excipients were added with flavouring being added as the final excipient.

3.4.2 Cyclodextrin Approach to Solubilising Ramipril

3.4.2.1 Phase Solubility

The phase solubility profile for Ramipril complexed with HP-β-CD is that of a A_L curve over the concentration ranges investigated (Figure 37).

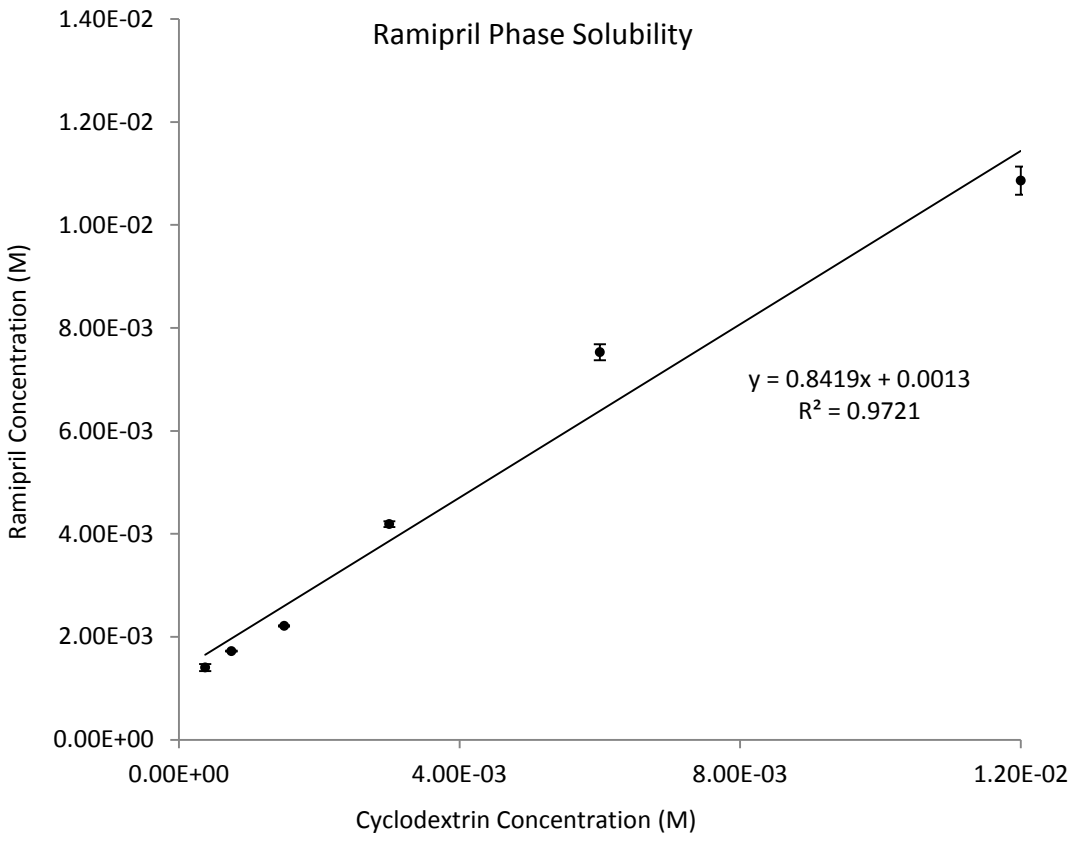


Figure 37 - Phase solubility profile for Ramipril complexed with HP-β-CD. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

This demonstrates a linear relationship between the increase in concentration of drug solubilised and the increase in cyclodextrin required to bring about this increase.

HP-β-CD is a modified β-CD and as a result has improved solubility. This improved solubility means that B_S profiles indicating limited solubility of the complex and B_I profiles indicating insolubility of the complex are rarely seen. More likely are A_L, A_P and A_N type profiles which indicate a linear relationship between the improvement in solubility with increasing cyclodextrin concentration, a positively deviating isotherm and a negatively deviating isotherm respectively (Brewster and Loftsson 2007). The Phase solubility profile for Ramipril complexed with HP-β-CD is that of the A_L type (Figure 37). This is particularly useful as the linear relationship allows for close estimation of the concentration of cyclodextrin needed to solubilise a chosen concentration of Ramipril.

Indications regarding the stoichiometry of the complexation can also be determined from the phase solubility profiles although this requires confirmation using conductometry. Using the gradient from the equation for the line it can be estimated that there is a 1:1 ratio of the binding between Ramipril and cyclodextrin molecules. As the target dosage of the formulation is 1mg/ml which equates to 0.24mM, it can be estimated that 0.24mM of cyclodextrin will be required to solubilise the drug. 0.24mM equates to 3.5mg/ml.

3.4.2.2 Effect of Temperature and pH on Solubilisation following complexation.

Increasing the temperature of the Ramipril-HP-β-CD system in the presence of excess Ramipril increased the amount of Ramipril solubilised over the whole temperature range and for all concentrations of cyclodextrin (Figure 38). The increase is greatest between 15°C and 30°C for the 1.2mM cyclodextrin solution.

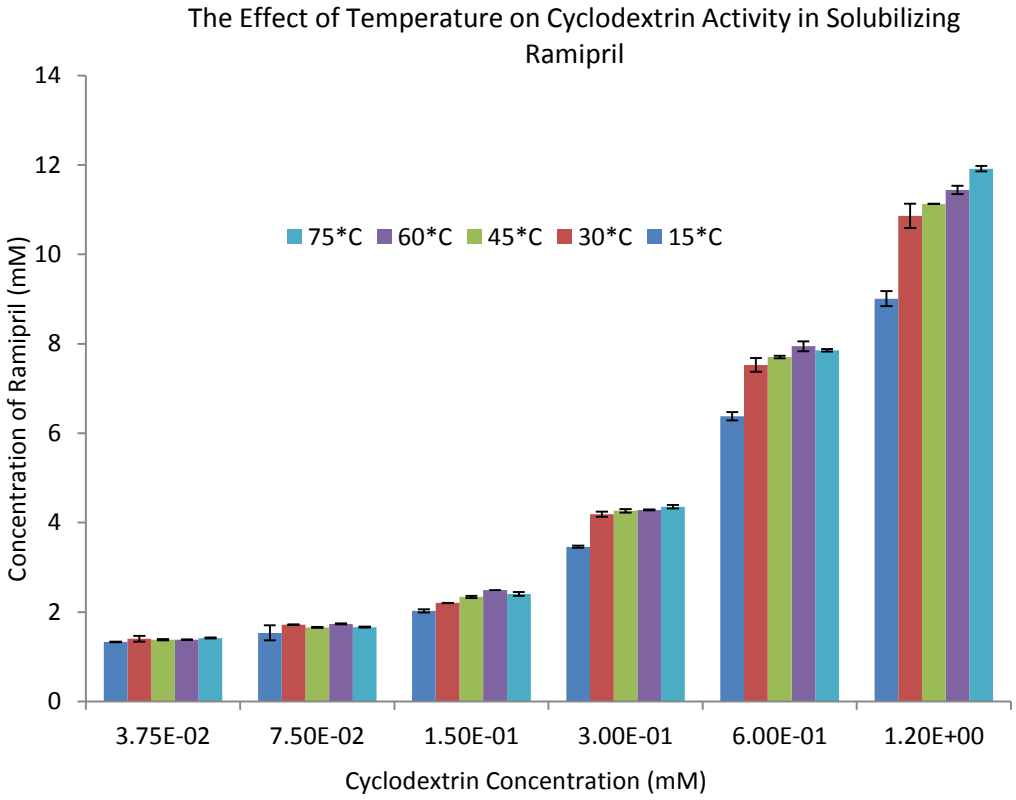


Figure 38 - Effect of temperature on Ramipril solubility in the presence of HP-β-CD. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

As shown below (Figure 39) the optimum pH for the solubilisation of Ramipril using HP-β-CD is pH 3. The pH 5 solution showed slightly poorer solubilisation of Ramipril with pH 7 showing the same reduction again.

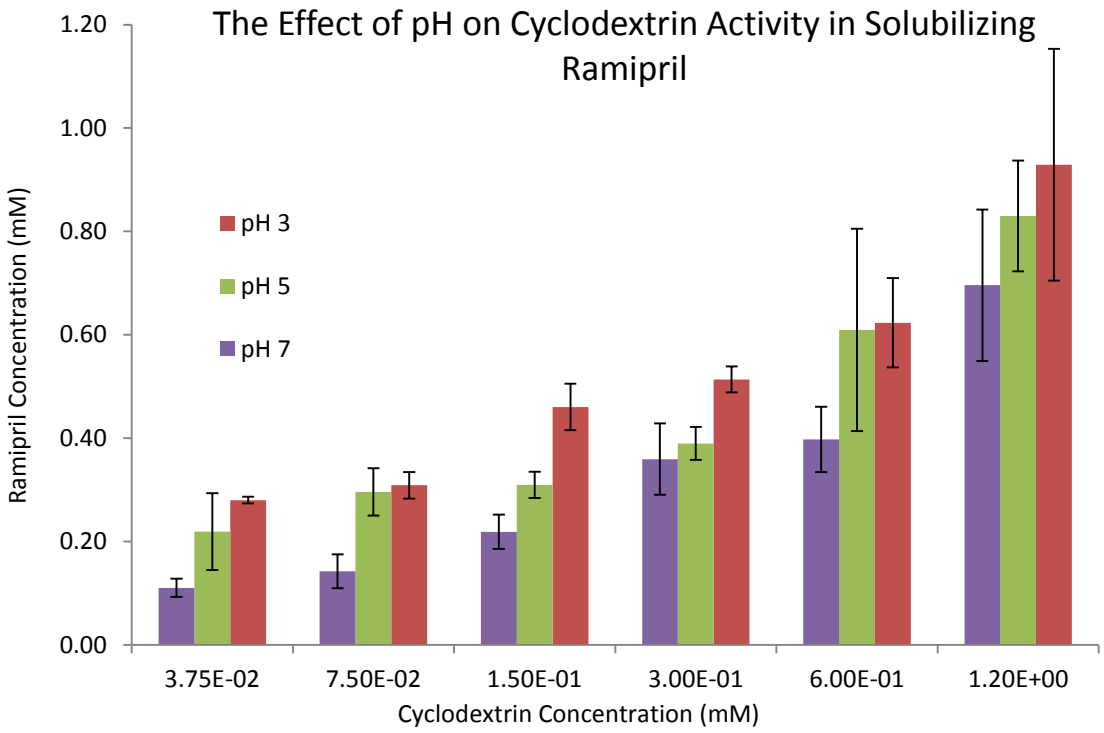


Figure 39 - Effect of pH on Ramipril solubility in the presence of HP-β-CD. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

It has been shown that in some cases, increasing temperature can decrease drug-cyclodextrin complex stability, possibly due to the loss of hydrophobic forces and other forces of interaction. This effect however is not universal and in situations where the drug-cyclodextrin complexation reaction is entropy driven then the effect may be negligible (Challa *et al*. 2005). As seen in Figure 6, increasing temperature does not decrease the amount of Ramipril solubilised meaning the drug molecules are forming complexes with HP-β-CD even at elevated temperatures. This would suggest that there is no decrease in hydrophobic forces or other forces of interaction up to and including 75°C. As the results show there is in fact an increase in the amount of drug solubilized at increasing temperature. This is again potentially further evidence of an entropy driven reaction and also may be contributed to by the increased kinetic energy in the system.

Drug solubility following complexation with cyclodextrin has been show to increase upon modification of the pH of a system. This occurs as a result of the alteration

to the complexation constant following the adjustment of pH. If solubility increases then this resembles an increase in the complexation constant (Kim *et al*. 1998). As (Figure 39) illustrates the amount of Ramipril solubilized by HP-β-CD increases with decreasing pH down to pH 3. This is possibly due to the drug existing in an increasingly more dissociated form at more neutral pH levels. HP-β-CD has been shown by Zia *et al* (2001) to complex to a lesser degree with ionised molecules when compared with their uncharged form. The negative charge present following ionisation of Ramipril could result in repulsion forces between the cyclodextrin and the drug molecule and also between drug molecules and this in turn could lead to a reduction in complexation.

3.4.2.3 Effect of Complexation on Ramipril Stability

As the below chart (Figure 40) shows there is no significant difference in the stability of Ramipril in acid or alkali conditions following complexation with HP-β-CD. Ramipril remains stable in both acidic solutions and Ramipril degrades rapidly in both alkali solutions.

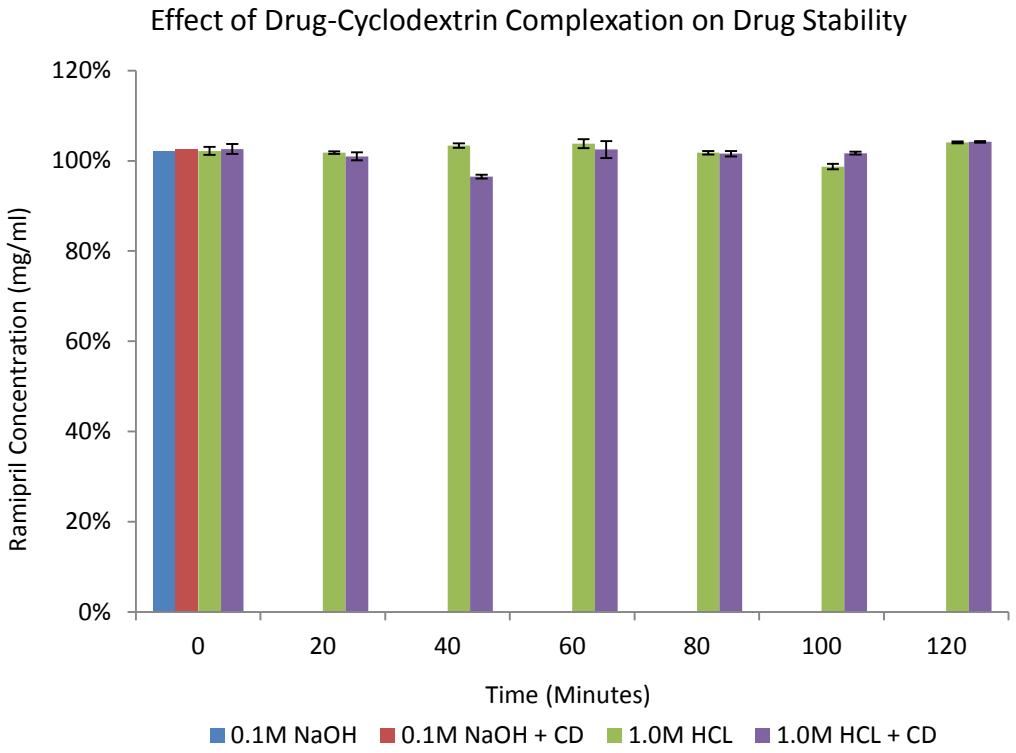


Figure 40 - Effect of complexation on Ramipril stability. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The photostability (Figure 41) of Ramipril is unaffected by complexation with HP-β-CD as there is no significant difference in the stability of Ramipril in either solution following exposure to UV light (254nm) for 24 hours.

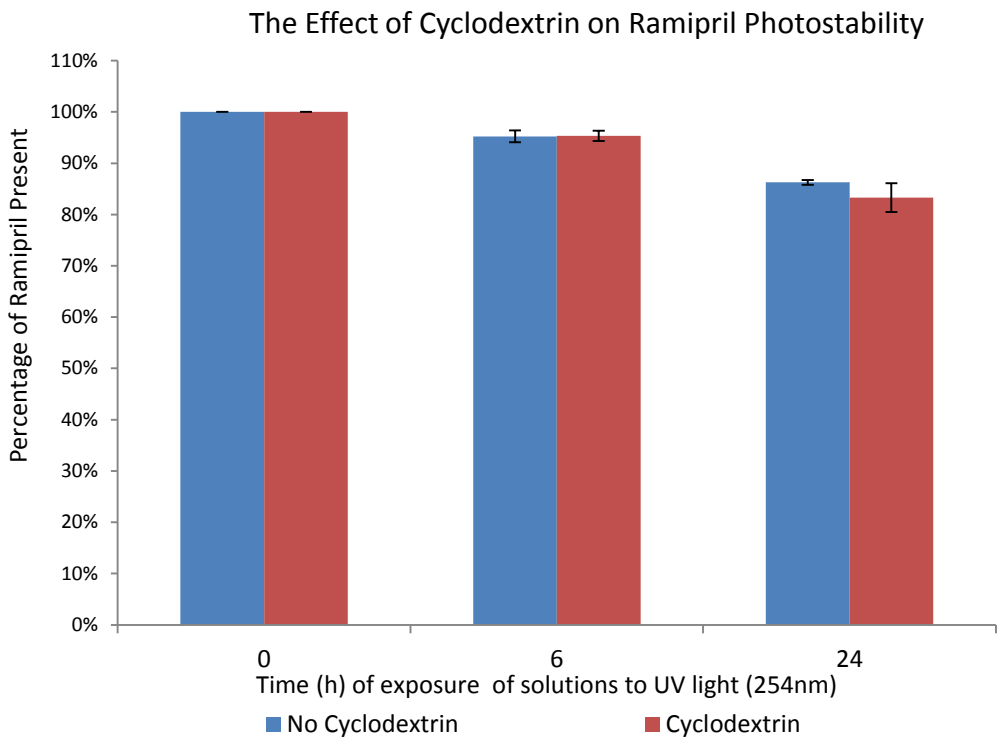


Figure 41 - Effect of HP-β-CD on Ramipril Photostability. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Cyclodextrins have in the past been shown to improve the stability of several liable drugs against dehydration, hydrolysis, oxidation, and photodecomposition (Challa *et al* 2005) (Zhang *et al* 2009). There have also been some cases in which complexation of a drug with a cyclodextrin has resulted in decreased stability when cyclodextrins have catalysed de-acetylation (Jarho *et al* 2000) or when drugs have undergone a structural change up on complexation with cyclodextrin (Sortino *et al* 2001). Other investigations have found the complexation of the drug with cyclodextrins to have no effect on stability. When this occurs it is possible that there is only partial inclusion of the drug inside the cyclodextrin (Koester *et al* 2001). Understanding the effect of complexation on the drug is important as if the stability of a drug is affected then this will have an impact on the shelf life of the product. As figure 40 shows, for the complexation of Ramipril with HP-β-CD there is no significant difference in drug stability in either 0.1M NaOH or 1.0M HCL over time. Ramipril is rapidly degraded in alkali conditions and this is confirmed in the

findings presented here. The fact that Ramipril is still rapidly degraded even in the presence of cyclodextrin suggests that the Ramipril molecules are only partly included in the cyclodextrin leaving some of the molecule susceptible. Ramipril is known to be stable in acid conditions, the findings here show that complexation with HP-β-CD does not contribute to the degradation of Ramipril. Figure 41 illustrates that following the complexation of Ramipril with HP-β-CD there is no significant effect on the Photostability of the drug. Degradation is seen to a small degree for both solutions which again lends strength to the argument that the drug is not completely encapsulated by HP-β-CD.

3.4.2.4 Determination of Ramipril – HP-β-CD Binding Constant and Stoichiometry

Solving the equations of the straight lines produced from the conductometry investigation (Figure 42) simultaneously allows for the accurate determination of the point of convergence. This yields an x value of 5.16mM which is the concentration of Ramipril.

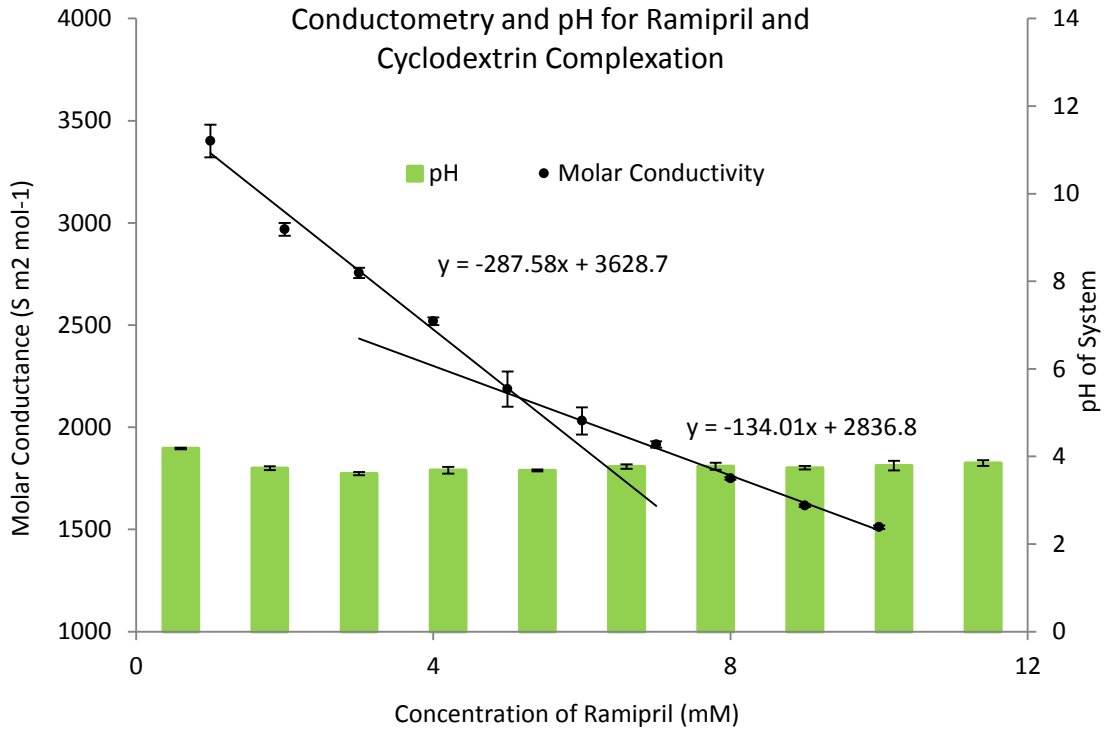


Figure 42 - Conductometry and pH for Ramipril and HP-β-CD complexation. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The stoichiometric ratio is then calculated using;

$$Drug{:}\,Cyclodextrin\ Ratio = \frac{[Drug\ complexed\ (mM)]}{[Cyclodextrin\ used\ (mM)]}$$

D:CD = 5.16/4.8

D:CD = 1.07

This leads to the determination of a 1:1 ratio for the complexation of Ramipril and HP-β-CD. It is now possible to calculate the binding constant (K) for the Ramipril-HP-β-CD system. Where a 1:1 stoichiometry is observed;

Drug (D) + HP-β-CD (CD)

⇅

Drug-CD Complex (D•CD)

With this considered, the equation for the binding constant becomes;

$$K_{1:1} = \frac{[\mathrm{D} \bullet \mathrm{CD}]}{[D][CD]}$$

[D] = Concentration of Free Drug (1.07M)

[CD] = Concentration of Free CD (1.00M)

[D•CD] = Concentration of Drug-CD complex (2.07M)

Applying this gives;

$$K_{1:1} = 1.93\ M^{-1}$$

In order to understand more about the drug-cyclodextrin interaction it is important to understand the stoichiometry of the complexation. That is the ratio of the number of drug molecules complexed with the number of cyclodextrin molecules. The ratio seen in most drug-cyclodextrin complexation is a 1:1 ratio although ratios of 2:1 and 1:2 are not uncommon. Ratios greater than this have been observed however are very rare. Once the stoichiometry is determined then calculations can be performed which allow for the determination of the binding constant (Zarzycki *et al* 1998). The findings from the conductometry investigation (Figure 42) show that Ramipril complexes with HP-β-CD in

a 1:1 ratio and this further supports the conclusions drawn from the phase solubility investigation.

There are a wide range of values for binding constants depending upon the host and guest molecules in question with the majority of the values falling between 50 and 2000M^{-1} with α-CD, β-CD and γ-CD having mean binding constant values of 130, 490 and 350M^{-1} respectively when a stoichiometric ratio of 1:1 is observed (Brewster and Loftsson 2007). Calculation of the binding constant is the main method by which host-guest affinity in solution is assessed. For Ramipril - HP-β-CD complexation the binding constant was calculated to be 1.93M^{-1}. The binding constant is also referred to as the stability constant and a value of 1.93M^{-1} is a relatively low value although there are other examples of low binding constants for example Imidazole with β-CD has a binding constant of 1.9M^{-1} (Tran and Lacerda, 2002). A low binding constant is not necessarily a detrimental factor as although complexation of Ramipril with HP-β-CD is necessary for solubilisation of the drug, the release of the drug from the cyclodextrin is essential for the absorption of the drug.

3.4.2.5 Final Formulation for Ramipril Solution Implementing a Complexation Approach to Achieve Solubilisation.

Following development of a Ramipril solution using cyclodextrins, the final formulation is as follows;

- Ramipril (1mg/ml)
- HP-β-CD (3.51mg/ml)
- Ascorbic Acid (0.1mg/ml)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (20%w/v)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

For the production of the formulation a cyclodextrin solution is first produced by dissolving 3.51mg/ml in distilled water. With this produced the next stage is to add the Ramipril until it is completely dissolved. Following this the other excipients are added until fully dissolved with flavour being added last.

3.4.3 Suspension Formulation Development

3.4.3.1 Selection of a Suspending Agent

Hydrophilic polymers including cellulose derivatives such as Methylcellulose, Ethylcellulose, Hydroxyethylcellulose, Hydroxypropylmethylcellulose and Sodium Carboxymethylcellulose along with Sodium Alginate, Acacia gum, Tragacanth Gum and Xantham Gum were investigated for their usefulness as a suspending agent.

Following investigation into the scientific literature Xantham gum looked the most promising for use in a Ramipril suspension having been proven to display pseudoplastic properties, produce colourless solutions and be soluble in water. Xantham gum is suitable for use in a pH range of 3-12 which has benefits over hydrophilic polymers such

as Ethylcellulose which are sensitive to acid conditions as Ramipril is most stable in an acidic environment. Also in comparison to preparation of some hydrophilic polymer solutions such as methylcellulose which requires careful heating and cooling cycles to achieve solubilisation, Xantham gum solutions can be prepared following simple addition and stirring.

A modified Xantham Gum is available from Azelis under the trade name of Xanatural 180 which provides improved aesthetics and is comparable to the Xantham gum from sigma. Xantham Gum (Table 26) and Xanatural 180 (Table 27) were analysed for viscosity to compare their viscosity modifying properties.

Table 26 - Viscosity table for Xantham Gum at concentrations between 0.5 and 0.1%.

Xantham Gum Viscosity Table (Temperature 25 degrees C)		
Concentration (%w/v)	**Viscosity (mPas)**	**STDEV**
0.5	21.6	0.3
0.4	19.8	0.25
0.3	15	0.36
0.2	5.3	0.04
0.1	2.2	0.01
0	0.3	0.03

Table 27 - Viscosity table for Xanatural 180 at concentrations between 0.5 and 0.1%.

Xanatural 180 Viscosity Table (Temperature 25 degrees C)		
Concentration (%w/v)	**Viscosity (mPas)**	**STDEV**
0.5	22.3	0.15
0.4	20.1	0.15
0.3	15.1	0.3
0.2	5.6	0.04
0.1	2.4	0.04
0	0.4	0.03

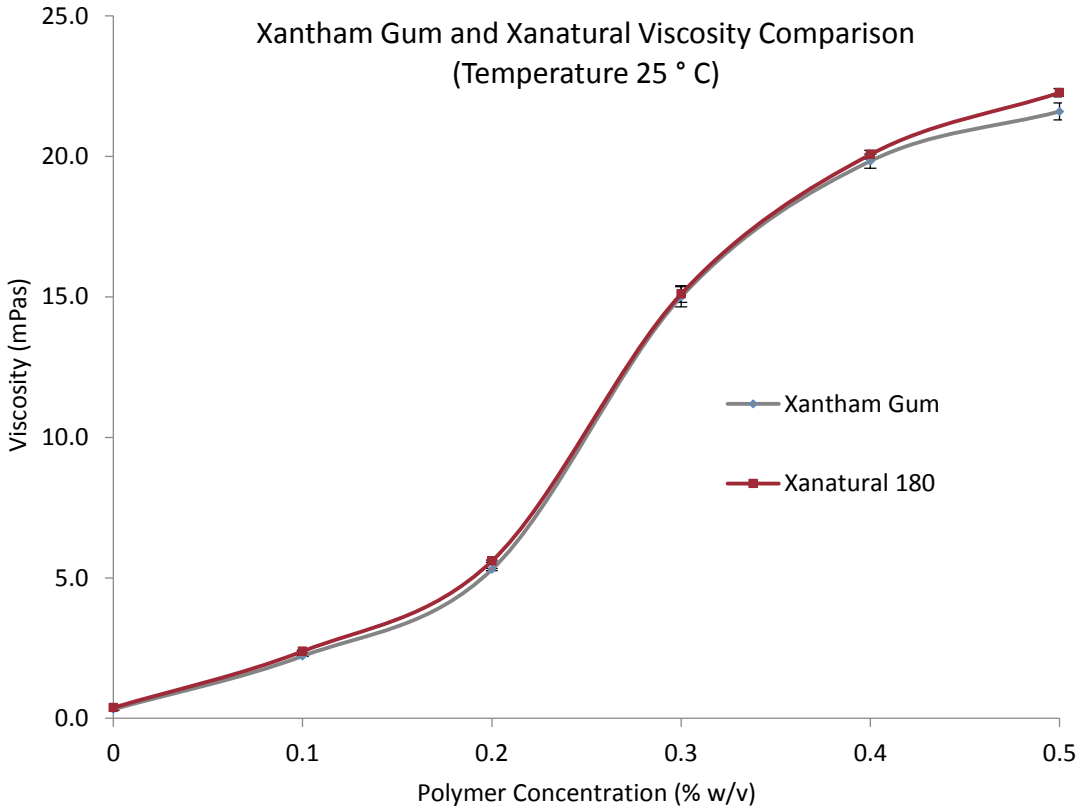


Figure 43 - Comparison of Xantham Gum and Xanatural 180 viscosity at 0.1, 0.2, 0.3, 0.4 and 0.5% w/v. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Following the comparison of Xantham gum and Xanatural 180 there was similar viscosity across the range of concentrations investigated (Figure 43). As the only difference between the two polymers was that Xanatural 180 produced a clear vehicle and Xantham gum produced a vehicle which was turbid in nature, Xanatural 180 was selected for use in the Ramipril suspension. One important concern of suspension production is their aesthetics and the clear suspension is more pleasing on the eye.

3.4.3.2 Suspension Rheology

For oral suspensions hydrophilic polymers such as Xanatural 180 are used as suspending agents and have the effect of increasing the viscosity of the liquid vehicle. This is useful for prolonging the suspension of particles but can at times in the same way make it more difficult to re-suspend any particles which do sediment.

To get the best results from the addition of hydrophilic polymers, the ideal properties for a suspensions resulting flow profile are pseudoplastic or shear thinning and thixotropic. These are non-Newtonian liquids for which the rate of shear is not proportional to the shear stress exerted. This is particularly useful as the relatively high viscosity under low shear conditions e.g. during storage slows the sedimentation process and the relatively low viscosity under high shear conditions e.g. shaking or pouring, allows for the easy re-dispersion of any sediment and also the easy dispensation of the medication.

The shear rate response to changes in shear stress is measured using a viscometer. Five different suspensions were produced using different concentrations of Xanatural between the recommended limits for use. These were analysed at 25°C and at 5 different shear stresses and the results are shown below (Figure 44).

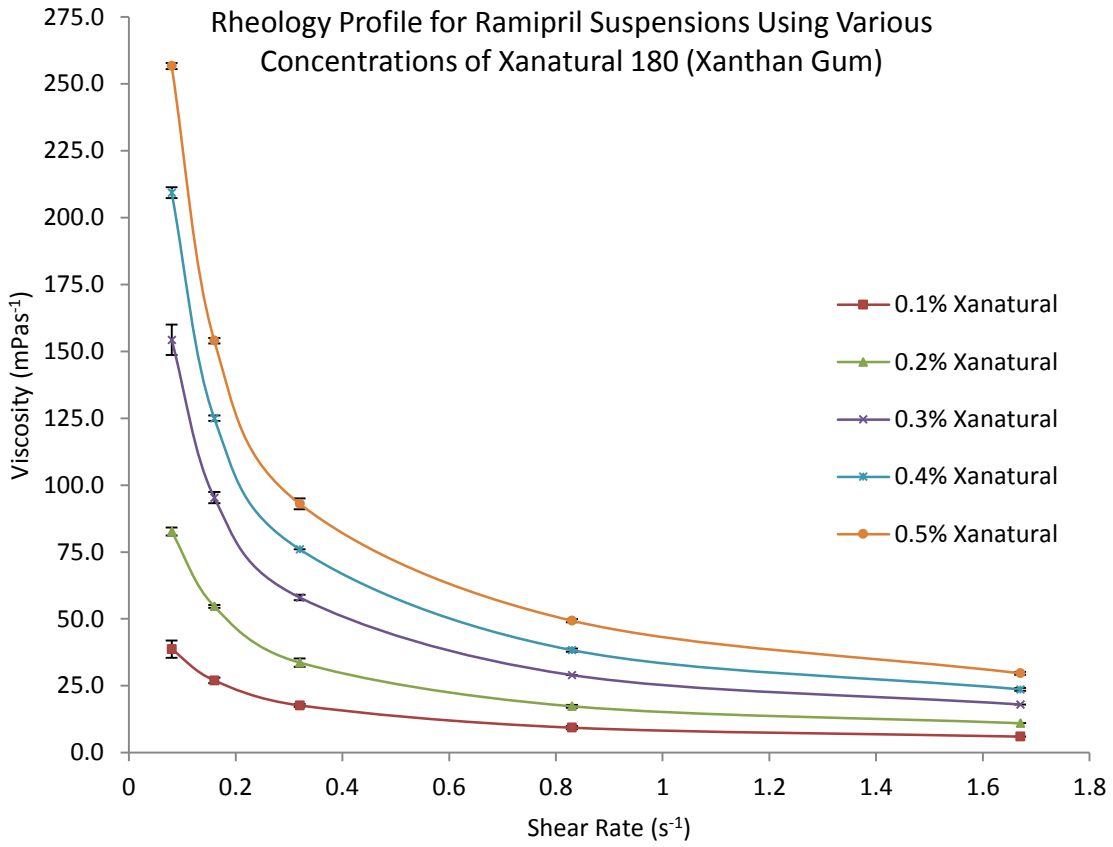


Figure 44 - Chart for the comparison of viscosity under variable shear stress in Ramipril suspensions produced using five different concentrations of Xanatural 180. As expected all concentrations of Xanatural 180 displayed pseudoplastic flow with the viscosity at each measurement point being proportional to the concentration of Xanatural 180 present in the solution. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

As the chart clearly shows, the suspensions produced using Xanatural 180 all display pseudoplastic properties for their rheology. These findings indicate desirable flow properties with the largest change in apparent viscosity observed for the highest concentration of Xanatural (0.5%). All concentrations of Xanatural produced suspension vehicles suitable for use and the selection of the concentration to be used required further development of the suspensions. As such the next stage was to investigate the rate and volume of sedimentation for Ramipril in each concentration of Xanatural.

3.4.3.3 Investigation into the Rate and Volume of Sedimentation

Before producing any suspensions in the lab the theoretical rate of sedimentation was calculated for Ramipril in each concentration of Xanatural (Table 28). This was calculated using Stokes' equation;

Table 28 - Theoretical Rate and Volume of Sedimentation of Ramipril in various concentrations of Xanatural 180.

Theoretical Rate and Volume of Sedimentation						
Xanatural (%w/v)	Particle diameter (cm)	Solid Particle Density (g/cm^3)	Vehicle Density (g/cm^3)	Vehicle Viscosity (g/cm sec)	Gravity (cm/s^2)	Theoretical Rate of Sedimentation (cm/sec)
0.1	0.007	0.43	1.009	0.4	980.7	-0.01
0.2	0.007	0.43	1.012	0.8	980.7	0
0.3	0.007	0.43	1.02	1.54	980.7	0
0.4	0.007	0.43	1.029	2.1	980.7	0
0.5	0.007	0.43	1.034	2.57	980.7	0

As the results suggest then there should be no sedimentation seen in the system however there are more than just the factors of density and viscosity which are responsible for sedimentation in a suspension. As such lab based experiments to confirm that there is no sedimentation were necessary. The suspensions were inspected at time points over a four week period and the results are as follows (Table 29);

Table 29 - Experimental Rate and Volume of Sedimentation of Ramipril in various concentrations of Xanatural 180.

Time Point	Volume Of Suspension (ml)	Volume Of Sediment (cm^2)	Ratio of Suspension volume to Sediment Volume
1 (20 Minutes)	1.5	0	0
2 (40 Minutes)	1.5	0	0
3 (60 Minutes)	1.5	0	0
4 (80 Minutes)	1.5	0	0
5 (100 Minutes)	1.5	0	0
6 (120 Minutes)	1.5	0	0
7 (140 Minutes)	1.5	0	0
8 (160 Minutes)	1.5	0	0
9 (180 Minutes)	1.5	0	0
10 (4 Hours)	1.5	0	0
11 (5 Hours)	1.5	0	0
12 (1 Day)	1.5	0	0
13 (2 Days)	1.5	0	0
14 (3 Days)	1.5	0	0
15 (4 Days)	1.5	0	0
16 (5 Days)	1.5	0	0
17 (1 Week)	1.5	0	0
18 (2 Weeks)	1.5	0	0
19 (3 Weeks)	1.5	0	0
20 (4 Weeks)	1.5	0	0

Following analysis of the sedimentation volume no sedimentation was observed at any of the time points. This has also been reported in previous suspensions using Xanthan Gum as a suspending agent (Nep and Conway 2011). This investigation showed the suspension to remain stable at up to four weeks in storage.

3.4.3.4 Particle Size and Investigation into Ostwald Ripening

A problem with particles in a suspension is the possibility of Ostwald Ripening or crystal growth occurring. This occurs when compounds are suspended in a vehicle in which they are slightly soluble. Upon a temperature increase the smaller particles in the system become soluble. This can lead to the crystallisation of the dissolved drug onto the surface of the larger drug molecules increasing the particle size further. As discussed

previously particle size is directly related to the rate of sedimentation and so an increase in particle size via Ostwald ripening will lead to destabilisation of a stable suspension.

Particle size measurement was performed after heating and cooling cycles to investigate the possibility of Ostwald ripening (Figure 45). The findings from this showed there was no increase in particle size.

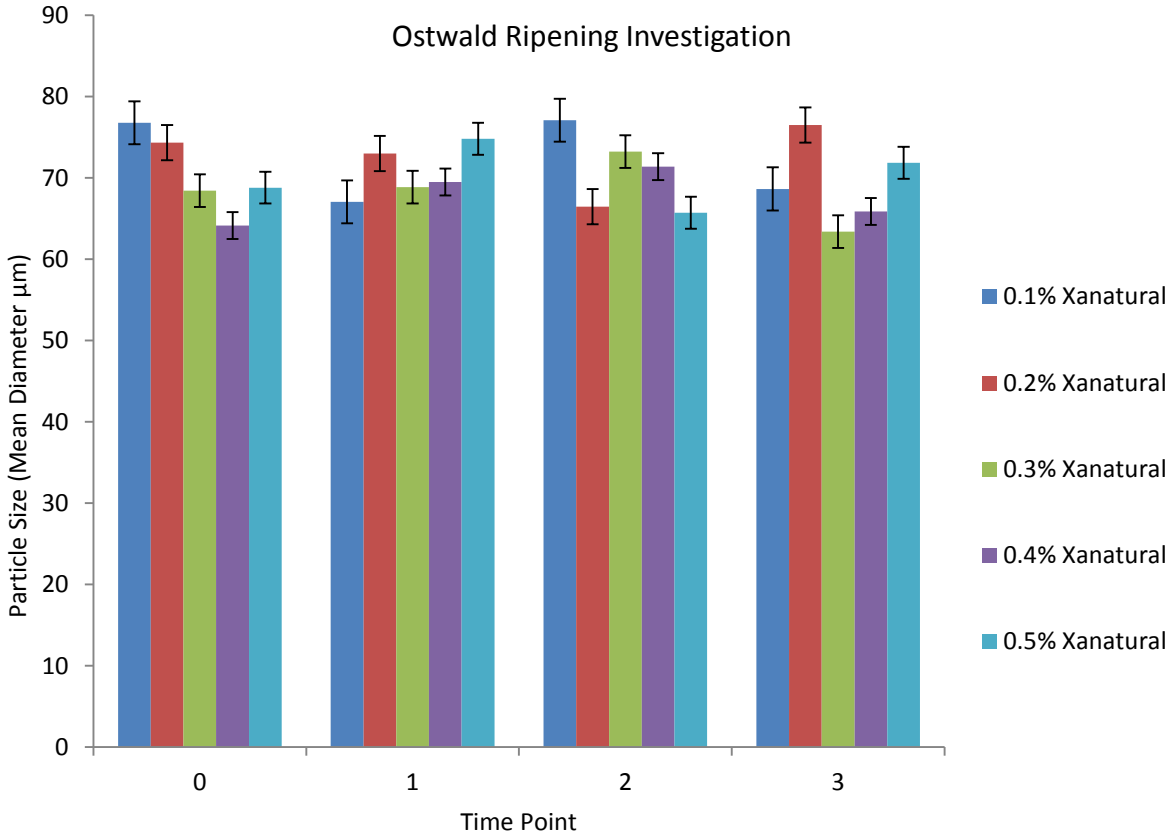


Figure 45 - Particle Size analysis for the investigation into Ostwald ripening. There is no statistical difference in the particle size at each concentration of Xanatural 180 for the duration of the investigation at each Xanatural concentration. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Images of the Ramipril crystals were also taken at each time point for the duration of the Ostwald ripening investigation in an effort to visibly identify any crystal growth that may have been observed. The microscope images follow below (Figures 46 – 65) and it is evident that there is no visibly discernable change in the particles supporting the findings from the particle size measurement.

Time Point 0

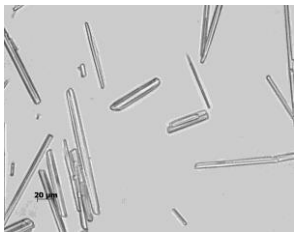


Figure 46 - Time Point 0 - 0.1% Xanatural

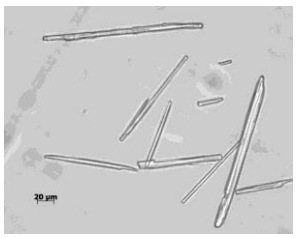


Figure 47 - Time Point 0 - 0.2% Xanatural

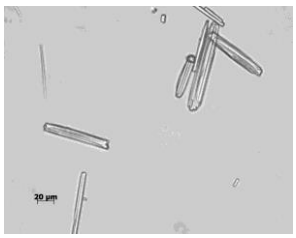


Figure 48 - Time Point 0 - 0.3% Xanatural

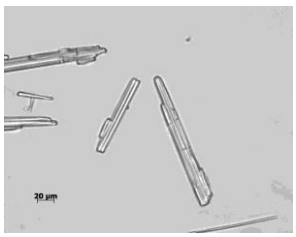


Figure 49 - Time Point 0 - 0.4% Xanatural

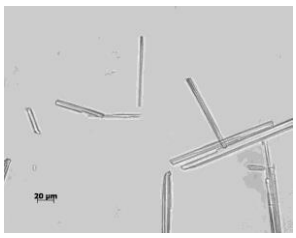


Figure 50 - Time Point 0 - 0.5% Xanatural

Time Point 1

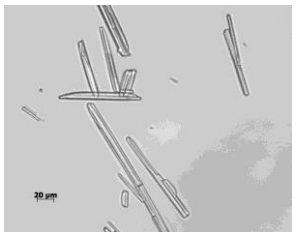


Figure 51 - Time Point 1 - 0.1% Xanatural

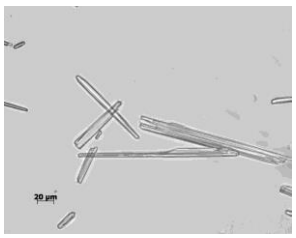


Figure 52 - Time Point 1 - 0.2% Xanatural

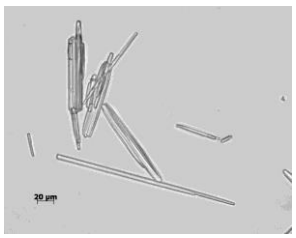


Figure 53 - Time Point 1 - 0.3% Xanatural

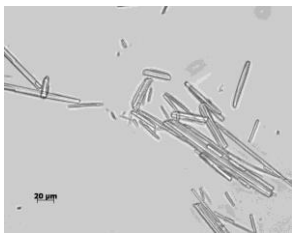


Figure 54 - Time Point 1 - 0.4% Xanatural

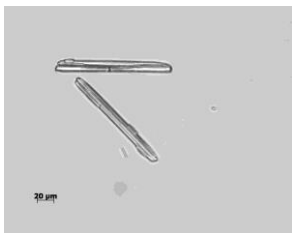


Figure 55 - Time Point 1 - 0.5% Xanatural

Time Point 2

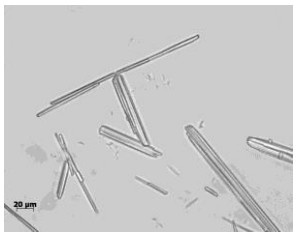


Figure 56 - Time Point 2 - 0.1% Xanatural

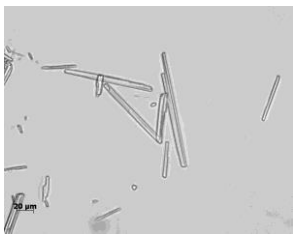


Figure 57 - Time Point 2 - 0.2% Xanatural

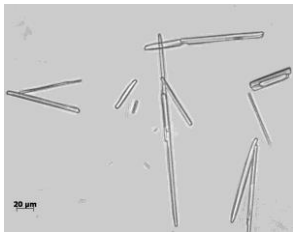


Figure 58 - Time Point 2 - 0.3% Xanatural

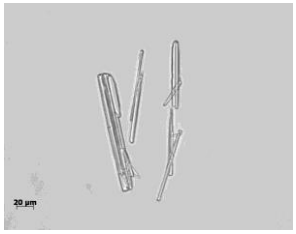


Figure 59 - Time Point 2 - 0.4% Xanatural

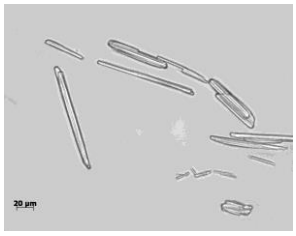


Figure 60 - Time Point 2 - 0.5% Xanatural

Time Point 3

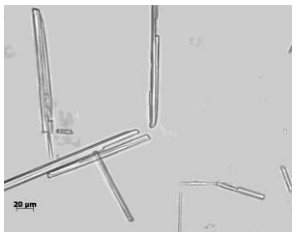


Figure 61 - Time Point 3 - 0.1% Xanatural

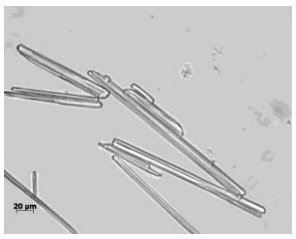


Figure 62 - Time Point 3 - 0.2% Xanatural

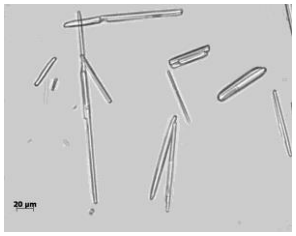


Figure 63 - Time Point 3 - 0.3% Xanatural

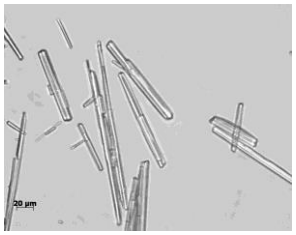


Figure 64 - Time Point 3 - 0.4% Xanatural

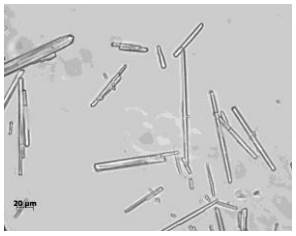


Figure 65 - Time Point 3 - 0.5% Xanatural

If Ostwald ripening had occurred then altering the amount of hydrophilic polymer or implementing the use of a different hydrophilic polymer may have been required for use in the formulation as hydrophilic polymers form a protective layer around drug molecules which should protect the formulation from Ostwald ripening.

3.4.3.5 Analysis of Zeta Potential

The Zeta potential of a suspension can be carefully modified if necessary in order to produce floccules which sediment reversibly and therefore stabilize formulations which display sedimentation. The use of electrolytes are the main method of modifying zeta potential. In order to identify the most useful concentration of electrolyte, the rate and volume of sedimentation must be assessed. Following electrolyte modification the suspension which displayed the largest sediment volume would be deemed the most stable as the particles in the suspension would be interacting the most weakly. There is however, no sedimentation observed for the Ramipril suspension using Xanatural 180 then this process cannot be investigated. The zeta potential for the suspension can however be measured in order to see if Xanatural concentration has any effect.

Measuring the Zeta potential in suspensions made using concentrations of Xanatural at 0.1, 0.2, 0.3, 0.4 and 0.5% would indicate if the concentration of hydrophilic polymer had any effect on the zeta potential. The larger the magnitude of the Zeta potential the better the stability of a suspension. Following analysis of the Zeta potential in suspensions made using concentrations of Xanatural at 0.1, 0.2, 0.3, 0.4 and 0.5% the results were as follows (Figure 66).

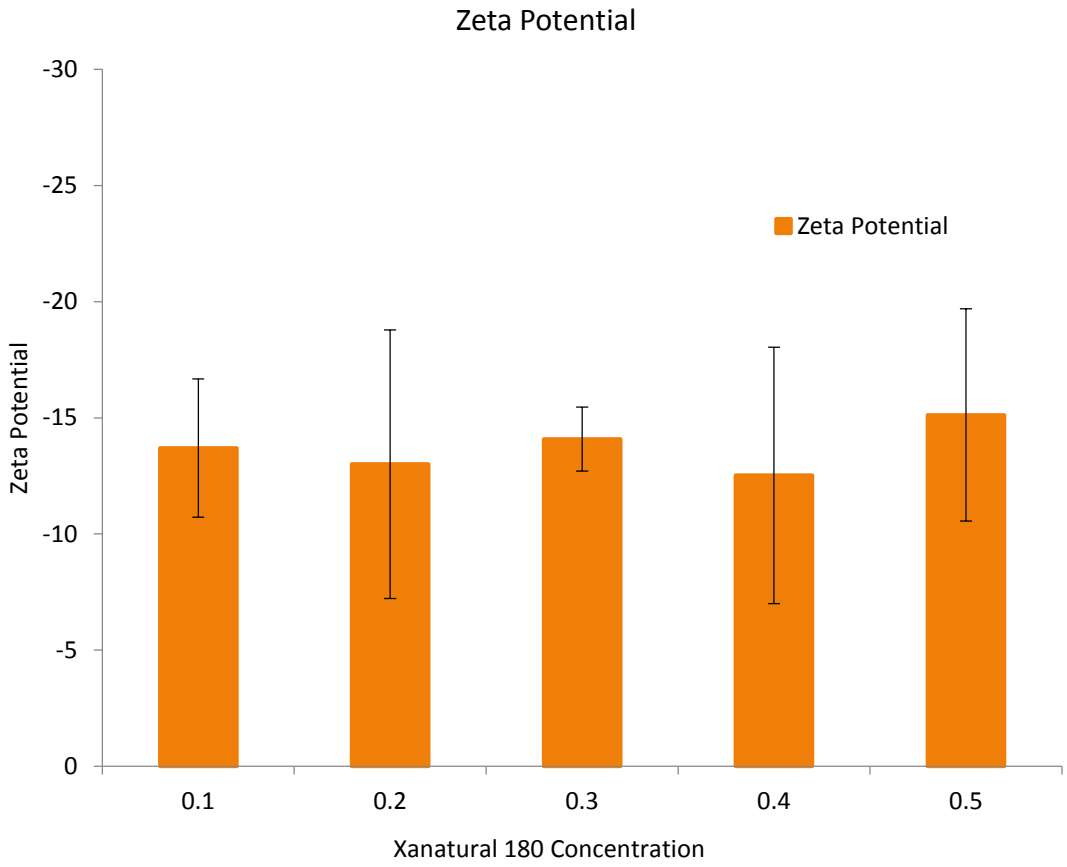


Figure 66 - Zeta potential of Ramipril suspension using different concentrations of Xanatural 180. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

There was no significant difference between the Zeta potential measured in each suspension regardless of the Xanatural concentration present. The Zeta potential measured is within +/-25mV and this is in the range where suspensions are known to produce non-caking suspensions (Greenwood & Kendall 1999). This is confirmed by the finding of a lack of sedimentation seen in the rate and volume of sedimentation investigation. As such the modification of the zeta potential to control flocculation is deemed to be unnecessary in the production of the Ramipril suspension.

3.4.3.6 Wettability and Investigation into Surfactant Use

An insoluble or poorly soluble drug may not be easily wetted, that is that the vehicle in which the particles are suspended may not easily form a layer around the suspended drug particle. In order for maximum Wettability the angle at which the liquid meets the solid particles surface needs to be low. The contact angle is defined in terms of the tensions between three phases, these being;

- Solid/Vapour ($\gamma_{s/v}$)
- Liquid/Vapour ($\gamma_{l/v}$)
- Solid/Liquid ($\gamma_{s/l}$)

Young's equation is used to describe Wettability;

$$\gamma_{l/v}\, cos\theta = \gamma_{s/v} - \gamma_{s/l}$$

Decreasing the interfacial tensions in the Liquid/Vapour and the Solid/Liquid will lead to a reduction in the contact angle. This is attained by the addition of surfactants into the formulation which adsorb at the Liquid/Vapour and the Solid/Liquid interfaces reducing the tension.

Poorly wetted drug particles have a tendency to clump together in an attempt to thermodynamically stabilise the system by lowering the Gibb's free energy. This clearly would cause problems in the stability of a suspension. Sufficiently wetted particles are also necessary for the production of homogenous suspensions and this is essential for the correct dosage. A final consideration is that increased wetting has the added benefit of improved absorption which can lead to better drug performance.

Wettability of a drug is investigated using a surface tensiometer to determine the contact angle for that compound. If the contact angle requires improvement then the use of surface active agents in the formulation may be necessary. If any alterations are made to the formulation the rate of sedimentation and zeta potential should be re-investigated to provide a comparison. The surfactants selected for use were SPAN 20, 40, 60 and

80 and also TWEEN 20, 40, 60 and 80. Both of these are widely used in the pharmaceutical industry and are readily available. The Wettability of Ramipril was first investigated using only distilled water to provide a reference. Surfactants producing a reduced receding contact angle would be considered to have improved the Wettability of the drug. The results are shown below (Figure 67);

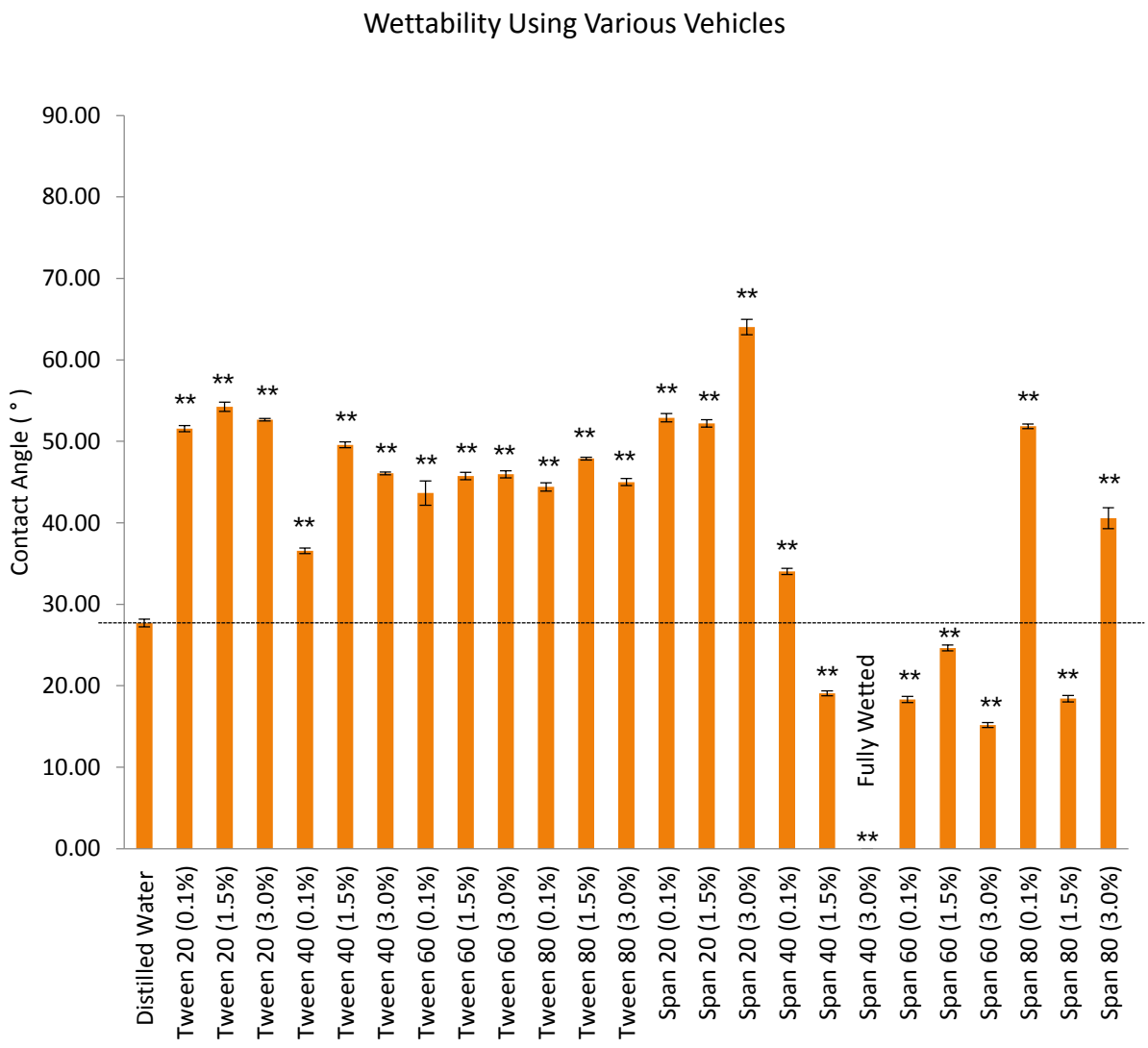


Figure 67 - Investigation into improving the wettability of Ramipril using various surfactants. (**) Indicates significant difference at p=0.001 when compared to distilled water. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

As the above chart indicates the wettability was improved most effectively by SPAN 40 at a concentration of 3% v/v. As such if there had been and problems experienced which involved the clumping of Ramipril in the suspension then the use of SPAN 40 as a surfactant to prevent this would be the first choice.

3.4.3.7 Final Suspension Formulation

The final composition of the suspensions are as follows;

Flavoured Suspension:

- Ramipril (1mg/ml)
- Xanatural 180 (0.1% w/v)
- Sodium Metabisulphate (1.0% w/v)
- Sodium Benzoate (0.5% w/v)
- Xylitol (40% w/v)
- Strawberry Concentrate (0.2%v/v)

Sweetened Suspension:

- Ramipril (1mg/ml)
- Xanatural 180 (0.1% w/v)
- Sodium Metabisulphate (1.0% w/v)
- Sodium Benzoate (0.5% w/v)
- Xylitol (40%w/v)

The suspensions are produced by first producing a 0.1% Xanatural 180 solution. Into this Ramipril is then added slowly during vigorous stirring and evenly distributed throughout the vehicle. The remaining excipients are then added to the formulation with the strawberry concentrate being added as the last ingredient.

3.4.4 Stability Analysis

3.4.4.1 Accelerated Conditions

Over the six month course of the stability testing in accelerated conditions with 40°C and 75% relative humidity, the drug content of the formulations fell below 95% of the starting dose indicating that the formulations failed to display adequate stability in accordance with ICH guidelines (Figure 68). The solutions produced behaved in a comparable manner however the suspension formulation showed a greater degree of degradation by the 6 month time point. The pH remained constant for the duration of the stability testing (Figure 69).

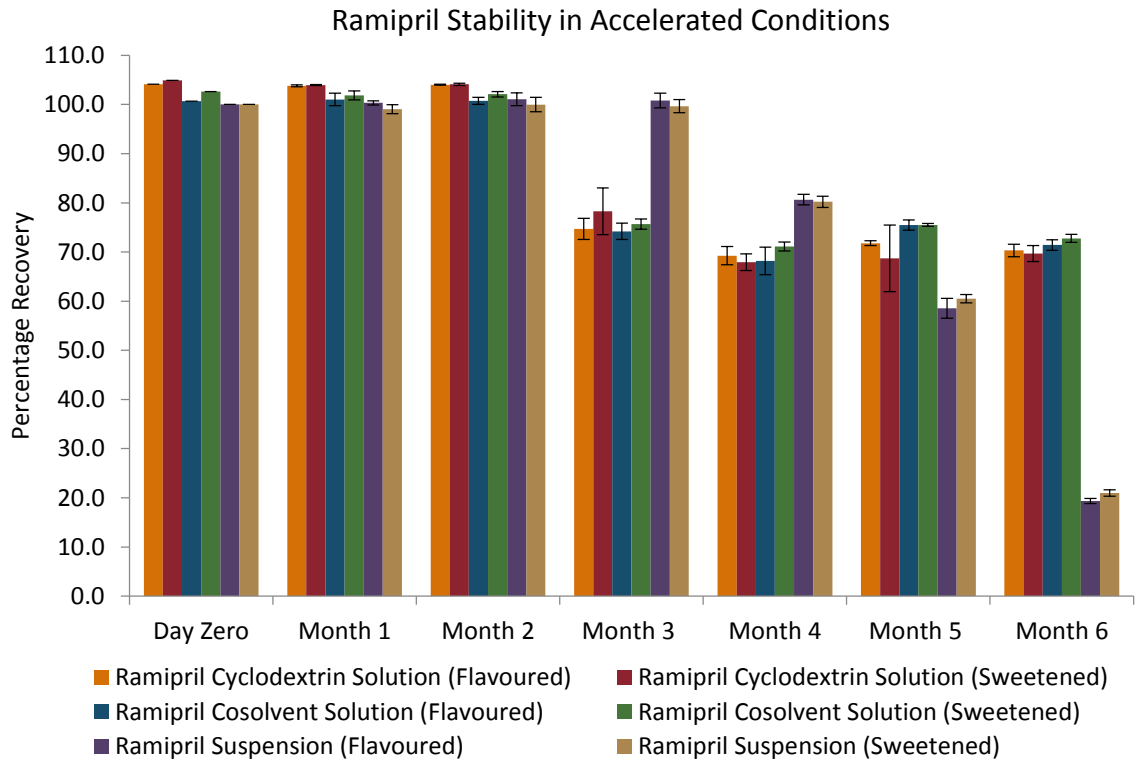


Figure 68 - Ramipril recovery following HPLC for formulations in Accelerated storage conditions. Before the third month of testing the formulations proved stable with little variation in drug content and consistent drug content between samples of the same batch. From the third month onwards a reduction of 30% to 80% of the drug content was seen when compared with the starting concentration. The variation between samples

also increased. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

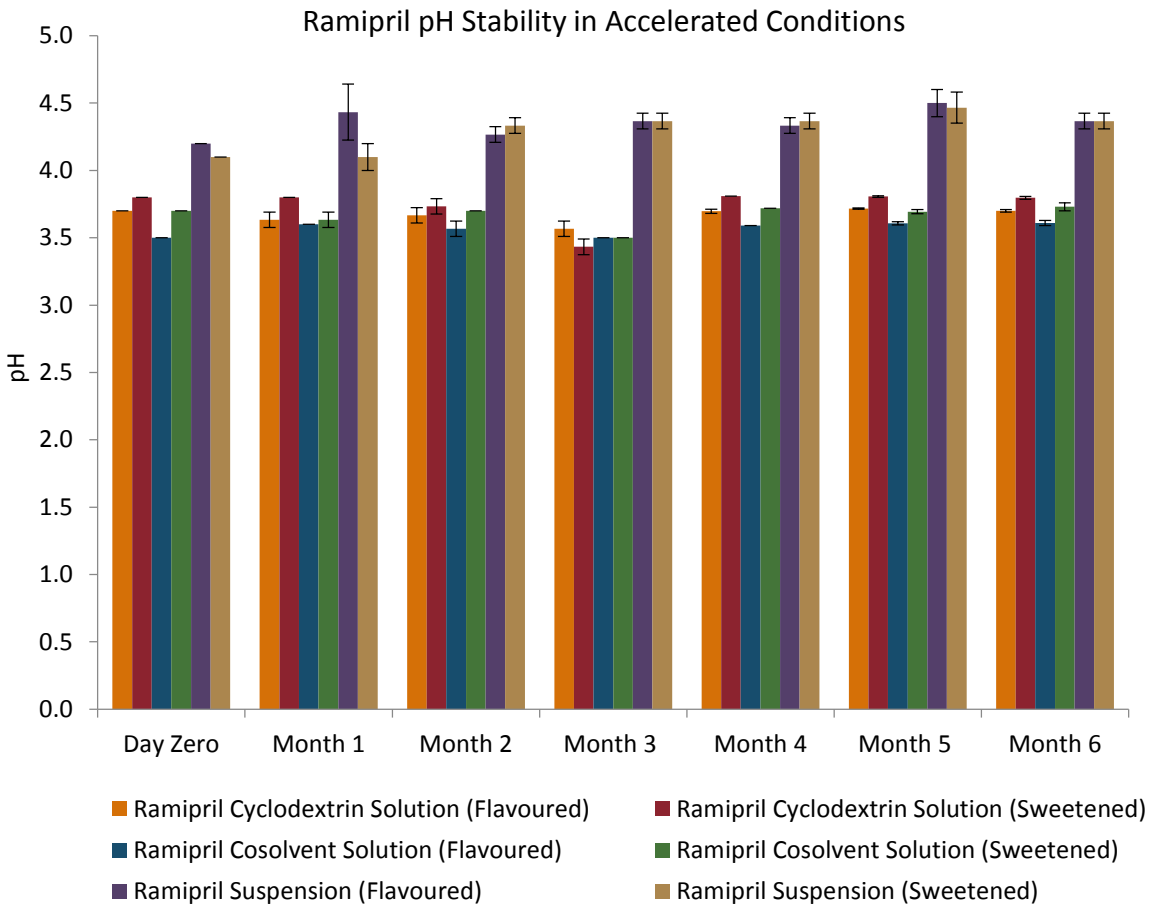


Figure 69 - Ramipril pH stability for formulations in Accelerated storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

3.4.4.2 Long Term Conditions

The twelve month testing course of the formulations stability in long term conditions, 25°C and 60% relative humidity, the drug content of all of the formulations remained >95% of the starting dose (Figure 70) and the pH remained constant for the duration (Figure 71).

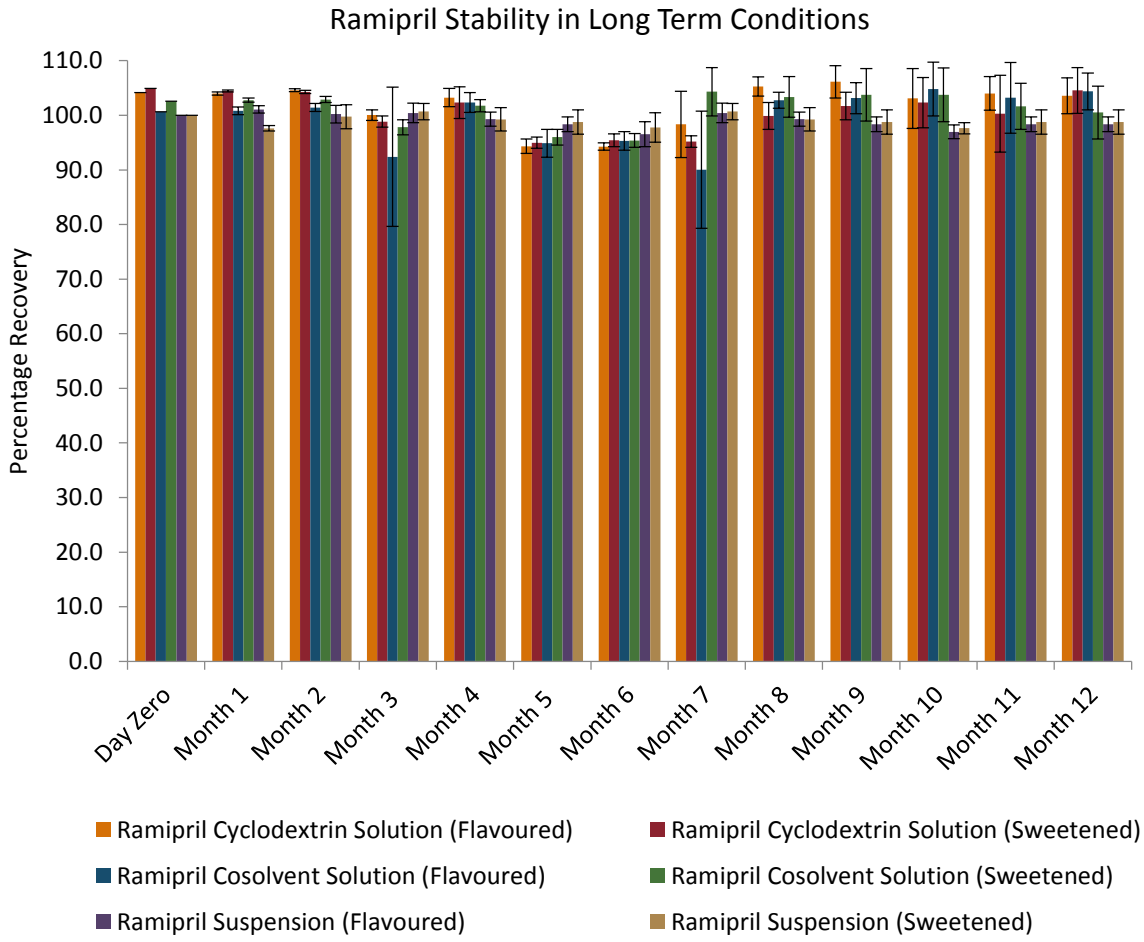


Figure 70 – Ramipril recovery following HPLC for formulations in Long Term storage conditions. For the duration of testing the formulations proved stable with little variation in drug content and consistent drug content between samples of the same batch. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

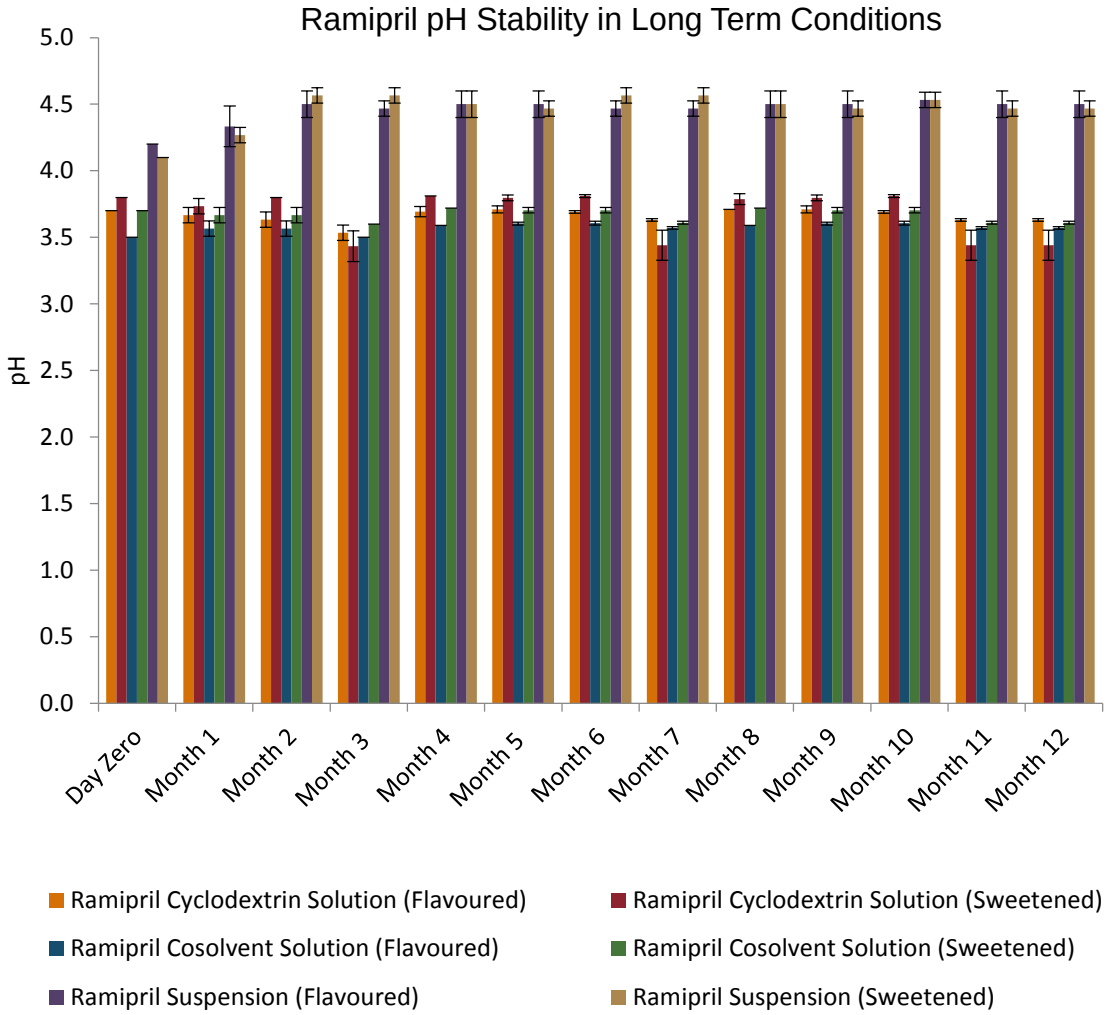


Figure 71 - Ramipril pH stability for formulations in Long Term storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

According to the parameters outlined by the ICH harmonised tripartite guideline stability testing of new drug substances and products Q1A(R2**)**, "significant change" for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation products exceeding its acceptance criterion;

3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., colour, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH; or
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

The Ramipril formulations tested under accelerated conditions showed significant decrease in the drug assayed in comparison to the day zero analysis (Figure 68). This was seen in all formulations regardless of the method of formulation. The formulations in long term storage conditions (Figure 70) did not show any significant decrease in the drug assayed even after 12 months in storage. This suggests that the degradation seen under accelerated conditions is a result of the elevated temperature. It is known that Ramipril diketopiperazine (DKP) degradate is the primary degradation compound for Ramipril. Degradation occurs through intramolecular condensation and sees the formation of a second amide bond (Figure 72).

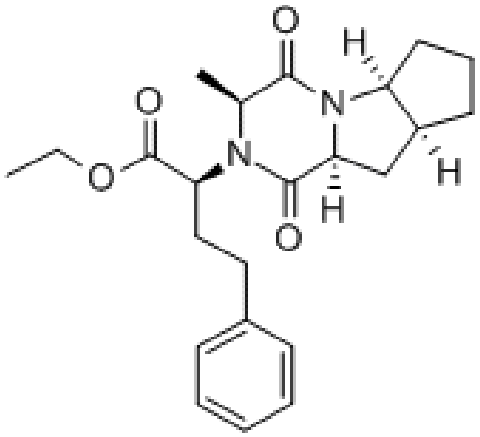


Figure 72 - Ramipril DKP – The primary degradation compound for Ramipril.

This reaction is known to occur with Ramipril as was seen with Lisinopril when the drug experiences conditions of elevated temperature (Diego *et al* 2010). The pH of the formulation in both accelerated (Figure 69) and long term conditions (Figure 71) did not change beyond the acceptance levels for the formulation. In addition, the appearance and odour of the formulations remained acceptable for the duration of the stability testing and in both long term and accelerated conditions. The advice for storage of the medication would have to emphasise storage in a cool location (<25°C), the humidity of the storage conditions is not a large issue as the medication will be contained within glass bottles impervious to moisture. With regards to excursions outside of the storage conditions such as transport during distribution, degradation due to temperature should not be a concern so long as excursions are short in duration and infrequent. This is as degradation was only considered significant after >1 month and the temperature at which degradation was seen is higher than most environmental temperatures the medication would experience.

4 Reformulation of Spironolactone to Produce an Age Appropriate Oral Liquid Dosage Form Targeting the Paediatric Patient Segment

4.1 Chapter Aims and Objectives

- Develop a HPLC method for the detection of Spironolactone and validate according to ICH guidelines.
- Formulate Spironolactone as an oral liquid dosage form with a particular focus on effectively targeting the paediatric patient segment.
- Characterise formulations including stability testing in long term and accelerated conditions as specified by ICH guidelines.

4.2 Introduction

Spironolactone is a competitive aldosterone antagonist, acting at the distal convoluting renal tubule, thus increasing sodium and water excretion and reducing potassium elimination. It is extensively used in medicine, though until recently it was considered only as a potassium-sparing diuretic and antihypertensive drug. Spironolactone is used in adults for the treatment of oedema and ascites in cirrhosis of the liver, malignant ascites, nephrotic syndrome, oedema in congestive heart failure, moderate to severe heart failure (adjunct), resistant hypertension (adjunct) and primary hyperaldosteronism in patients awaiting surgery. It is used in neonates, infants and children with congestive heart failure secondary to congenital heart disease. In children under 18 years of age it is used for oedema in heart failure and in ascites, nephrotic syndrome and reduction of hypokalaemia induced by diuretics or amphotericin. In addition to licenced uses, it may also reverse aldosterone-induced cardiac fibrosis, improve morbidity and survival of patients with congestive heart failure (Paediatric Formulary Committee 2012).

As with many other frequently used drugs, spironolactone is available only as tablets (Table 30), rather than in liquid dosage form suitable for paediatric use. Over the last 25 years, many extemporaneously prepared spironolactone containing oral liquid formulations have been reported in the literature, as well as their physical and chemical

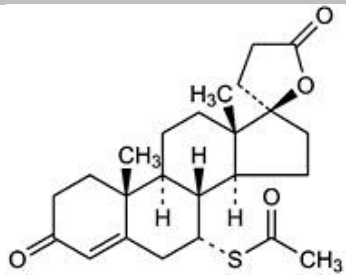
stability. The latter is an important issue in pharmaceutical compounding because medicines must remain stable and efficacious during the course of their use. To improve stability, or even drug solubility, most of the described spironolactone formulations contain complex vehicle mixtures, including propyleneglycol, glycerine and alcohol. However, the use of these excipients in the concentrations described is not desirable in infants.

Table 30 - Currently Available Spironolactone Dosage Forms

Available Dosage Forms	
Aldactazide 25-25 mg tablet	Novo-Spiroton 100 mg Tablet
Aldactazide 25-25 tablet	Novo-Spiroton 25 mg Tablet
Aldactazide 50-50 mg tablet	Spironolactone 100 mg tablet
Aldactazide 50-50 tablet	Spironolactone 25 mg tablet
Aldactone 100 mg tablet	Spironolactone 50 mg tablet
Aldactone 25 mg tablet	Spironolactone powder
Aldactone 50 mg tablet	Spironolactone-HCTZ 25-25 mg tablet

As discussed in the introduction, given the recommendation for the production of an age appropriate formulation, coupled with the need for dose flexibility and the lack of an oral liquid formulation, the production of an oral liquid formulation created with a focus on paediatric use would be highly advantageous. The physiochemical properties of Spironolactone (Table 31) as with any drug guide the formulation development.

Table 31 - Spironolactone Monograph listing important physiochemical properties which should be considered before formulation.

Spironolactone Monograph	
Chemical Name	(7α,17α)-7-(Acetylthio)-17-hydroxy-3-oxopregn-4-ene-21-carboxylic acid γ-lactone
Other Names	Aldactone
Molecular Formula	$C_{24}H_{32}O_4S$
Molecular Weight	416.6
CAS Number	52-01-7
Spironolactone Structure	
Appearance	Light cream to light tan crystalline powder
Solubility	Spironolactone is practically insoluble in water but is soluble in alcohol.
Sensitivities	Spironolactone is sensitive to strong oxidising agents.

The aim of the presented research encapsulates the formulation of, and characterization of an oral Spironolactone suspension, including the production of a

suitable HPLC method, excipient selection and stability assessment of the formulations in accordance with ICH guidelines.

4.3 Materials and Methods

4.3.1 HPLC Method Development for the Detection of Spironolactone

The Spironolactone HPLC method was developed using a Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler. The stationary phase is a Phenomenex Gemini 5µ C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size). The detection wavelength was set at 254nm. The injection volume is 20µl with a run time of 10 minutes. Preparation of Calibration Standards involves the production of six standards produced via serial dilution in mobile phase. Standards at 2.0, 1.0, 0.5, 0.25, 0.125 and 0.0625mg/ml and samples are prepared for analysis by dilution so as to fall within the calibration range and were diluted with Spironolactone mobile phase. Method validation was carried out following ICH Guidelines (Q2(R1)).

4.3.2 Formulation Development

The Spironolactone for the formulation was purchased from Discovery fine chemicals while citric acid, Propyl Paraben, Butyl Paraben and Xylitol were all supplied by Sigma. The flavour concentrates used in the formulation development were free samples provided by S. Black's (Azelis). Formulations were produced using volumetric glassware. Ultrapure water was first added into which excipients were dissolved with the aid of a magnetic stir bar before it was removed and the solutions made up to volume. Upon production formulations were transferred to amber glass bottles.

4.3.3 Sol-Gel Transition Temperature

Pluronic was used at concentrations of 0, 1, 2, 3, 4 and 5%w/v and Xanatural was used at concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5%w/v. Samples were heated to 15, 25, 35, 45, 55, 65, 75 and 85°C and observed for any change in the liquid state.

4.3.4 Suspension Rheology

Suspension Rheology was investigated to provide information on flow properties by measuring suspension viscosity at different shear rates using a Brookfield DV – I + Viscometer at shear rates of 0.08, 0.16, 0.32, 0.83 and 1.67 reciprocal seconds.

4.3.5 Rate and Volume of Sedimentation

For investigation into the rate and volume of sedimentation spironolactone suspensions in concentrations of Xanatural from 0.1-0.5%w/v and Pluronic F127 from 1-5%w/v were prepared and were allowed to stand undisturbed for 24 hours. Assessment of the actual sedimentation is obtained by measuring the depth of sedimentation.

4.3.6 Particle Size Measurement and Investigation into Ostwald Ripening

Ramipril suspensions were produced using concentrations of Xanatural 180 at concentrations of 0.1-0.5% w/v and Pluronic F127 from 1-5%w/v, these were measured for particle size using a Sympatec HELIOS/BF particle size analyser. These suspensions were then be heated and cooled repeatedly with particle size being measured after each cycle. Microscope images were also recorded to document any crystal growth that may have been evident. Each cycle involves heating to 40°C of 24 hours then cooling to 4°C for 24 hours. Three cycles were completed.

4.3.7 Analysis of Zeta Potential

The Zeta potential of Ramipril suspensions produced using 0.1-0.5% Xanatural 180 Pluronic F127 from 1-5%w/v were analysed using a Brookhaven instruments

corporation zeta plus zeta potential analyser in conjunction with a BI-ZEL electrode assembly (Brookhaven Instruments).

4.3.8 Stability Analysis

The conditions for storage are set out in the ICH Harmonisation Guidelines (Q1A(R2)) (2003) (Table 32) which states that 'a drug substance should be evaluated under storage conditions that test its thermal stability, and if applicable its sensitivity to moisture'. The conditions are listed below;

Table 32 - Storage conditions for stability testing as outlined in the ICH guidelines (ICH Guidelines Q1A(R2) 2003). (R.H = Relative Humidity)

Study	Storage Conditions	Minimum Time Period
Long Term	25°C ± 2°C at 60% R.H ± 5% or 30°C ± 2°C at 65% R.H ± 5%	12 Months
Accelerated	40°C ± 2°C at 75% R.H ± 5%	6 Months

Samples were stored in Firlabo SP-BVEHF stability cabinets and analysed on a monthly basis, quantitatively for drug recovery via HPLC and pH and qualitatively for colour and odor.

4.4 Results and Discussion

4.4.1 HPLC Method Development for the Detection of Spironolactone

The Spironolactone HPLC method developed was based loosely around the method developed by Allen and Erickson (1996) in which using HPLC, the stability of Spironolactone 5 mg/mL in extemporaneously compounded oral liquids was evaluated. The system was a Hewlett-Packard series 1050 liquid chromatograph, including a multisolvent mixing and pumping system, an autoinjector, a diode-array detector, and a computer with Chem Station software. The stationary phase was a Bakerbond C_{18}

analytical column (250 × 4.6 mm, 5-µm particle size). The mobile phase was 79% methanol in water; it was delivered isocratically at 1.0 mL/min.

Initial runs involved alterations to the Allen method described above in that the mobile phase was changed from methanol and water to acetonitrile and water (70:30). It is widely recognized that acetonitrile is a preferential solvent over methanol in HPLC applications thanks to its reduction of retention time and improvement in UV absorption properties. Also the preliminary injection volume was 20µl. Using a Thermofisher C18 column and a flow rate of 1.00ml/ml with the absorption wavelength set to 254nm the following trace was produced (Figure 73);

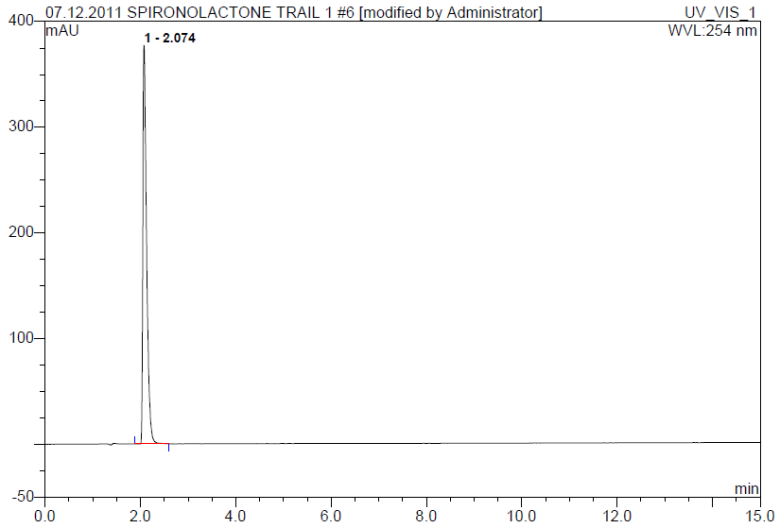


Figure 73 – Spironolactone HPLC method development. Trace produced following modification to the method described by Allen and Erickson (1996).

As is evident this trace produced a tall narrow peak at shortly after 2 minutes. This is however relatively close to the solvent front indicating poor retention of the compound on the column. This can be problematic when analysing samples consisting of multiple compounds as poor retention can lead to co-elution of multiple compounds. In order to improve the retention of the column, the mobile phase can be altered to be more polar. This involves reducing the acetonitrile content from 70% to 50%. This will result in the Spironolactone having a lower affinity for the mobile phase and thus a higher

apparent affinity for the column. The injection volume was also increased to produce a greater peak area. This is possible thanks to the narrow peaks. If broad peaks were in presence then increasing the injection volume may increase the peak width more than the peak height and so increase the chance of peaks overlapping.

Following modification of the mobile phase to acetonitrile and water (50:50), and increasing the injection volume to 50µl, the following trace was produced (Figure 74);

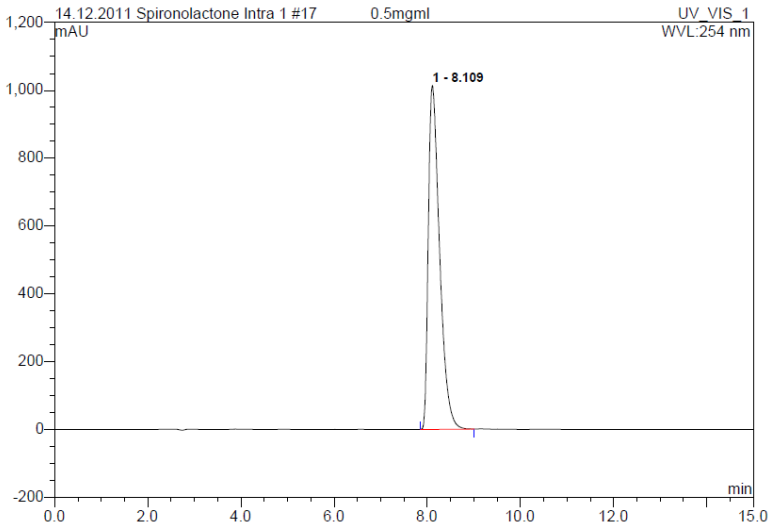


Figure 74 - Spironolactone HPLC method development following modification of the mobile phase to acetonitrile and water (50:50), and increasing the injection volume to 50µl

The changes made had the desired effect with the retention time increased slightly but still providing a relatively fast analysis procedure. This will maximize separation of the drug peak from peaks produced by other excipients in the formulation. Coupled with the modified mobile phase, the increased injection volume also had a large impact increasing the peak area from 34mAU*min to 284mAU*min for the 0.5mg/ml standard. This increase in peak area will improve LOD and LOQ by increasing the gradient of the calibration curve.

With a suitable method developed the next stage was to validate the method via intraday and interday repeats (Figures 75 – 80). Producing a final calibration chart (Figure 81) and validation (Table 33).

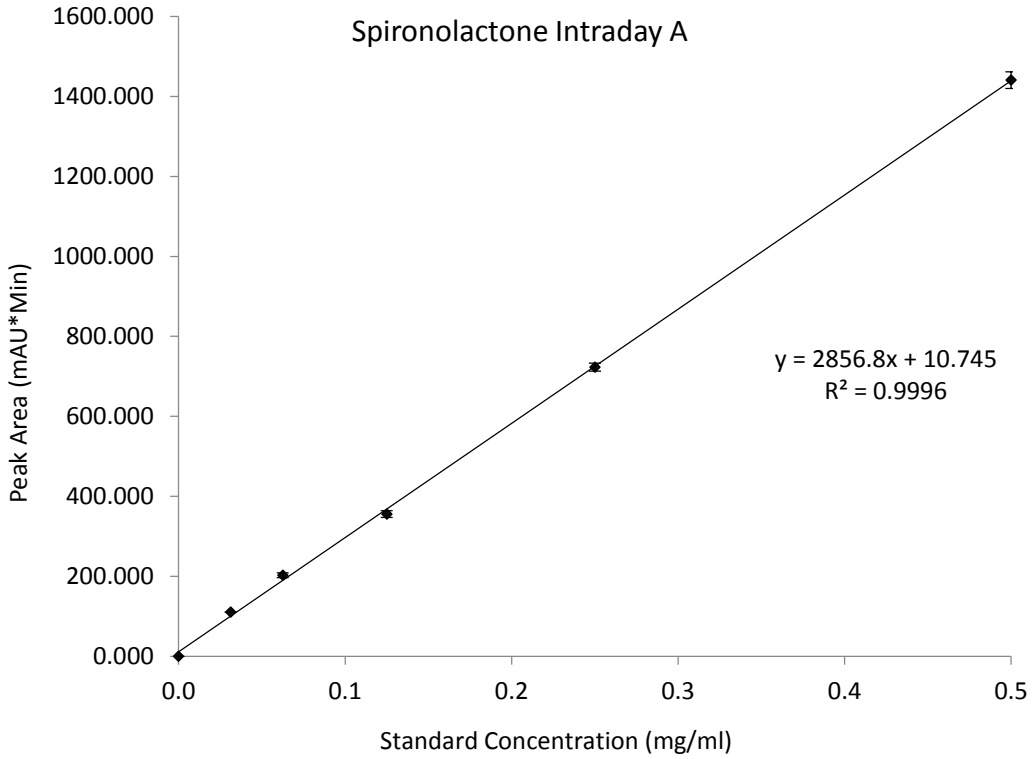


Figure 75 - Spironolactone Validation - Intraday Run A. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

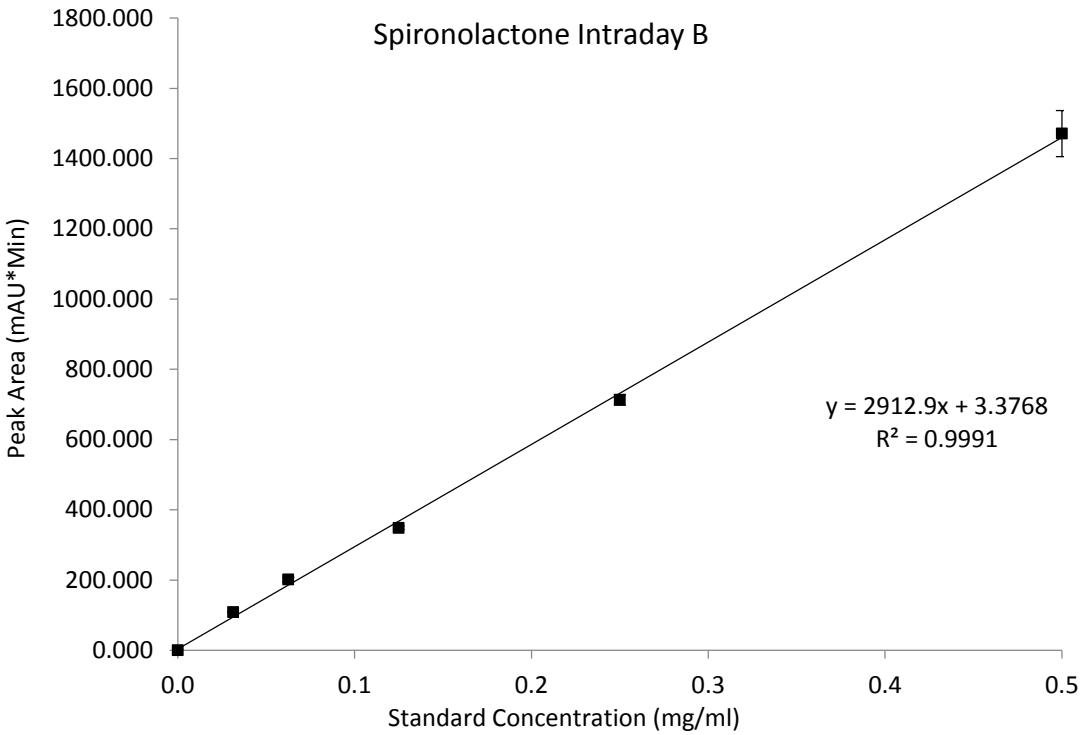


Figure 76 - Spironolactone Validation - Intraday Run B. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

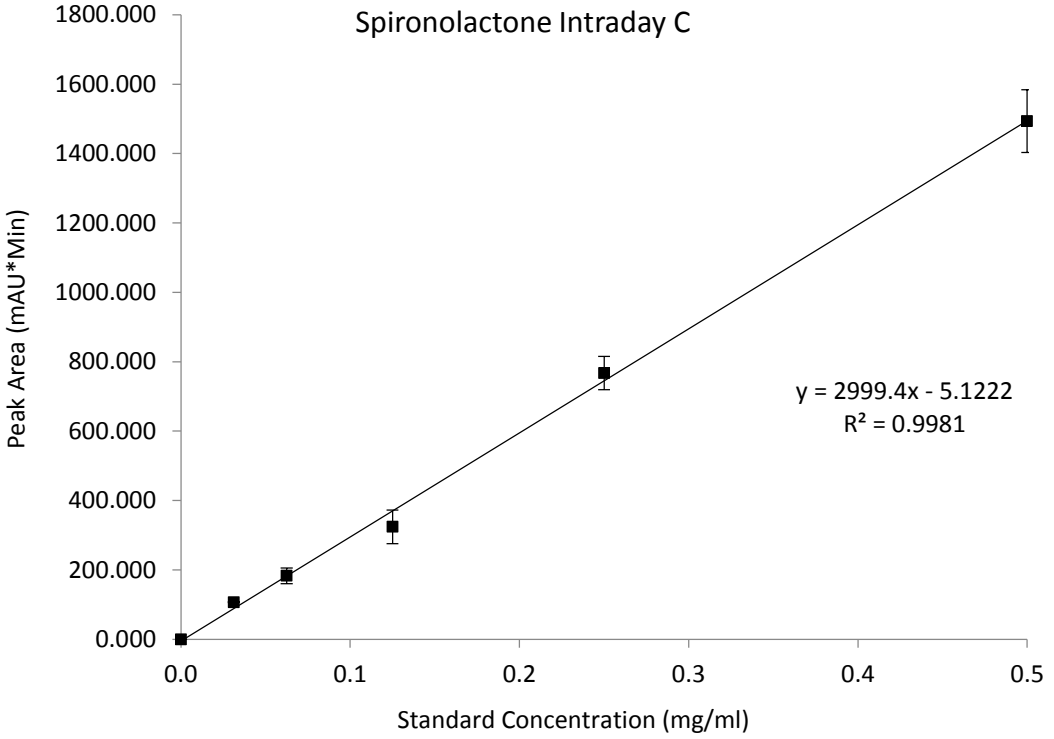


Figure 77 - Spironolactone Validation - Intraday Run C. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

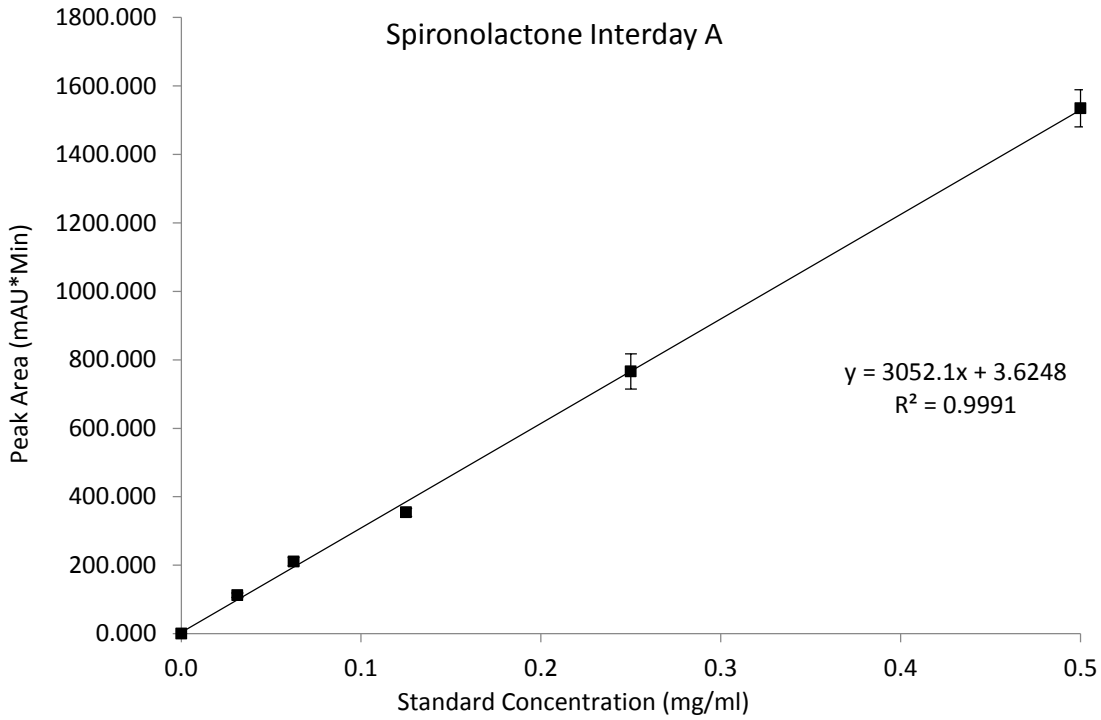


Figure 78 - Spironolactone Validation - Interday Run A. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

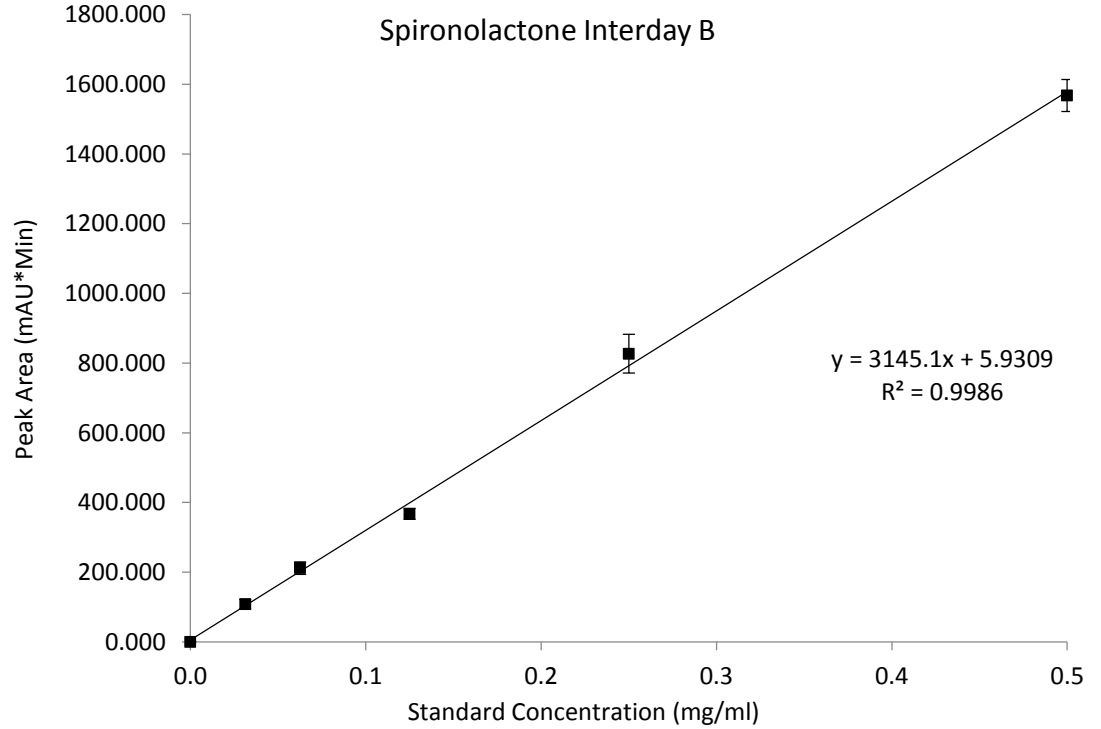


Figure 79 - Spironolactone Validation - Interday Run B. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

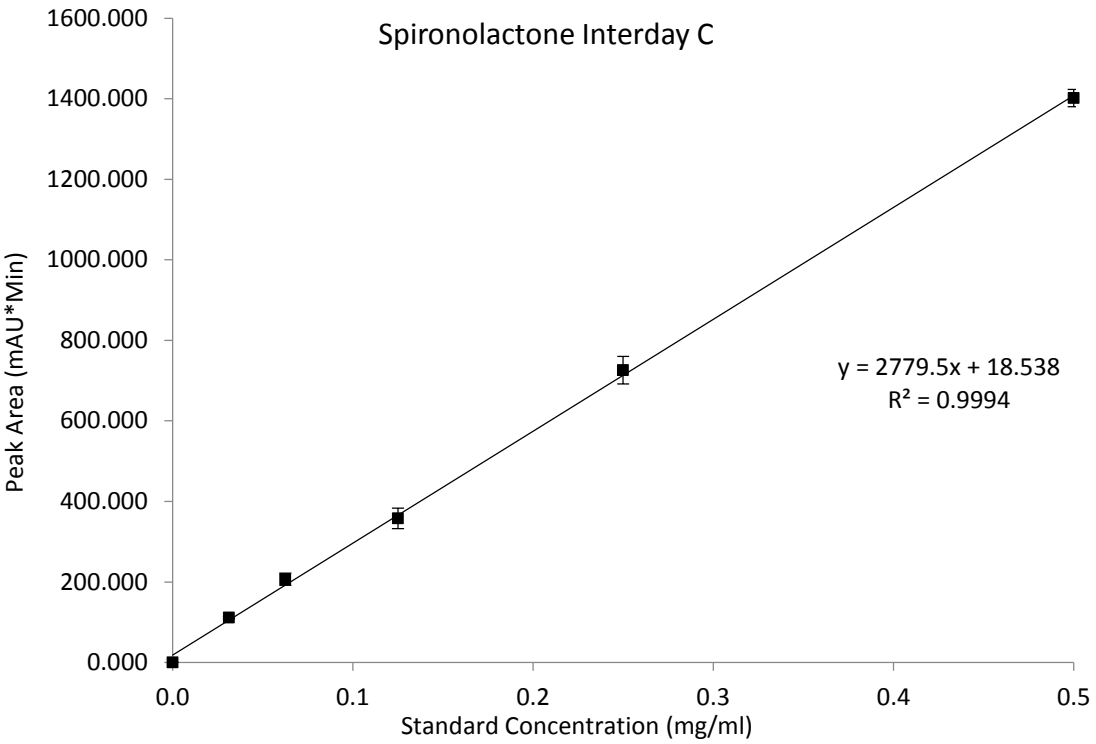


Figure 80 - Spironolactone Validation - Interday Run C. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

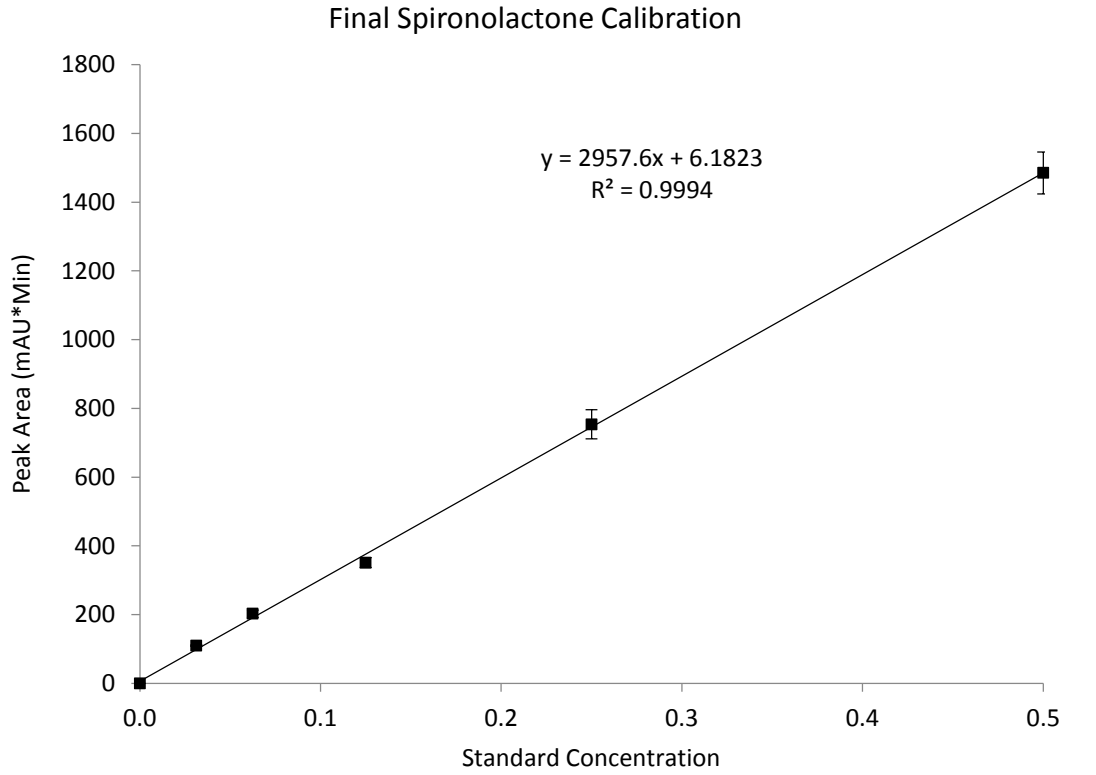


Figure 81 – Final calibration for Spironolactone HPLC method. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Table 33 – Spironolactone Validation Summary

Spironolactone Validation	
Selectivity	The Method was deemed to be selective for Spironolactone by running 'blank' samples and Spironolactone spiked samples
Accuracy	Determined by spiking mobile phase with known amounts of Spironolactone (0.5mg/ml, 0.25mg/ml and 0.125mg/ml). The recoveries ranged from 97.18% to 102.84% with an average standard deviation of 4.13%.
Linearity	Response for the detector was determined to be linear over a range of 0mg/ml to 0.5mg/ml. Correlation coefficients and slopes were obtained by plotting standard concentration (mg/ml) against peak area (mAU*min). R^2=0.9994 m=2957.6
Precision	Precision and intermediate precision were tested via the comparison of three intraday and interday calibration curves. These showed suitable repeatability.
Limit Of Detection	LOD is 0.4µg/ml.
Limit Of Quantification	LOQ is 1.39µg/ml

With the Spironolactone HPLC method validated then next stage was to produce an oral liquid formulation. As Spironolactone is insoluble the formulation will have to allow for this. Also the concentrations at which the formulation must be produced were also cause for consideration. 10mg/ml and less so 5mg/ml are high concentrations of drug to be included in a formulation.

4.4.2 Formulation Development

Spironolactone has a solubility of only 22µg/ml in water and the desired dosages for a liquid Spironolactone formulation are 10mg/ml and 5mg/ml, in order to produce a solution the solubility would need to be increased 227 fold. Additionally, Spironolactone has been shown to be degraded by cyclodextrins meaning that this common method of solubilising insoluble drugs cannot be used. As such and with an onus on simplicity, complex methods of solubilising the insoluble drug were rejected in favour of producing a suspension.

4.4.2.1 Buffer Production

It is known that Spironolactone is most stable at pH4.5 (Pramar & Gupta 1991) and as such the formulation pH was controlled so as to maintain optimum conditions for drug stability. There are many buffer systems which could be implemented for this task however it was decided that a citric acid/trisodium citrate buffer would be the best option as this system has a buffering capacity in the range of pH3-6.2 and is widely used in oral liquid formulations. Orally ingested citric acid is absorbed and is generally regarded as a nontoxic material, when used as an excipient trisodium citrate is generally regarded as a nontoxic and non-irritant excipient. Excessive consumption may cause gastrointestinal discomfort or diarrhoea however, concentrations used in the production of a buffer will be far too low to induce these adverse effects. Investigation into the buffer production revealed that the ratio of 0.1M citric acid to 0.1M trisodium citrate which produced a pH in the desired range was between 49.5:50.5 and 44.5:55.5 (Table 34).

Table 34 – Buffer production – Ratios of citric acid and trisodium citrate.

pH	x ml 0.1M-citric acid	y ml 0.1M-trisodium citrate
3	82	18
3.2	77.5	22.5
3.4	73	27
3.6	68.5	31.5
3.8	63.5	36.5
4	59	41
4.2	54	46
4.4	49.5	50.5
4.6	44.5	55.5
4.8	40	60
5	35	65
5.2	30.5	69.5
5.4	25.5	74.5
5.6	21	79
5.8	16	84
6	11.5	88.5
6.2	8	92

4.4.2.2 Inclusion of Pluronic F127 and Xanatural 180.

During preliminary work carried out with the view to solubilising Spironolactone it was observed that upon addition to water the Spironolactone powder clumped and a homogenous mix was impossible to achieve. Pluronic F127 was used in an effort to solubilise the Spironolactone and although it failed to achieve this it proved very useful as a dispersing agent. In the presence of Pluronic F127, Spironolactone dispersed well and produced homogenous suspensions. Although the inclusion of Pluronic F127 prevented clumping, sedimentation occurred rapidly and irreversibly. To combat the sedimentation Xanatural 180 was implemented as a suspending agent. Pluronic was used at concentrations of 0, 1, 2, 3, 4 and 5%w/v and Xanatural was used at concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5%w/v.

Pluronic is known to undergo transition from a solution to a gel at the sol-gel transition temperature. This normally only occurs at high concentrations, well over 5% and increased temperature. Although the pluronic solution itself would not be expected to form a gel, the presence of Xanatural may alter its behaviour. All combinations of Pluronic and Xanatural were investigated at temperatures up to 85°C (Table 35).

All of the various combinations remained liquid throughout the temperature range and so were then investigated at both 10mg/ml and 5mg/ml concentrations of Spironolactone for the Rate and Volume of sedimentation, Particle Size and Zeta potential. From this the Optimum combination of dispersing agent and suspending agent was determined.

Table 35 – Sol-gel transition temperature investigation for all combinations of Pluronic and Xanatural investigated up to 85°C

Mix	Xanatural (%w/v)	Pluronic F127 (%w/v)	Temperature (*C)						
			15	25	35	45	55	65	75	85
1	0.1	1	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
2	0.1	2	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
3	0.1	3	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
4	0.1	4	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
5	0.1	5	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
6	0.2	1	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
7	0.2	2	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
8	0.2	3	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
9	0.2	4	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
10	0.2	5	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
11	0.3	1	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
12	0.3	2	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
13	0.3	3	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
14	0.3	4	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
15	0.3	5	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
16	0.4	1	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
17	0.4	2	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
18	0.4	3	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
19	0.4	4	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
20	0.4	5	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
21	0.5	1	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
22	0.5	2	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
23	0.5	3	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
24	0.5	4	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
25	0.5	5	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid

4.4.2.3 Rate and Volume of Sedimentation

The rate and volume of sedimentation was investigated at a range of Pluronic and Xanatural concentrations for 10mg/ml (Table 36) and 5mg/ml (Table 37) Spironolactone suspensions. Measurements were taken at 5, 10, 15, 20, 30, 60, 120, 240 and 480 minutes.

Table 36 – Rate and volume of sedimentation for 10mg/ml Spironolactone Suspension

10mg/ml Spironolactone		**Sedimentation depth (mm) after time (Minutes)**								
Pluronic Conc (%w/v)	**Xanatural Conc (%w/v)**	**5**	**10**	**15**	**20**	**30**	**1h**	**2h**	**4h**	**8h**
0	0	2	4	6	8	8	8	8	8	6
1	0	0	1	1	2	2	3	3	2	2
2	0	0	1	1	2	2	3	3	2	2
3	0	0	1	1	2	2	3	3	2	2
4	0	0	1	1	2	2	3	3	2	2
5	0	0	1	1	2	2	3	3	2	2
1	0.1	0	0	2	2	3	3	3	4	4
2	0.1	0	0	2	2	3	3	3	4	4
3	0.1	0	0	2	2	3	3	3	4	4
4	0.1	0	0	2	2	3	3	3	4	4
5	0.1	0	0	2	2	3	3	3	4	4
1	0.2	0	0	0	2	2	3	3	4	4
2	0.2	0	0	0	2	2	3	3	4	4
3	0.2	0	0	0	2	2	3	3	4	4
4	0.2	0	0	0	2	2	3	3	4	4
5	0.2	0	0	0	2	2	3	3	4	4
1	0.3	0	0	0	0	0	2	2	3	3
2	0.3	0	0	0	0	0	2	2	3	3
3	0.3	0	0	0	0	0	2	2	3	3
4	0.3	0	0	0	0	0	2	2	3	3
5	0.3	0	0	0	0	0	2	2	3	3
1	0.4	0	0	0	0	0	0	0	0	0
2	0.4	0	0	0	0	0	0	0	0	0
3	0.4	0	0	0	0	0	0	0	0	0
4	0.4	0	0	0	0	0	0	0	0	0
5	0.4	0	0	0	0	0	0	0	0	0

Table 37 - Rate and volume of sedimentation for 5mg/ml Spironolactone Suspension

5mg/ml Spironolactone		Sedimentation depth (mm) after time (Minutes)								
Pluronic Conc (%w/v)	**Xanatural Conc (%w/v)**	**5**	**10**	**15**	**20**	**30**	**1h**	**2h**	**4h**	**8h**
0	0	0	1	1	1	1	2	2	1	1
1	0	0	1	1	1	1	2	2	1	1
2	0	0	1	1	1	1	2	2	1	1
3	0	0	1	1	1	1	2	2	1	1
4	0	0	1	1	1	1	2	2	1	1
5	0	0	0	1	1	2	2	2	3	3
1	0.1	0	0	1	1	2	2	2	3	3
2	0.1	0	0	1	1	2	2	2	3	3
3	0.1	0	0	1	1	2	2	2	3	3
4	0.1	0	0	1	1	2	2	2	3	3
5	0.1	0	0	0	1	1	2	2	3	3
1	0.2	0	0	0	1	1	2	2	3	3
2	0.2	0	0	0	1	1	2	2	3	3
3	0.2	0	0	0	1	1	2	2	3	3
4	0.2	0	0	0	1	1	2	2	3	3
5	0.2	0	0	0	0	0	1	1	2	2
1	0.3	0	0	0	0	0	1	1	2	2
2	0.3	0	0	0	0	0	1	1	2	2
3	0.3	0	0	0	0	0	1	1	2	2
4	0.3	0	0	0	0	0	1	1	2	2
5	0.3	0	0	0	0	0	0	0	0	0
1	0.4	0	0	0	0	0	0	0	0	0
2	0.4	0	0	0	0	0	0	0	0	0
3	0.4	0	0	0	0	0	0	0	0	0
4	0.4	0	0	0	0	0	0	0	0	0
5	0.4	0	0	0	0	0	0	0	0	0

From the observation of the Spironolactone sedimentation in different concentrations of Pluronic and Xanatural it was determined that increasing the concentration of Pluronic above 1% to 2, 3, 4 or 5% had no significant effect on sedimentation. Increasing the Xanatural concentration up to 0.4% gradually reduces the amount of sedimentation until at 0.4%w/v sedimentation no longer occurs. The differences in the formulations are illustrated below (Figure 82 and 83).

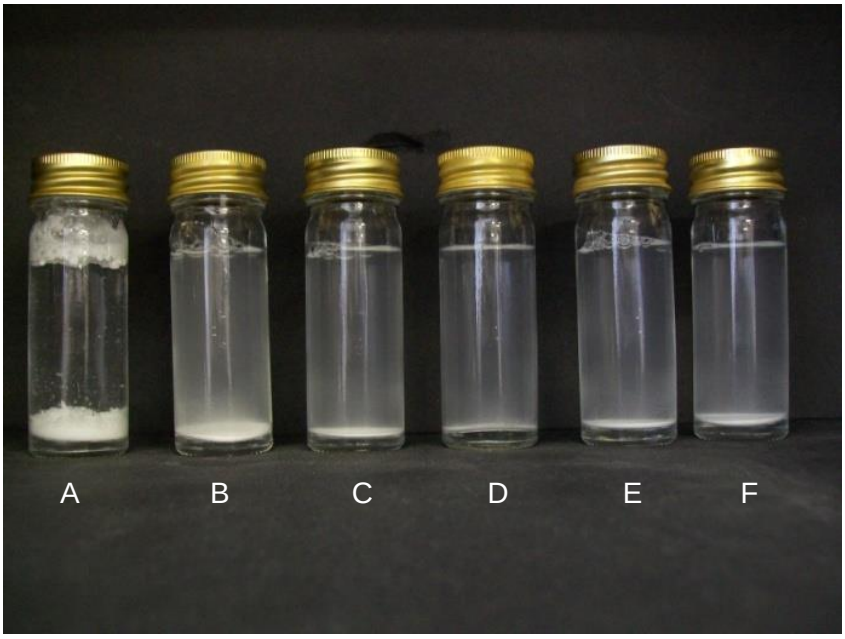


Figure 82 - Spironolactone in (A) Buffer Solution, (B) 1% Pluronic, (C) 2% Pluronic, (D) 3% Pluronic, (E) 4% Pluronic and (F) 5% Pluronic after 24h of standing following vigorous initial shaking

Figure 83 - Spironolactone in (A) Buffer Solution + 0.4% Xanatural, (B) 1% Pluronic + 0.4% Xanatural, (C) 2% Pluronic + 0.4% Xanatural, (D) 3% Pluronic + 0.4% Xanatural, (E) 4% Pluronic + 0.4% Xanatural and (F) 5% Pluronic + 0.4% Xanatural after 24h of standing following vigorous initial shaking.

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4.4.2.4 Particle Size

The particle size is also an important consideration and as such the particle size was investigated for all concentration combinations of Pluronic and Xanatural (Table 38 and 39).

Table 38 – Particle size analysis for 10mg/ml formulation at different ratios of Pluronic and Xanatural.

Particle Size (µm) (10mg/ml Spironolactone)	**Xanatural Concentration**					
Pluronic F127 Concentration	0.00%	0.10%	0.20%	0.30%	0.40%	0.50%
0.00%	42	44	42	43	42	42
1.00%	22	23	22	23	23	22
2.00%	21	23	22	21	22	23
3.00%	22	22	23	22	21	22
4.00%	24	22	21	22	24	22
5.00%	23	22	22	24	25	22

Table 39 - Particle size analysis for 5mg/ml formulation at different ratios of Pluronic and Xanatural.

Particle Size (µm) (5mg/ml Spironolactone)	**Xanatural Concentration**					
Pluronic F127 Concentration	0.00%	0.10%	0.20%	0.30%	0.40%	0.50%
0.00%	42	44	42	43	42	42
1.00%	21	22	23	22	22	22
2.00%	21	20	22	21	23	22
3.00%	22	23	22	22	22	21
4.00%	23	23	22	21	23	21
5.00%	21	20	23	22	23	21

The presence of pluronic reduced the particle size of the Spironolactone by approximately 50% in both the 10mg/ml and 5mg/ml suspensions, all concentrations of Pluronic had the same effect and so the concentration of choice is 0.1%. The presence and concentration of Xanatural had no impact on the particle size and so from the sedimentation work the 0.4% concentration still appears to be the optimum concentration.

4.4.2.5 Zeta Potential

Zeta potential analysis for all combinations of the Pluronic and Xanthan Gum showed no significant difference in response to increasing concentrations (Table 40 and 41). With the information gathered so far it was decided to carry forward the use of Pluronic F127 and Xanatural at concentrations of 1%w/v and 0.4%w/v.

Table 40 – Zeta potential analysis for 10mg/ml formulation at different ratios of Pluronic and Xanatural

Zeta Potential (mV) (10mg/ml Spironolactone) Pluronic F127 Concentration	**Xanatural Concentration**					
	0.00%	**0.10%**	**0.20%**	**0.30%**	**0.40%**	**0.50%**
0.00%	-8	-7	-7	-8	-7	-7
1.00%	-7	-8	-8	-7	-8	-7
2.00%	-7	-6	-8	-7	-7	-6
3.00%	-8	-7	-7	-8	-7	-8
4.00%	-7	-6	-8	-6	-7	-7
5.00%	-6	-7	-8	-7	-8	-7

Table 41- Zeta potential analysis for 5mg/ml formulation at different ratios of Pluronic and Xanatural

Zeta Potential (mV) (5mg/ml Spironolactone) Pluronic F127 Concentration	**Xanatural Concentration**					
	0.00%	**0.10%**	**0.20%**	**0.30%**	**0.40%**	**0.50%**
0.00%	-8	-7	-7	-8	-7	-7
1.00%	-8	-7	-7	-7	-8	-7
2.00%	-6	-7	-7	-6	-7	-6
3.00%	-7	-8	-6	-6	-7	-7
4.00%	-7	-6	-7	-7	-8	-7
5.00%	-7	-6	-7	-8	-8	-6

4.4.3 Final Formulations

From the investigations carried out it was clear that Xanatural 180 and Pluronic F127 would be essential components in the formulation to produce a vehicle providing suitable characteristics to maintain stability of the dosage form through the control of sedimentation and the prevention of caking. Based on knowledge gained in the Ramipril and Lisinopril formulations, the other excipients selected for the formulation were Sodium

Metabisulphate and Sodium benzoate to act as an antioxidant and preservative respectively. Flavouring would be present in the form of Xylitol as a sweetener and strawberry flavour included in a flavoured variety.

The final ingredients for the formulation are as follows;

- Spironolactone (10mg/ml or 5mg/ml)
- Xanatural 180 (0.4%w/v)
- Pluronic F127 (1.0%w/v)
- Sodium Metabisulphate (1.0%w/v)
- Sodium Benzoate (0.5%w/v)
- Xylitol (40%w/v)
- Strawberry Flavour (0.2%) (Flavoured Formulation Only)

4.4.4 Formulation Characterisation

With the formulation completed, investigation into its physical properties were carried out with investigations into the suspension rheology, Ostwald ripening and suspension stability.

4.4.4.1 Suspension Rheology

For oral suspensions hydrophilic polymers such as Xanatural 180 are used as suspending agents and have the effect of increasing the viscosity of the liquid vehicle. This is useful for prolonging the suspension of particles but can at times in the same way make it more difficult to re-suspend any particles which do sediment. To get the best results from the addition of hydrophilic polymers, the ideal properties for a suspensions flow profile are pseudoplastic or shear thinning. These are non-Newtonian liquids for which the rate of shear is not proportional to the shear stress exerted. This is particularly useful as the relatively high viscosity under low shear conditions e.g. during storage slows the sedimentation process and the relatively low viscosity under high shear

conditions e.g. shaking or pouring, allows for the easy re-dispersion of any sediment and also the easy dispensation of the medication.

The Spironolactone suspensions showed the desired pseudoplastic flow properties for both dosage concentrations with the 10mg/ml dosage resulting in higher viscosity than the 5mg/ml dosage (Figure 84).

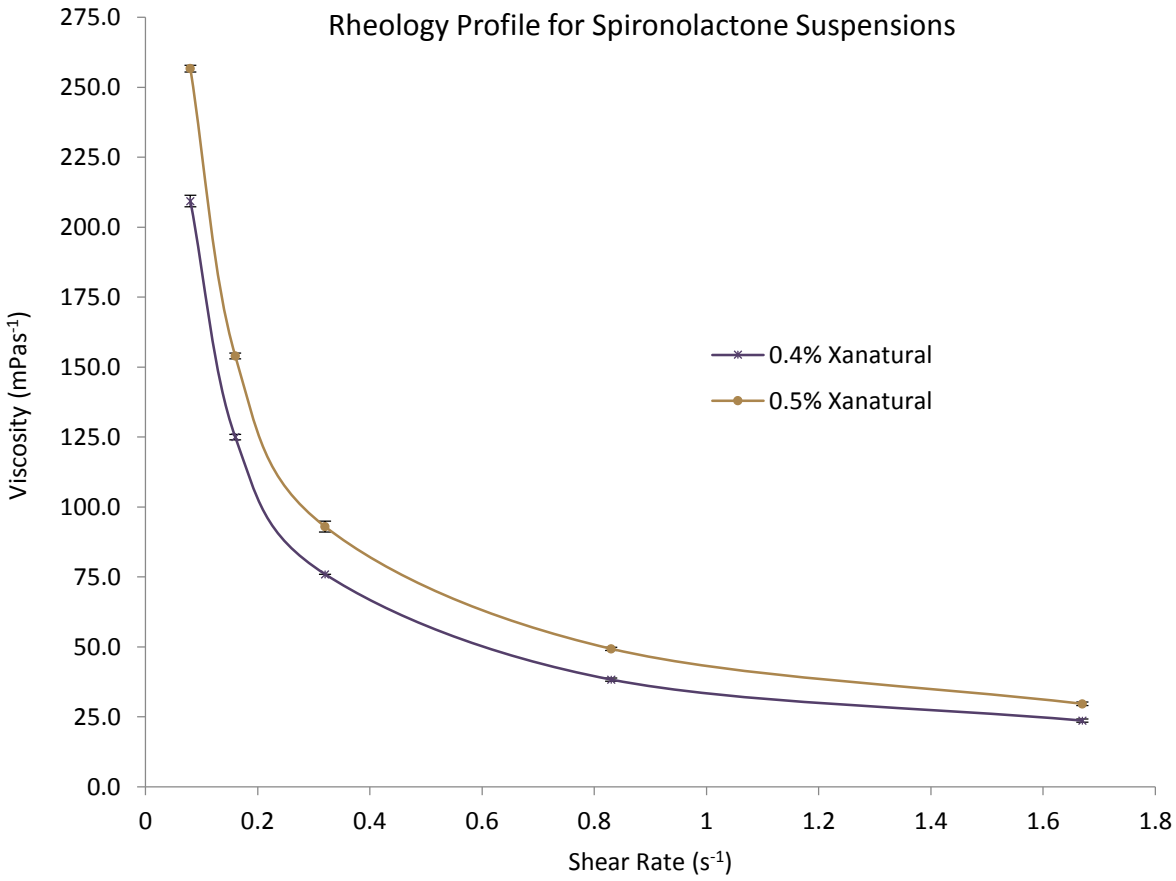

Figure 84 - Pseudoplastic flow properties for both dosage concentrations with the 10mg/ml dosage resulting in higher viscosity than the 5mg/ml dosage. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

4.4.4.2 Ostwald Ripening

A common problem with particles in a suspension is the possibility of Ostwald ripening (crystal growth) occurring. This has been shown to be a problem previously with Spironolactone and occurs when compounds are suspended in a vehicle in which they

are slightly soluble. Upon a temperature increase the smaller particles in the system become soluble. This can lead to the crystallisation of the dissolved drug onto the surface of the larger drug molecules increasing the particle size further. As particle size is directly related to the rate of sedimentation an increase in particle size via Ostwald ripening will lead to destabilisation of a stable suspension. As Ostwald ripening has been shown to affect Spironolactone in the past, an increased number of heat/cool cycles was performed (Figure 85 and 86);

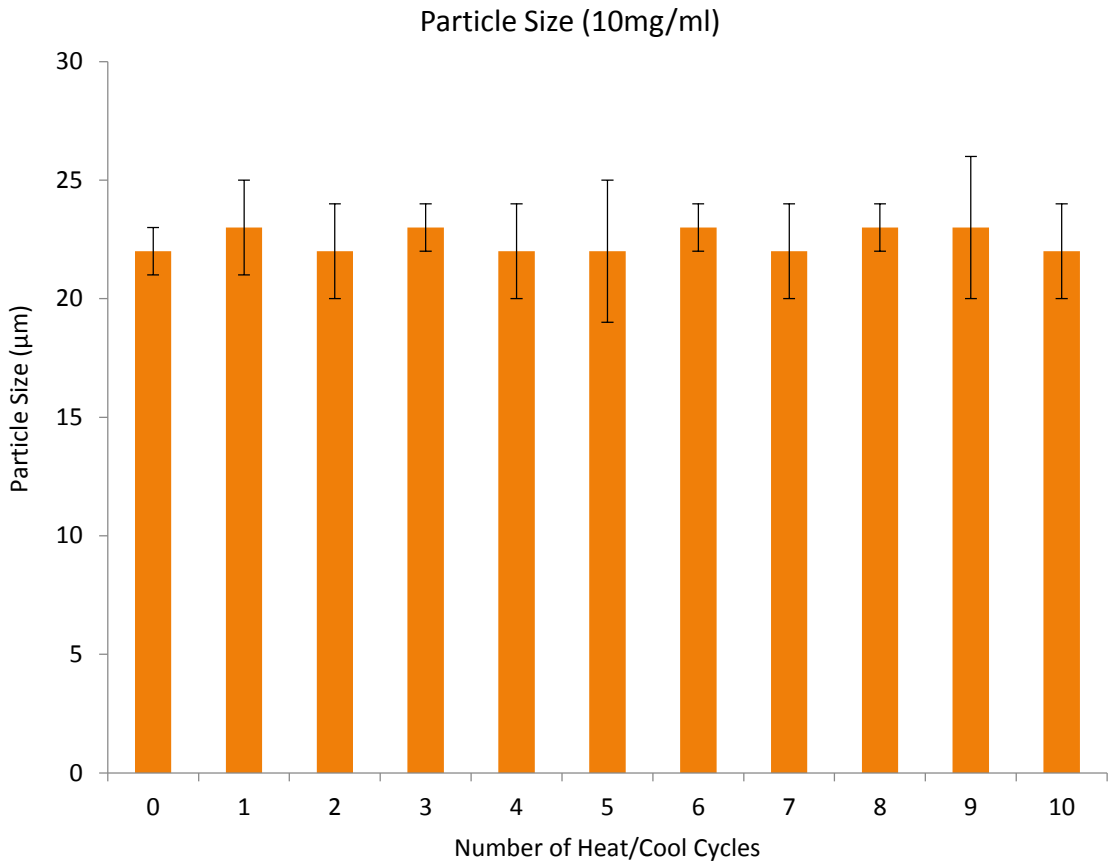

Figure 85 – Particle size analysis for Ostwald ripening observation in 10mg/ml Spironolactone suspension. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

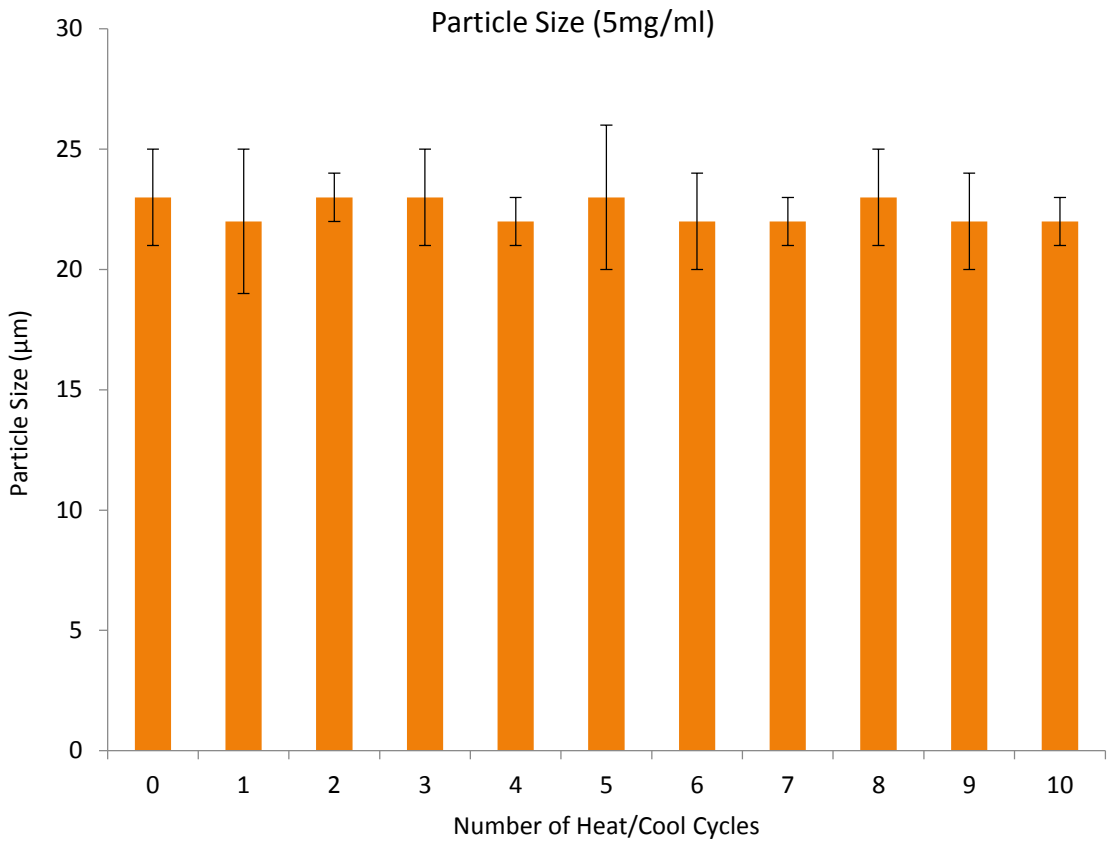

Figure 86 - Particle size analysis for Ostwald ripening observation in 5mg/ml Spironolactone suspension. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Ostwald Ripening was shown not to be a problem for either of the Spironolactone dosage concentrations. It has been previously reported that hydrophobic polymers can be implemented to prevent Ostwald ripening, as such it is reasonable to conclude that the Xanatural 180 present in the Spironolactone formulations has provided a degree of protection against the crystal growth which has been reported in previous attempts to formulate spironolactone.

4.4.5 Stability Analysis

4.4.5.1 Accelerated Conditions

Over the six month course of the stability testing in accelerated conditions with 40°C and 75% relative humidity, the drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines (Figure 87). The pH remained constant for the duration of the stability testing (Figure 88).

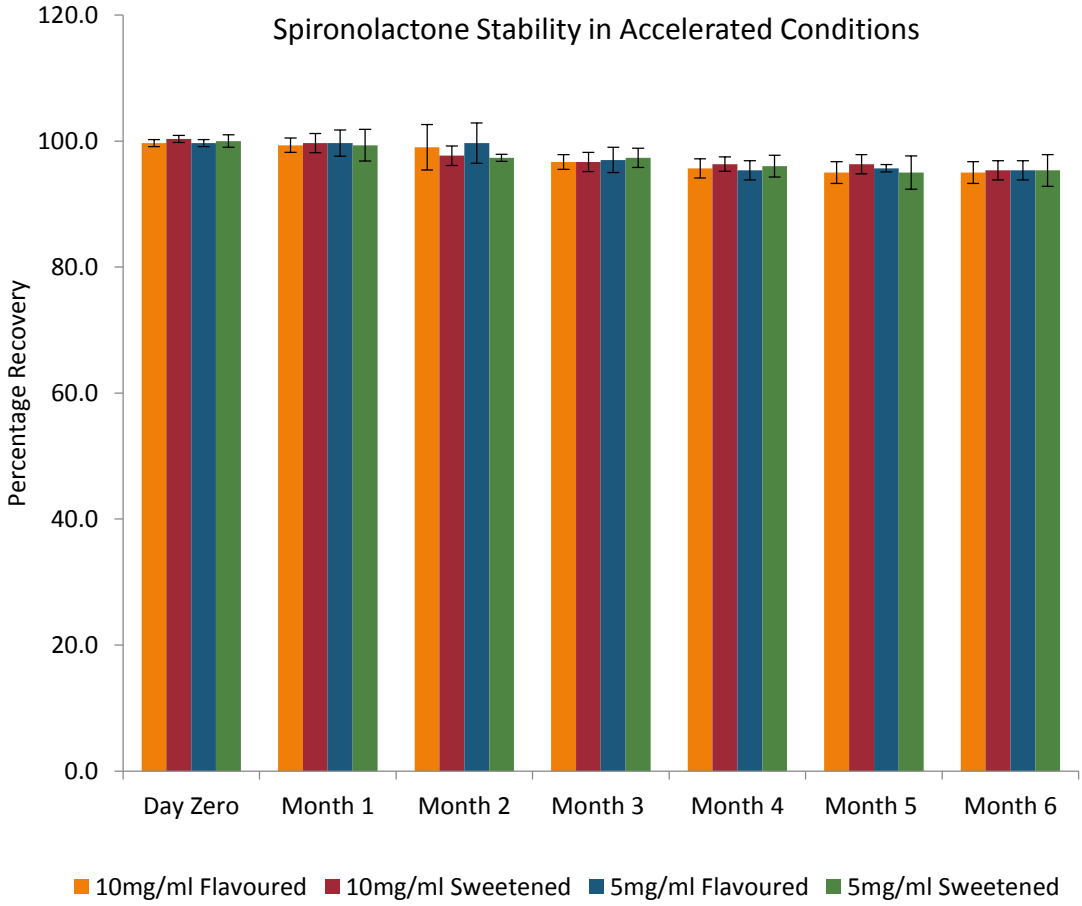

Figure 87 – Spironolactone recovery following HPLC for formulations in accelerated storage conditions. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

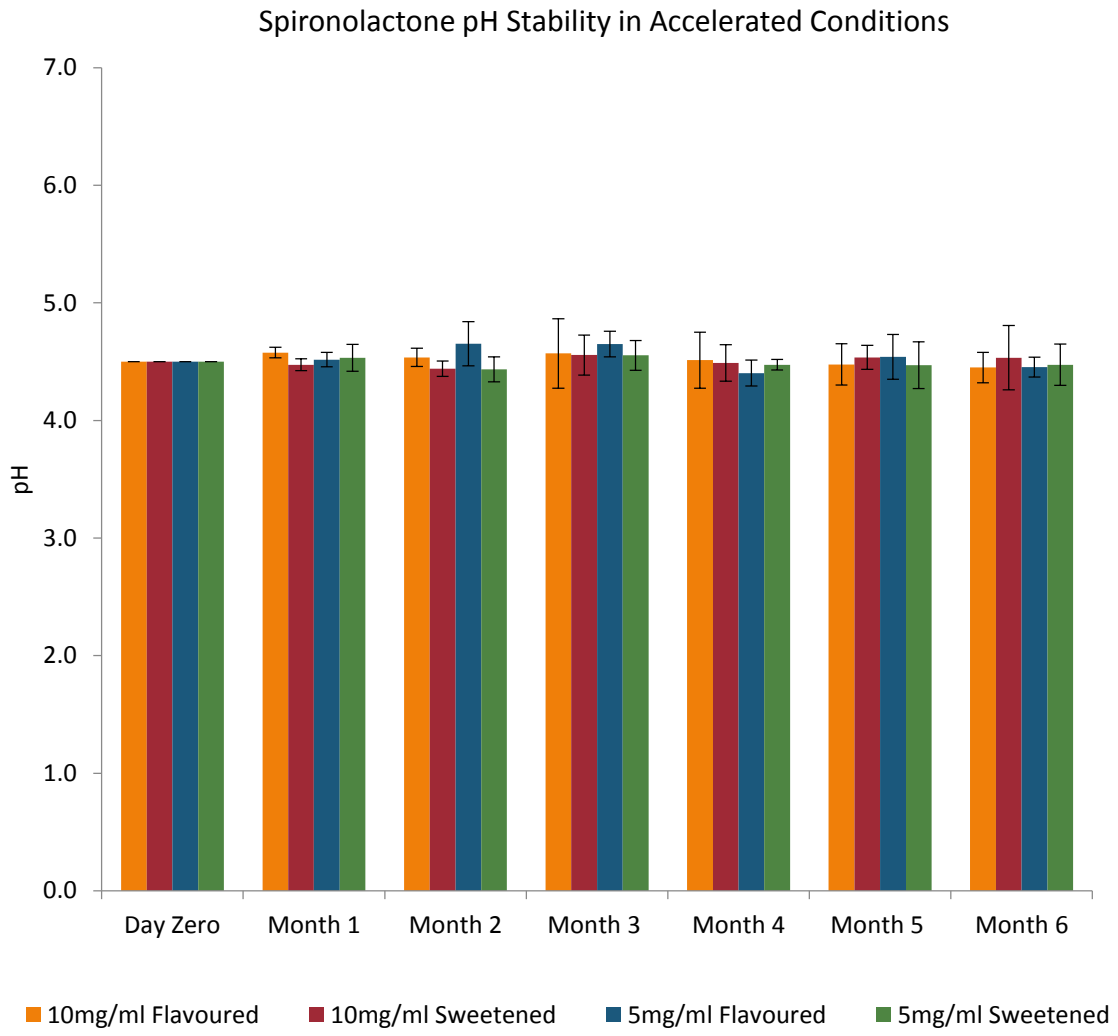

Figure 88 – Spironolactone pH stability for formulations in accelerated storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

4.4.5.2 Long Term Conditions

As seen in the accelerated conditions, for the twelve month testing course of the formulations stability in long term conditions, 25°C and 60% relative humidity, the drug content of all of the formulations remained >95% of the starting dose and the pH remained constant for the duration.

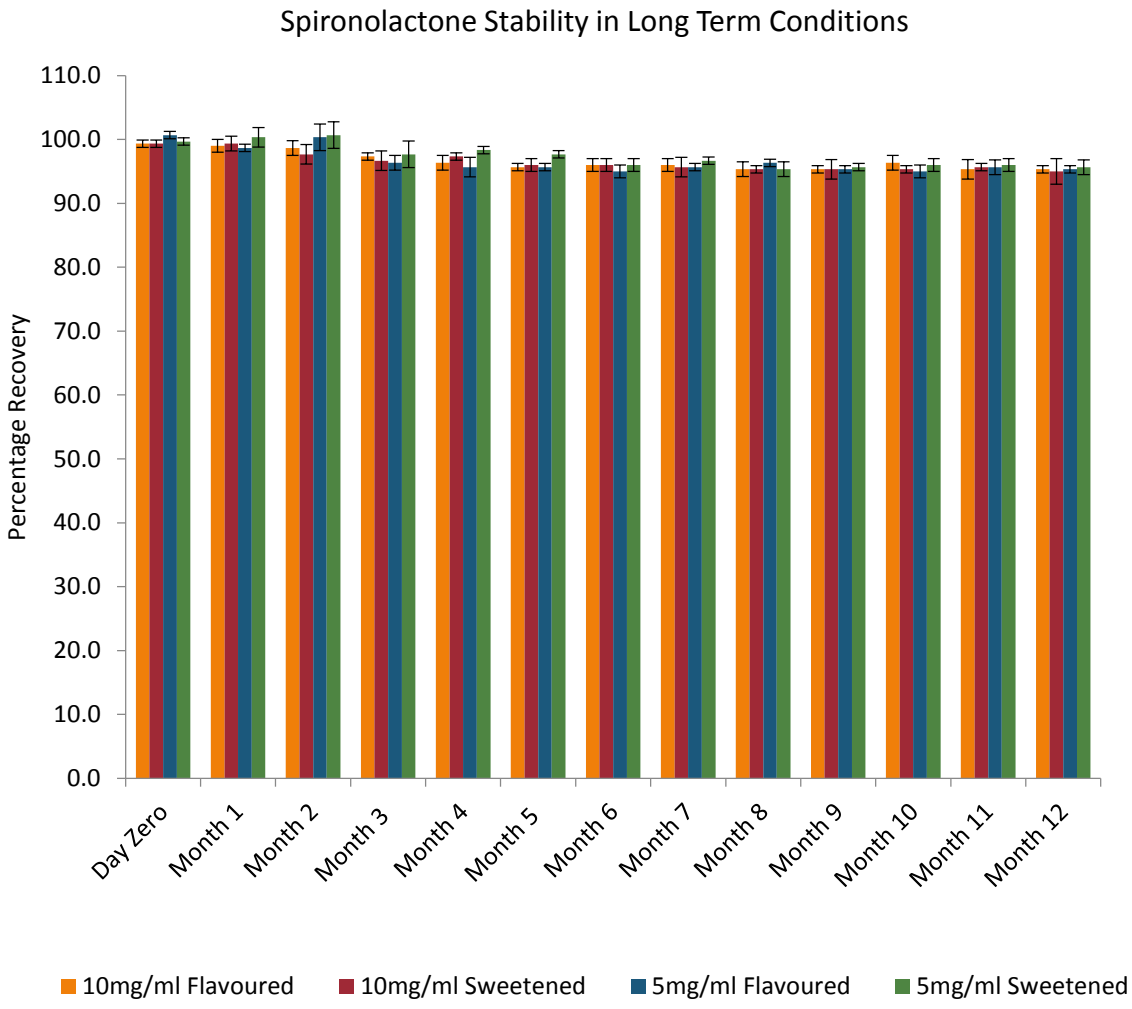

Figure 89 – Spironolactone recovery following HPLC for formulations in long term storage conditions. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

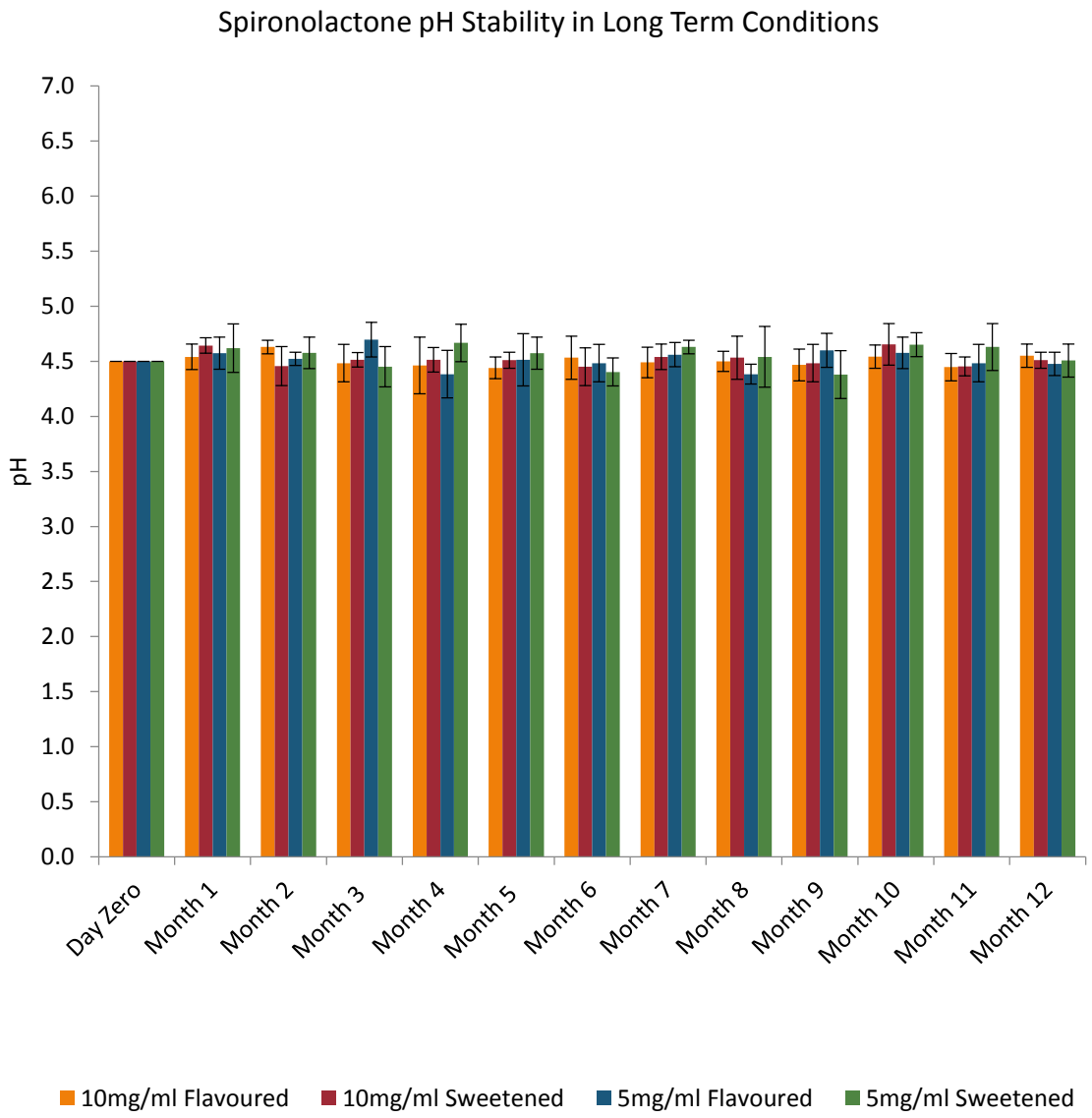

Figure 90 - Spironolactone pH stability for formulations in long term storage conditions. For the duration of testing the formulations proved stable with little variation in pH seen for all samples. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

According to the parameters outlined by the ICH harmonised tripartite guideline stability testing of new drug substances and products Q1A(R2**)**, "significant change" for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;

2. Any degradation product's exceeding its acceptance criterion;
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., colour, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH; or
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

The Spironolactone formulations tested under all conditions showed no significant decrease in the drug assayed in comparison to the day zero analysis. This was seen in all formulation types and at all dosages. In addition, the appearance and odour of the formulations remained acceptable for the duration of the stability testing and in both long term and accelerated conditions. Sedimentation did not occur in formulations stored in long term storage conditions and some degree of sedimentation was observed in formulations stored in accelerated storage conditions. The reason behind the sedimentation seen is most likely the increased temperature decreasing the viscosity of the vehicle and thus allowing particles to gravitate more easily. Caking did not occur however and the suspension was returned to a homogenous state easily and with minimal agitation meaning that the physical stability of the formulations in all conditions was maintained for the duration of testing. The advice for storage of the medication would have to advise against storage in hot conditions and directions for the procedure of re-suspension would have to be provided to ensure formulation homogeneity immediately prior to administration. With regards to excursions outside of the storage conditions such as transport during distribution, degradation and lack of physical stability should not be a concern.

5 *IN VITRO* AND *IN VIVO* PERMEABILITY ASSESSMENT OF LIQUID RAMIPRIL, LISINOPRIL AND SPIRONOLACTONE FORMULATIONS

5.1 CHAPTER AIMS AND OBJECTIVES

- Caco-2 cells grown on transwell inserts are used to perform *in vitro* drug transport studies for the formulations developed in chapters 2, 3 and 4.
- Wistar rats are used to perform *in vivo* drug transport studies for the formulations developed in chapters 2, 3 and 4.
- Analyse samples from drug transport studies via HPLC.
- Compare *in vitro* and *in vivo* findings to identify any *in vitro – in vivo* correlation which can later be combined with genomic studies to investigate the application of microarrays in the production of an *in vitro – in vivo* correlation screening toolbox.

5.2 INTRODUCTION

Where drug absorption is concerned, the barrier function of human intestine is the largest obstacle in the absorption of orally administered drugs. There are two components to this barrier function which can be classified as physical and biochemical. The physical barrier is present as a result of tight junctions which form between the enterocytes in the intestine and also the hydrophobic nature of the cell membranes while the biochemical barrier is a result of enzymes and transporter proteins. A model which predicts the absorption of a drug needs to replicate these barriers as closely as possible (Mohammed *et al* 2011).

Varieties of cell monolayer models that mimic *in vivo* intestinal epithelium in humans have been developed and are now used in many applications. Unlike enterocytes, human immortalized (tumor) cells grow rapidly into confluent monolayers that exhibit several characteristics of differentiated epithelial cells. As such the cell culture model provides an ideal system for the rapid assessment of the intestinal permeability of drug candidates. The Caco-2 cell model has been the most extensively characterized and useful cell model in the field of drug permeability studies. Caco-2 cells,

a human colon adenocarcinoma, undergo spontaneous enterocytic differentiation in culture and are polarized with well-established tight junctions. Due to the permeation characteristics of drugs across Caco-2 cell monolayers correlating with their human intestinal mucosa permeation characteristics, Caco-2 cells can be used to predict the oral absorption of drugs in humans. In the past 10–15 years, there has been a tremendous growth in the use of Caco-2 cells for mechanistic studies, and as a rapid *in vitro* screening tool in support of drug discovery within the pharmaceutical industry. In order to assay the permeability of drugs, Caco-2 cells are grown on membrane supports which create an apical and basal chamber inside each well of a six well plate. Following addition of the drug substance to the apical chamber, its arrival in the basal chamber is measured at various time points to produce a profile for the drug's permeability. This is expressed as apparent permeability which correlates to *in vivo* absorption.

One of the limitations of a cell model is that the cells only represent one obstacle in the absorption of the drug and the model requires correlations to be produced with the *in vivo* performance. Factors such as gastrointestinal transit time, first pass metabolism, fasted/fed absorption differences and mucus production are all largely unaccounted for. *In vivo* permeability analysis is the most 'true to life' means of modelling permeability and for the most part involves the use of animal models, rats are the preferred species used to model the human intestine, Zhao *et al* 2003 reported a correlation of R^2=0.88 when they investigated the correlation of drug absorption between humans and rats. Also in 2003, Cao *et al* reported that drug absorption in the small intestine of humans and rats showed a linear correlation (R^2=0.8) for both passive and carrier mediated absorption pathways. They also investigated the genetic expression levels of intestinal transporters in the duodenum for both species, this did not correlate as well as the values for drug absorption however an R^2 value of 0.56 indicated that there was some level of correlation. Transporters such as PepT1, SGLT-1, GLUT5, MRP2, NT2, and high affinity glutamate transporter were shown to display similar expression levels in both humans

and rats while in contrast, other transporters such as MDR1, MRP3, GLUT1, and GLUT3 were shown to be expressed at different levels. Finally they investigated the expression levels of metabolising enzymes present in the intestine of both species such as CYP3A4/CYP3A9 and UDPG and it was revealed that here there was no correlation, providing reasoning for differences seen in drug metabolism and oral bioavailability seen between rats and humans (Cao *et al* 2003). These correlations and the similarity between rats and humans in the undelaying mechanisms playing a part in oral drug absorption through the intestinal membrane are the reason the rat model is preferred for use *in vivo* and widely used.

Results generated from *in vivo* permeability experiments can provide information on a variety of parameters which are dependent upon experimental design, three functions which are common to almost all *in vivo* data analysis are the area under the curve (AUC), peak concentration (C_{MAX}) and peak time (T_{MAX}).

The area under the plasma drug concentration-time curve (AUC) reflects the actual body exposure to drug after administration of a dose of the drug and is expressed in mg*h/L. The AUC is governed by the initial dose given and the rate at which the drug in question is eliminated from the body. Total drug elimination may be calculated through the cumulative amount eliminated in each time interval, from time zero (time of the administration of the drug) to time infinity. This total amount corresponds to the fraction of the dose administered that reaches the systemic circulation. The AUC is directly proportional to the dose when the drug follows linear kinetics and the AUC is inversely proportional to the clearance of the drug (Urso *et al* 2002).

Following oral administration C_{MAX} and T_{MAX} are dependent on the extent, and the rate of drug absorption and on the disposition profile of the drug. The peak time (T_{MAX}) and the peak concentration (C_{MAX}) may be directly obtained from the experimental observations (Urso *et al* 2002).

In this chapter, investigation into the *in vitro* and *in vivo* drug absorption of Lisinopril, Ramipril and Spironolactone from developed formulations is reported with the aim of finding a correlation between the Log P_{app} produced from *in vitro* assays and the information derived from *in vivo* assays.

5.3 Materials and Methods

5.3.1 Assessment of Absorption *In Vitro*

5.3.1.1 Culture of Caco-2 Cells for permeability assay

Caco-2 cells (passage 45) (A kind gift from Dr A. Collett and Dr Daniel Patten at the University of Huddersfield) were seeded at a density of $1.3x10^5$ cells/cm^2 onto permeability supports (Appleton Woods Ltd). Cells were cultured in an incubator (Sanyo) 37°C in a humidified 5% CO_2/95% air atmosphere. Media was changed every 2-3 days over a three week period. During this time TEER measurements were taken following each media change using an EVOM – Epithelial Voltohmmeter (World Precision Instruments Ltd) to develop a profile for tight junction formation and development and to ensure monolayer integrity. Additionally, TEER was measured immediately before and after experiments to ensure that monolayer integrity had been maintained for the duration of the investigation.

For the determination of Trans-epithelial flux of Ramipril, Lisinopril and Spironolactone across Caco-2 monolayers, culture media was removed and the monolayers washed with HBSS before the monolayers were incubated at 37°C for 30 minutes with 2.5ml of HBSS in the basolateral compartment and 1.5ml of HBSS in the apical compartment. After 30 minutes the HBSS was removed from the apical compartment and replaced with 1.5ml of the formulation to be tested. 200µl samples were then taken from the basolateral compartment of each well at time points of 5, 10, 15, 20, 30, 60, 90, and 120 minutes. 200µl samples were also taken from the apical

compartment of each well at the 120 minute time point. In each case where sample was taken from the basolateral compartment a matching volume of HBSS was added. This was corrected for in calculations. The samples were analysed via HPLC. All of the experiments were performed in triplicate and the level of transport is described as the percentage of drug arriving in the basolateral compartment.

5.3.1.2 HPLC Methods Adapted for inclusion of HBSS

HPLC Methods were repeated using HBSS in standard production to account for any changes to the retention time, peak size or peak shape that the addition of a salt solution may cause.

5.3.1.3 Lisinopril HPLC Method

The Lisinopril HPLC method for permeability assays was modified from a method previously developed in house and used a Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler. The stationary phase is a Phenomenex Gemini 5µ C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size). The detection wavelength was set at 228nm. The injection volume is 20µl with a run time of 10 minutes. Preparation of Calibration Standards involves the production of a stock solution 4mg/gml in HBSS followed but the production of six standards, initially with a 1:1 dilution and then subsequent serial dilution of the stock solution using mobile phase. Standards at 2.0, 1.0, 0.5, 0.25, 0.125 and 0.0625mg/ml. Samples were prepared for analysis by dilution so as to fall within the calibration range and were diluted with Lisinopril mobile phase.

5.3.1.4 Ramipril HPLC Method

The HPLC method for Ramipril permeability assays was adapted from the method previously developed in house which had been based upon a combination of the stability indicating HPLC methods described by Allen et al (1995) and Patil et al (2008). The method developed used a Perchlorate Buffer and Acetonitrile (55:45) mobile

phase and C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size) with a flow rate of 1mg/min and a detection wavelength of 216nm. The injection Volume was 50µl and the run time was 10 minutes. Six calibration standards produced via serial dilution in HBSS. The standards were then diluted 1:5 in water and acetonitrile (45:55). Samples were diluted to be within calibration standards using water and acetonitrile (45:55).

5.3.1.5 Spironolactone HPLC Method

The Spironolactone HPLC method for permeability assays was developed in house using from the existing method using a Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler. The stationary phase is a Phenomenex Gemini 5µ C18 reverse phase HPLC column (150 x 4.5mm with 5µm Particle Size). The detection wavelength was set at 254nm. The injection volume is 20µl with a run time of 10 minutes. Preparation of Calibration Standards involves the production of six standards. A 4mg/ml Spironolactone stock is produced in HBSS followed but the production of six standards, initially with a 1:1 dilution and then subsequent serial dilution of the stock solution using mobile phase. Standards at 2.0, 1.0, 0.5, 0.25, 0.125 and 0.0625mg/ml. serial dilution in mobile phase. Samples were prepared for analysis by dilution so as to fall within the calibration range and are diluted with mobile phase.

5.3.1.6 Calculation of apparent permeability (P_{app})

The apparent permeability of a molecule (P_{app}) determined following permeability assay through Caco-2 has been shown in previous studies to correlate reasonably well with the associated human absorption coefficients. Studies to determine P_{app} of a molecule in formulation are incredibly useful in the drug discovery and development area as P_{app} is often the first indicator of a molecule's potential to pass from the intestine into the blood. As a result P_{app} can be used to identify molecules which are suitable for oral

administration and also it allows for the comparison of different formulations containing the same active ingredient.

Following the production of a transport/time graph which displays the rate of drug transport from the apical to basolateral chamber of the permeability support over the time of the assay, P_{app} is calculated using the following equation (Yang 2007);

$$P_{app} = \left(\frac{V_r}{AC_o}\right)\left(\frac{dC}{dt}\right)$$

Where;

V_r – is the volume of the basolateral chamber (ml)

A – is the filter surface area available for transport (cm^2).

C_0 – is the initial concentration of drug (mg/µl).

$\frac{dC}{dt}$ - is the initial slope of the cumulative concentration (dC) of drug in the basolateral chamber (mg/µl) with time (dt) (s).

5.3.2 Assessment of Absorption *in Vivo*.

Rats were Wistar rats with a body weight of approximately 250g-300g. Once prepared the formulations were loaded into a 1ml syringe and administered via oral gavage. Dosing was calculated depending upon the weight of each individual animal and formulations were diluted in H_2O, Lisinopril was dosed at 10mg/kg, Ramipril was dosed at 5mg/kg and Spironolactone was dosed at 40mg/kg. Following dosing, an additional 1ml of H_2O was administered to rinse in the formulations. Blood samples (45µl) were taken via tail bleeds at 15 minutes, 30 minutes, 45 minutes, 1 hour, 1.5 hour, 2 hour, 2.5 hour, 3 hour and 4 hour time points following administration. Plasma was extracted from blood samples by centrifugation at 2800 x g for 10minutes followed by

liquid-liquid extraction using hexane and methylene chloride. Samples were then diluted 1:5 in mobile phase and analysed using HPLC.

5.3.2.1 Calculation of AUC

Area under the curve for drug absorption *in vivo* was performed using the trapezoidal rule where the plasma concentration against time profile is split into multiple trapezoids and the area of each trapezoid is added to provide the overall AUC (Figure 91).

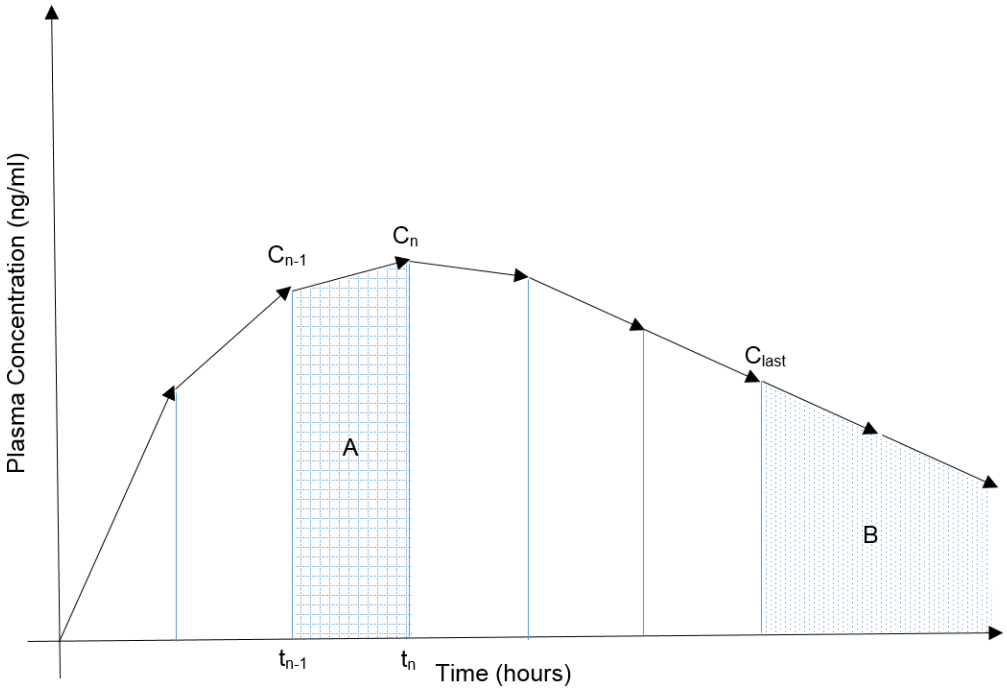

Figure 91 - Trapezoidal calculation of the area under the curve (AUC). The area under the curve is separated into trapezoids where each time point forms the vertical boundaries. To calculate the area of each trapezoid for example trapezoid A is given by $(C_{n-1} - C_n) \times (t_n - t_{n-1})/2$. *Where the plasma concentration has not returned to 0 before the final time point, extrapolation to work out AUC to time infinity, trapezoid B, is calculated using* $(C_{last}/0.693/t_{1/2})$. *AUC is given by the sum of all of the trapezoids and the units are expressed as ng*h/ml.*

5.4 RESULTS AND DISCUSSION

5.4.1 Culture of Caco-2 cells for permeability investigation.

For the permeability investigation of Lisinopril, Ramipril and Spironolactone, membrane supports were seeded at a density of $1.3x10^5$ cells/cm^2 and allowed to develop. TEER was measured at each media change to monitor the integrity of the developing monolayers. At a density of $1.3x10^5$ cells/cm^2 it takes roughly 7 days for Caco-2 cells to reach confluence in 6 well and 24 well plates, however for the tight junctions to form fully between these cells producing a fully selectively permeable membrane it takes much longer. Sources in literature generally report that Caco-2 monolayers grown on permeable supports are suitable for use between 2 and 4 weeks following seeding (Fleck *et al* 2014, Parker et al 2014, Inaba *et al* 2014). As it is not possible to inspect the Caco-2 cells on permeability supports using a microscope the least invasive technique to monitor cell monolayer development is a technique known as trans-epithelial electronic resistance (TEER) measurement. This is widely implemented to indicate confluence and ensure integrity of the monolayer prior to permeability assessment. The TEER of the seeded membranes was measured following each media change to provide a profile for the cell development (Figure 92) and provide indication as to when the cells would be suitable for use when grown under the specified conditions

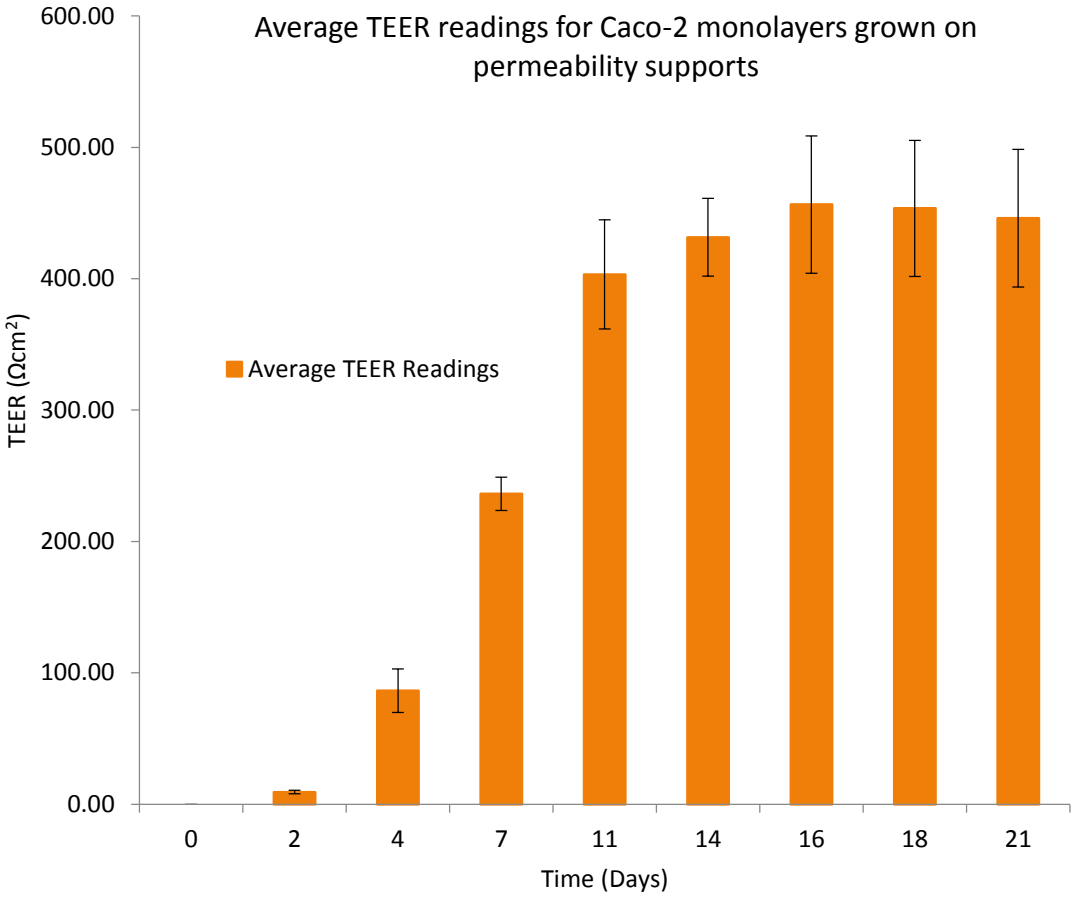

Figure 92 – TEER profile as Caco-2 monolayers develop over time when grown on transwell inserts. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

In keeping with literature (Tokumura *et al* 2014) the TEER value for the monolayers peaked at approximately 425Ω at 11 days following the seeding of the membranes. At this point the cells are confluent and TEER readings level off. The TEER values remained constant confirming that the monolayers were suitable for use. Permeability experiments were performed after 21 days of culture to ensure stable TEER values over 400Ω and also to ensure the enzymes and transporters which have a roll in drug absorption have adequate time to be fully expressed.

5.4.2 HPLC Methods Accounting for HBSS Inclusion

Prior to undertaking the permeability experiments, calibration curves for each of the Lisinopril (Figure 93), Ramipril (Figure 94) and Spironolactone (Figure 95) HPLC methods were required to be produced integrating HBSS into the production of calibration standards. This is necessary as samples collected from the basolateral compartment of the permeability assay transwell set up would be in HBSS and even a small concentration of salt solution can potentially have significant effects on the behaviour of HPLC separation and detection. Retention time, peak size and peak shape can all be affected. HBSS is a salt solution designed to maintain optimum physiological pH for the cultured cells during permeability experiments and the salt concentrations are relatively low.

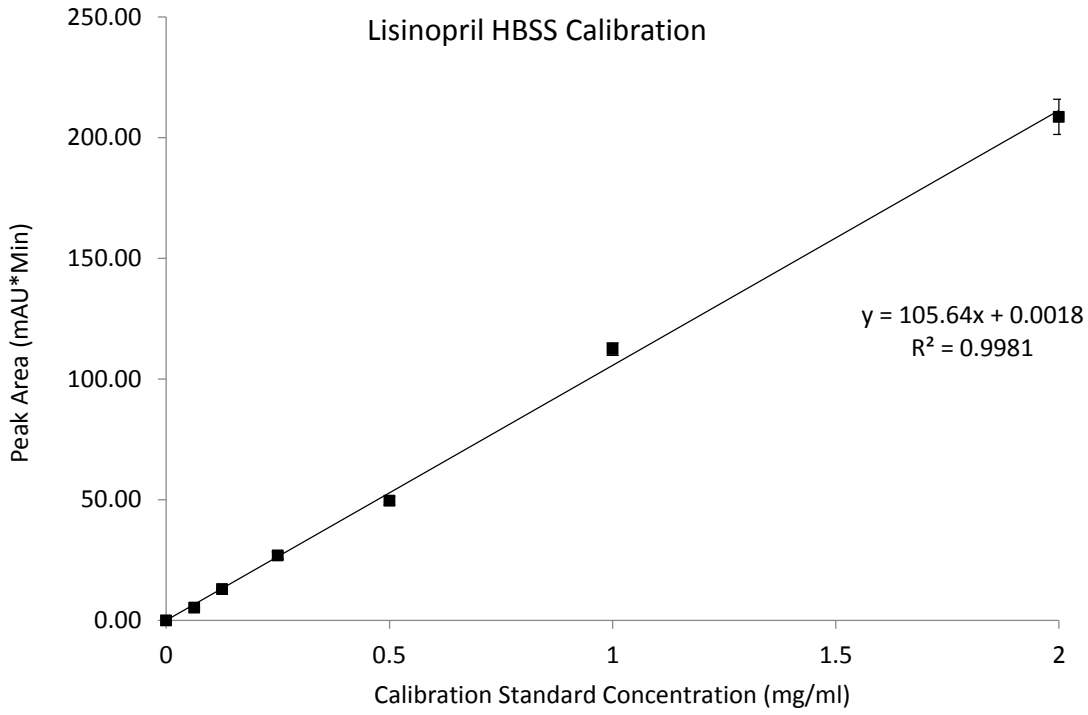

Figure 93 – Lisinopril HPLC calibration using standards produced in HBSS. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

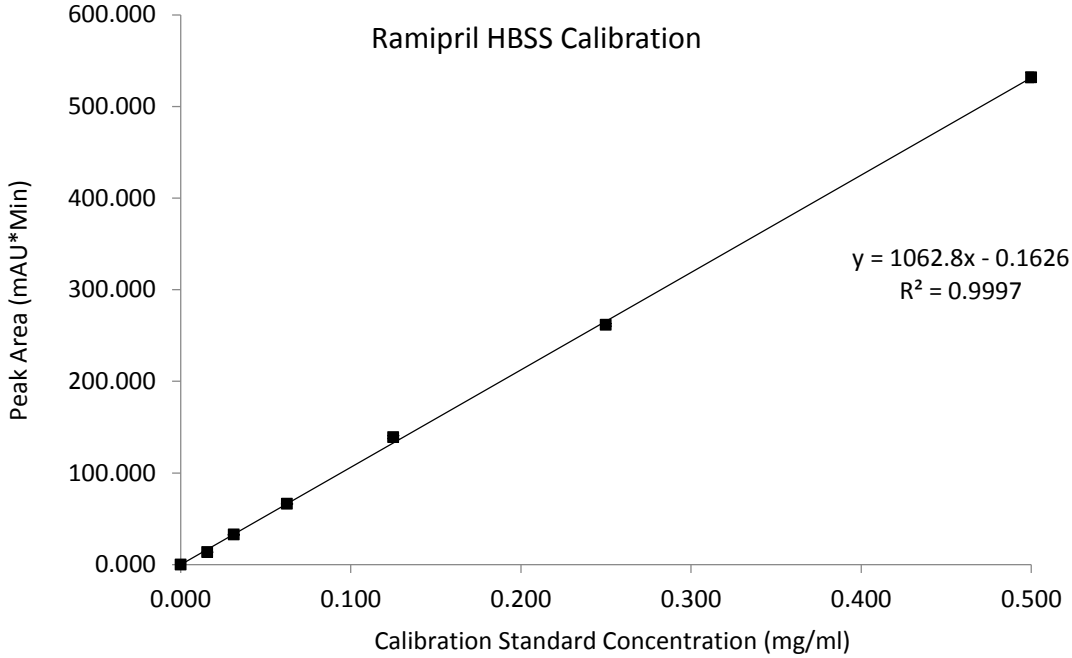

Figure 94 – Ramipril HPLC calibration using standards produced in HBSS. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD) N.B. some error bars are obscured by the data point.

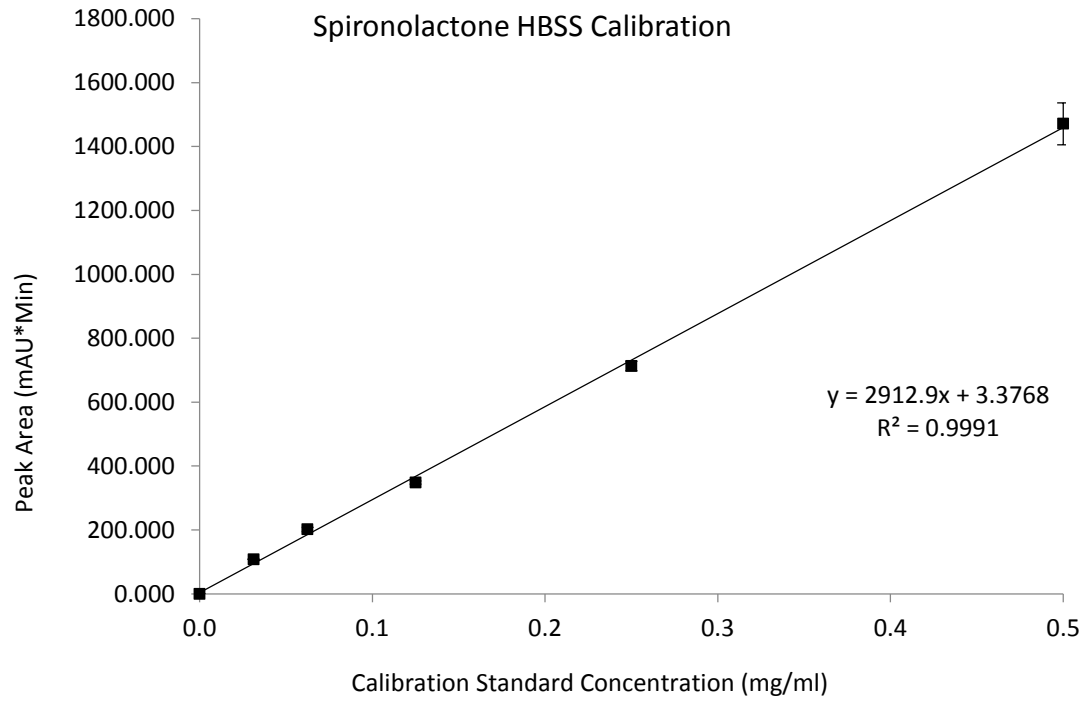

Figure 95 – Spironolactone HPLC calibration using standards produced in HBSS. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD) N.B. some error bars are obscured by the data point.

All of the calibrations produced following the incorporation of HBSS into standard production were found to produce curves with comparable equations and R^2 values to the validated HPLC methods produced in house. This is most likely due to the effect of the standards undergoing dilution in mobile phase for all of the methods prior to running reducing the salt solution to a point where it would have a negligible effect on the method performance. The dilution in mobile phase also has the effect of reducing the presence of a solvent front as the refractive index of the injected sample more closely matches that of the mobile phase already passing through the detector.

5.4.3 *In Vitro* Drug Transport and Calculation of P_{app}

TEER values were measured before and after the permeability experiments for each transwell used to ensure that the TEER did not drop. If this had been observed then it would have indicated that the integrity of the monolayer had been compromised. In all cases the TEER remained above an acceptable level of 300Ω (Figure 96).

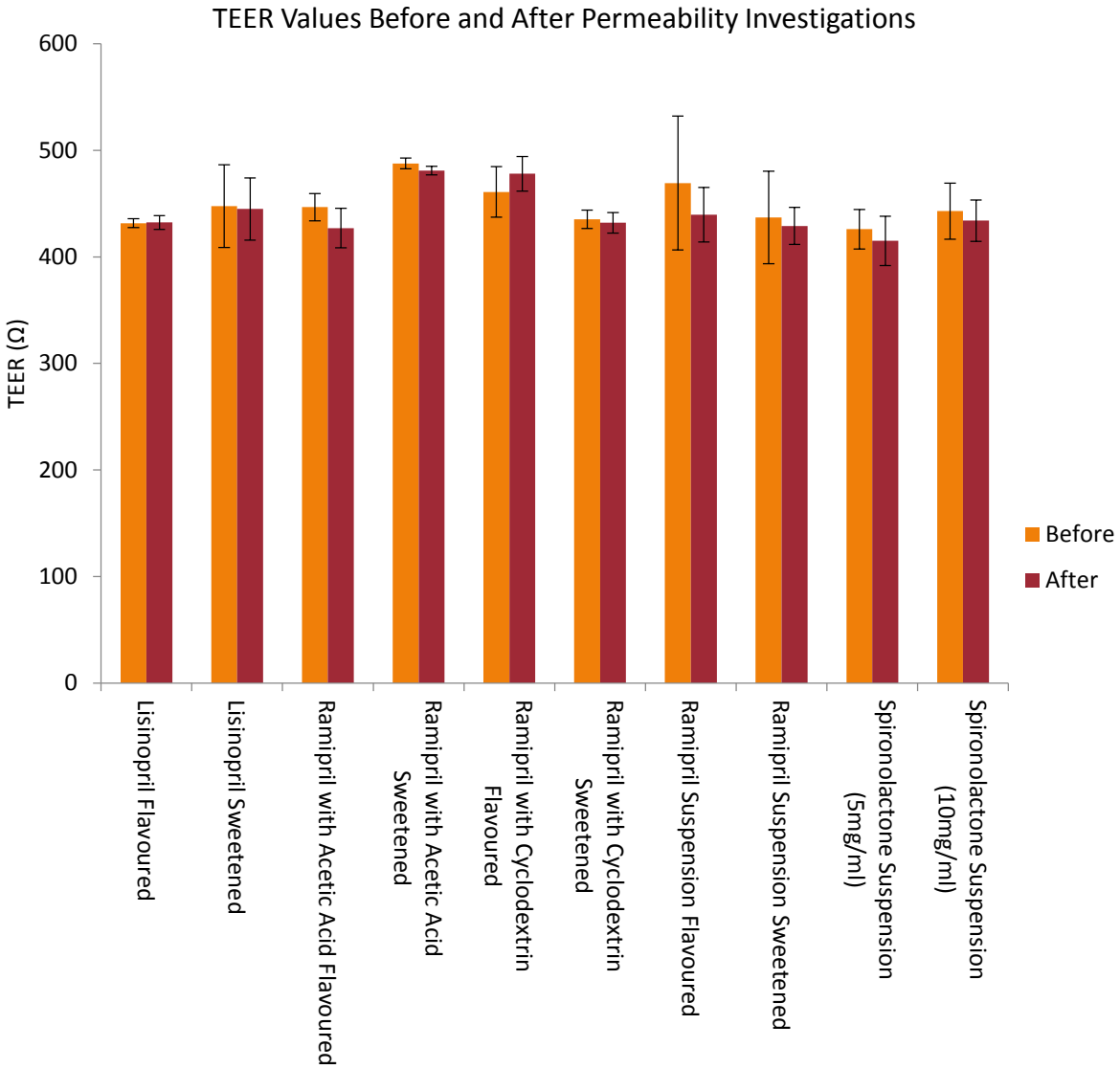

Figure 96 - TEER values before and after permeability investigations for all formulations tested. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

Following permeability experiments, samples were analysed via HPLC and the findings from which were plotted to reveal the percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time (Figures 97-108).

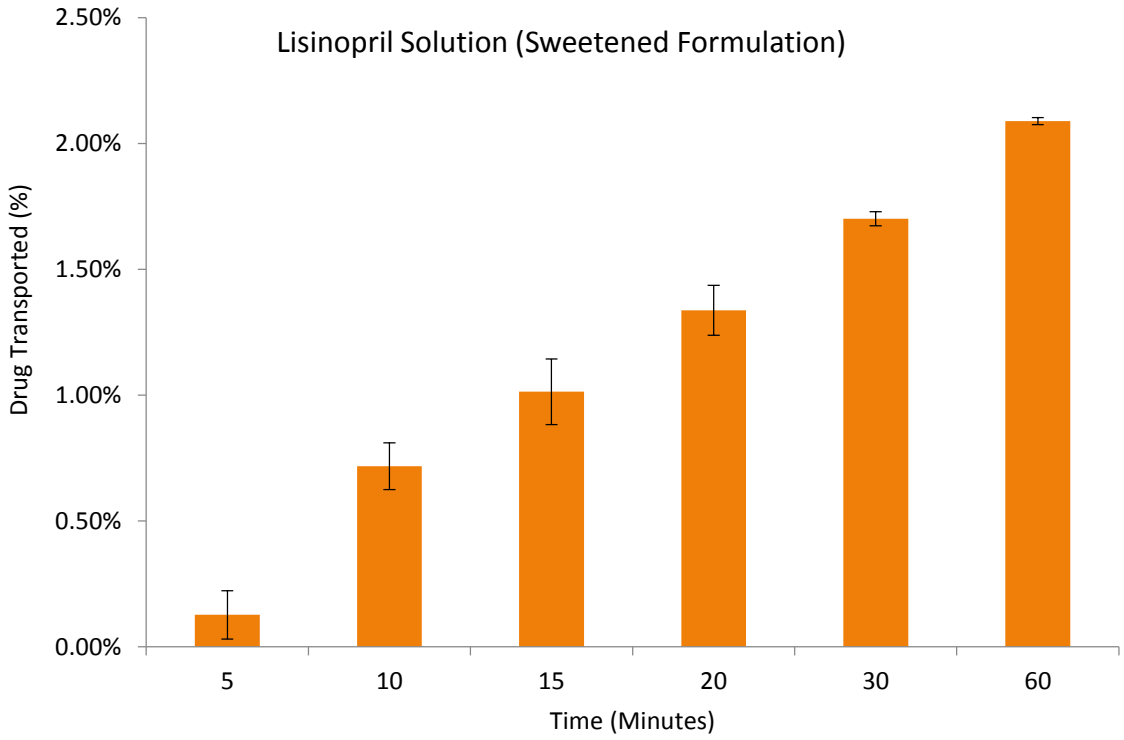

Figure 97 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Lisinopril solution. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

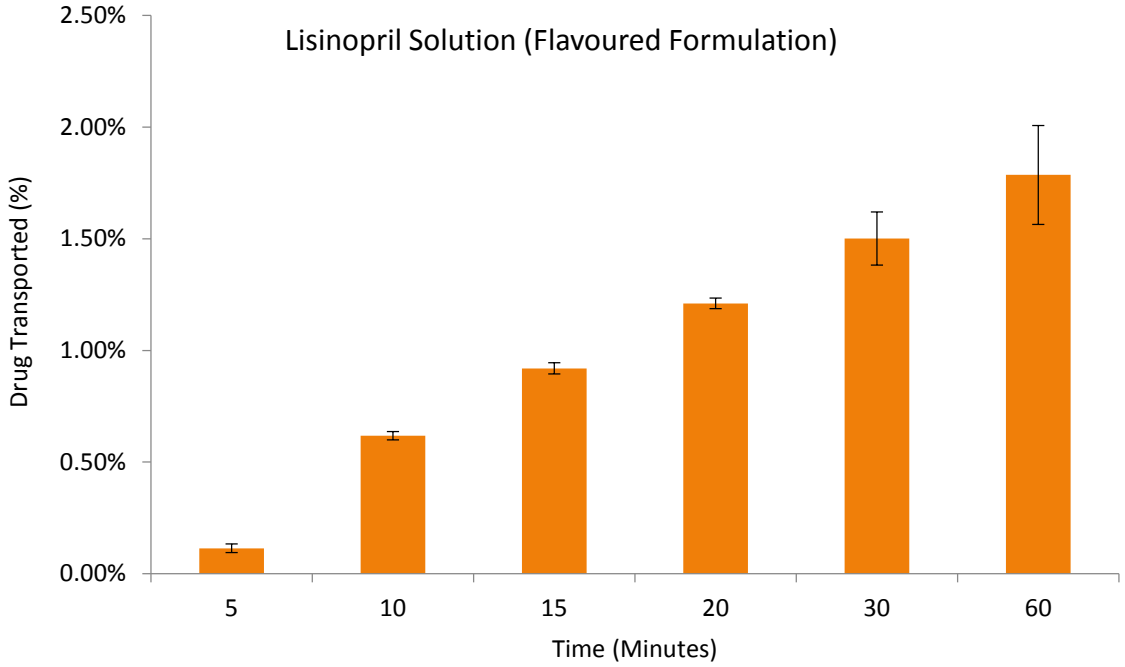

Figure 98 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Lisinopril solution. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

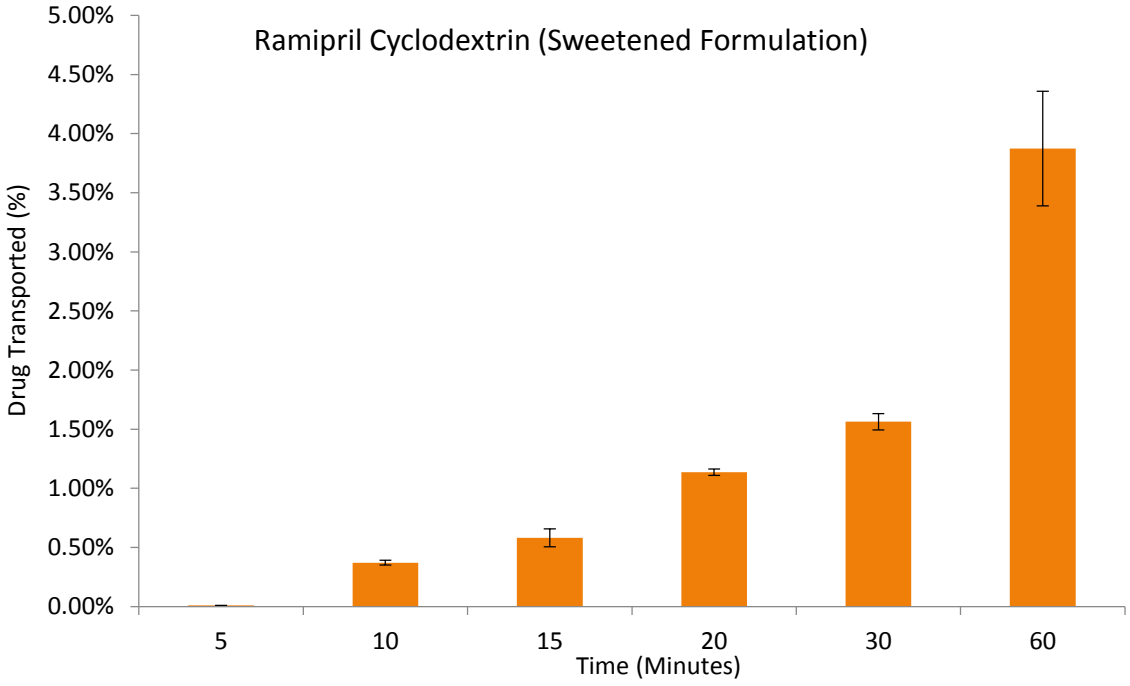

Figure 99 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Ramipril solution produced using HP-β-CD. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

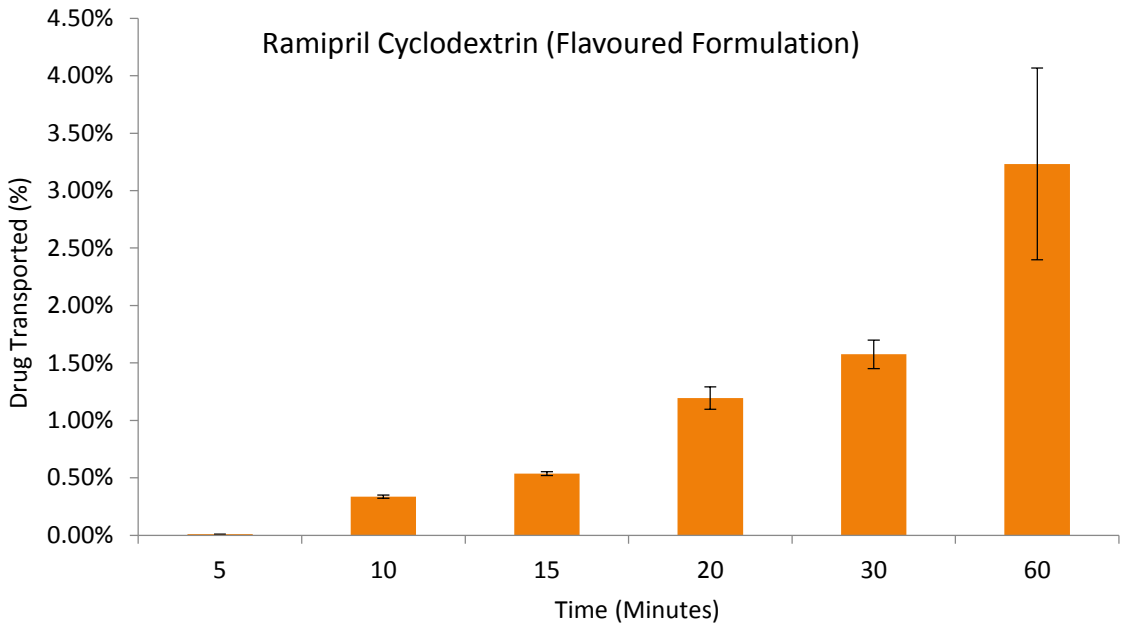

Figure 100 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Ramipril solution produced using HP-β-CD. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

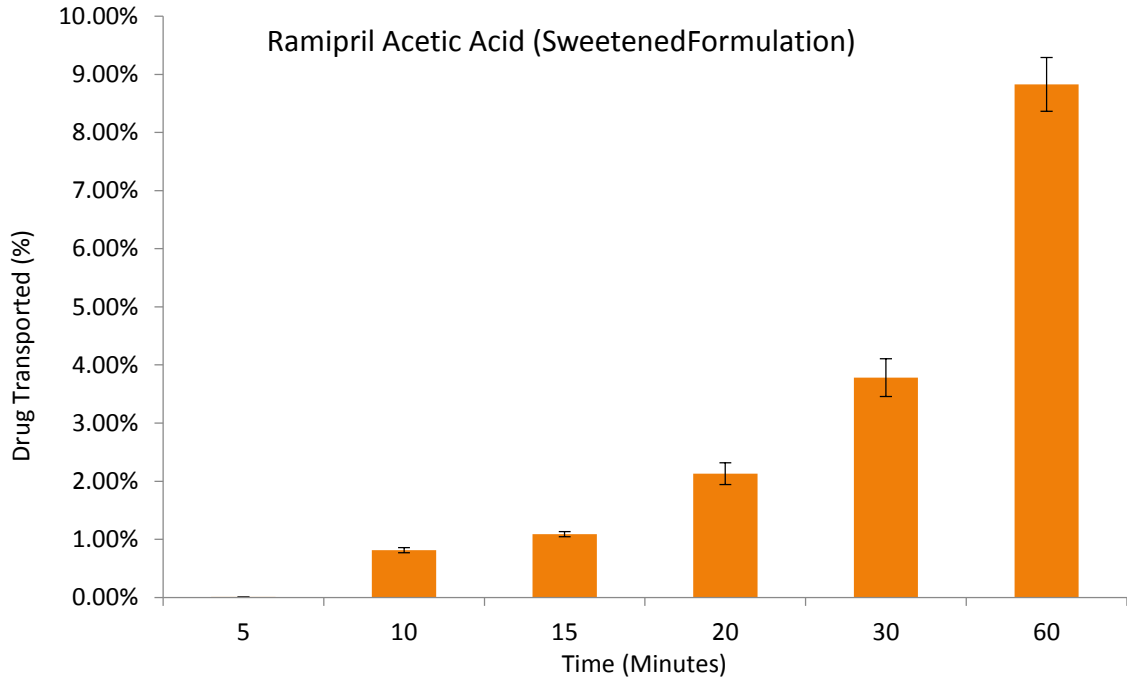

Figure 101 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Ramipril solution produced using acetic acid as a co-solvent. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

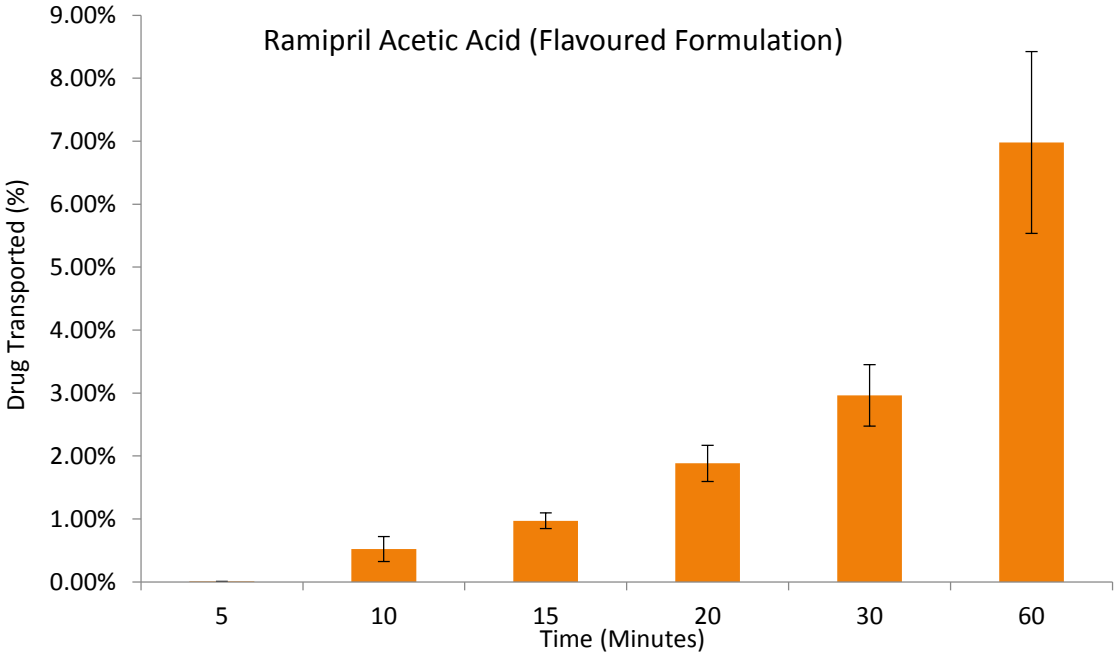

Figure 102 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Ramipril solution produced using acetic acid as a co-solvent. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

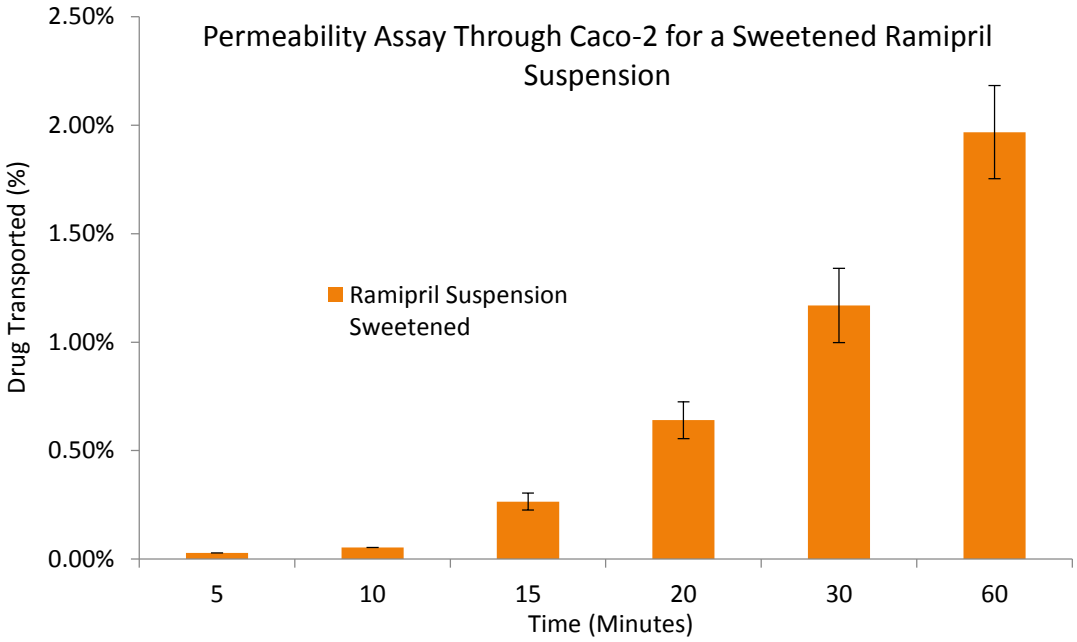

Figure 103 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Ramipril suspension. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

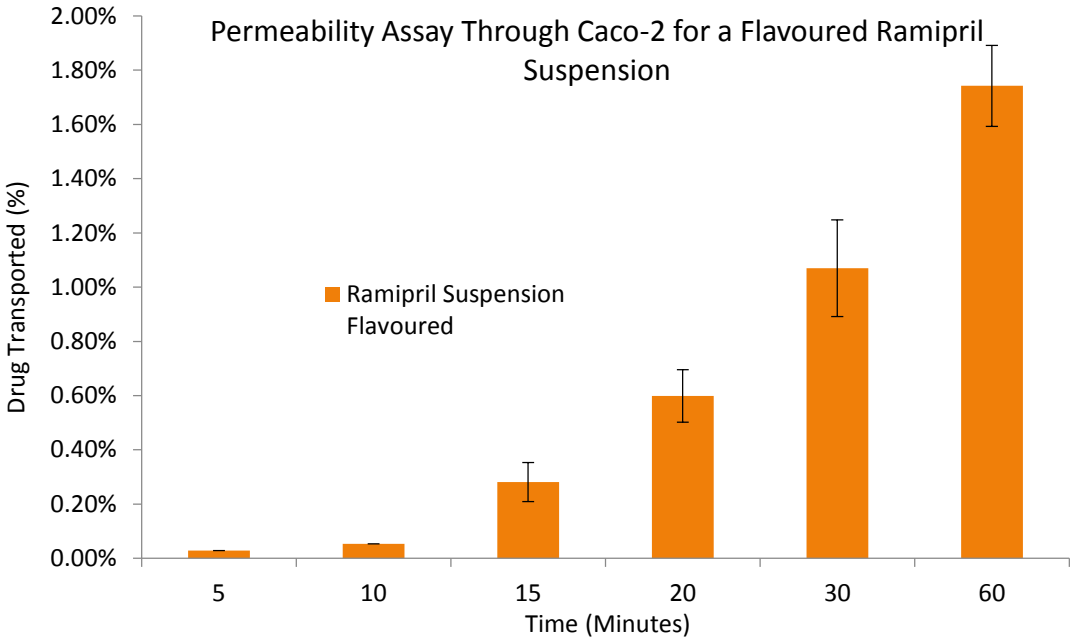

Figure 104 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Ramipril suspension. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

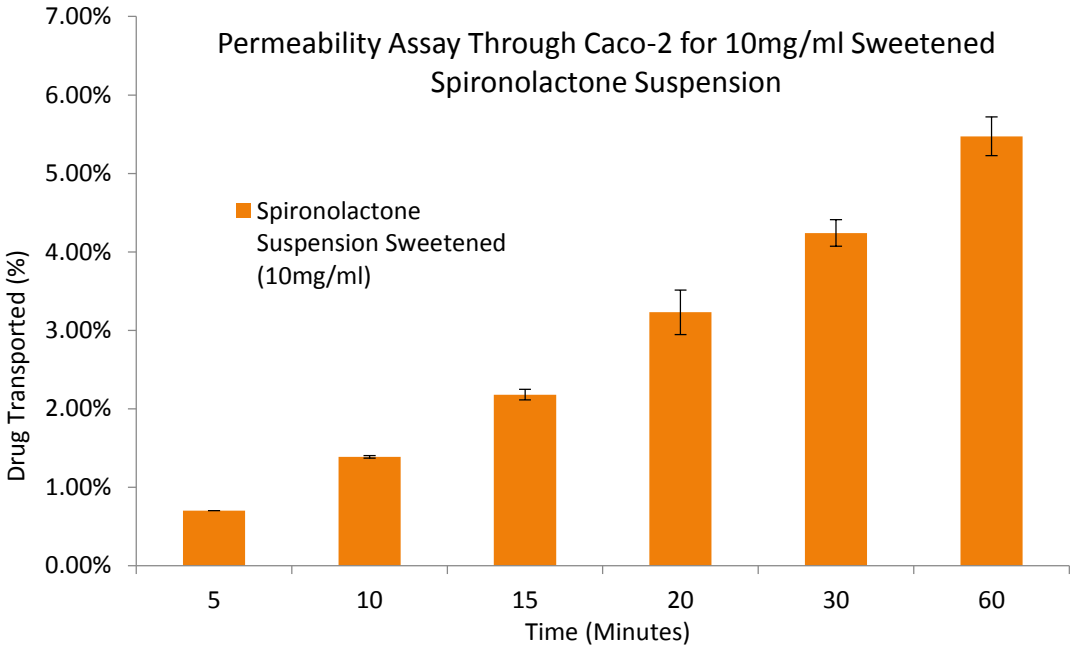

Figure 105 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Spironolactone suspension (10mg/ml). Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

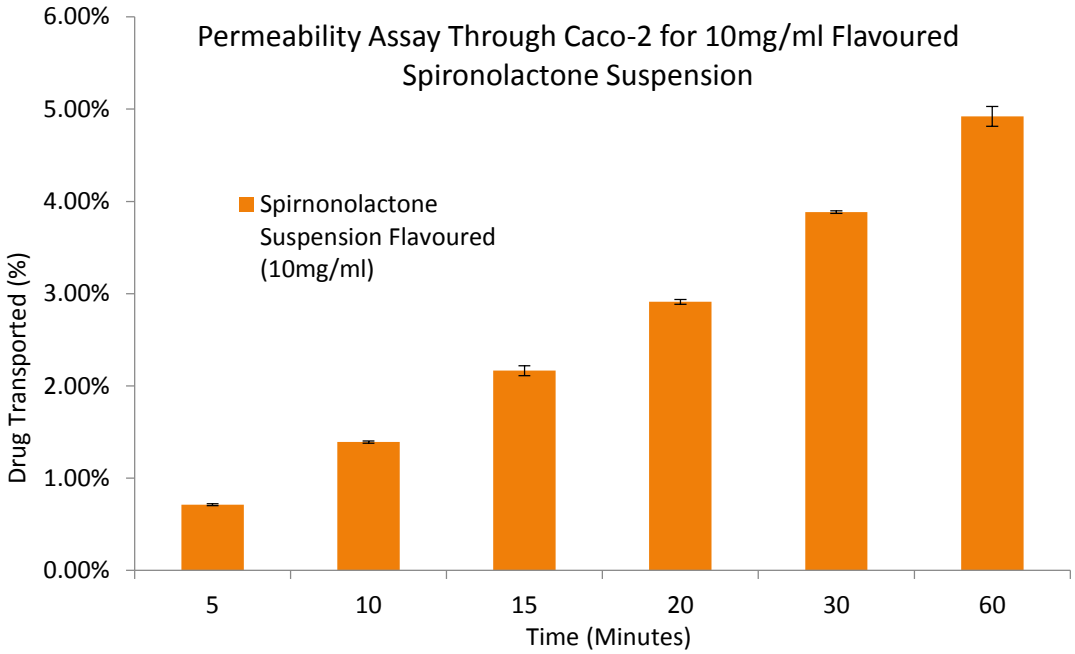

Figure 106 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Spironolactone suspension (10mg/ml). Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

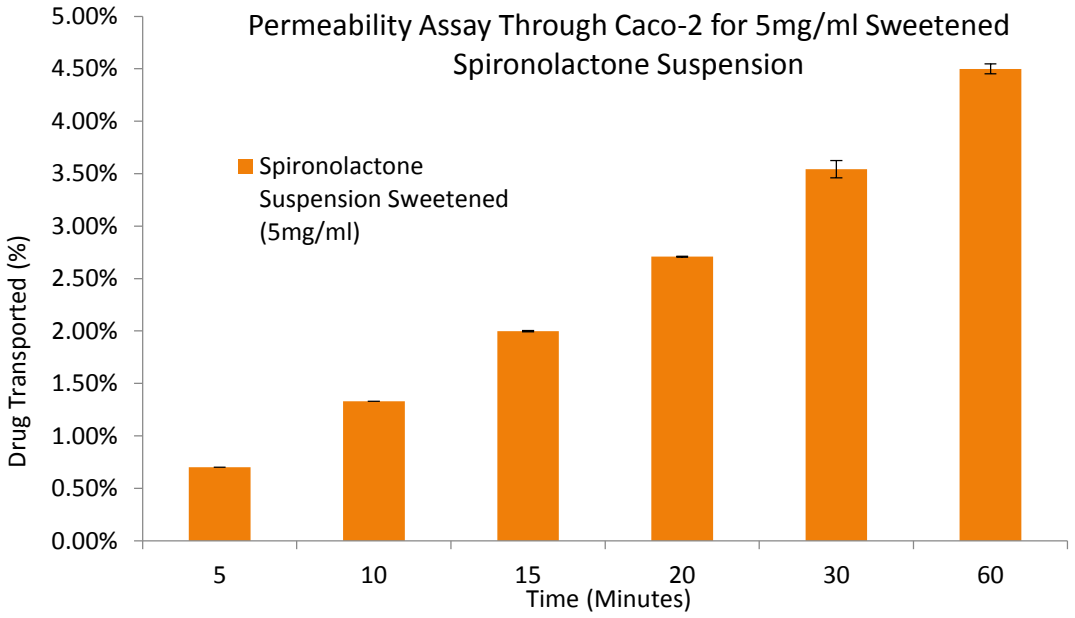

Figure 107 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the sweetened Spironolactone suspension (5mg/ml). Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

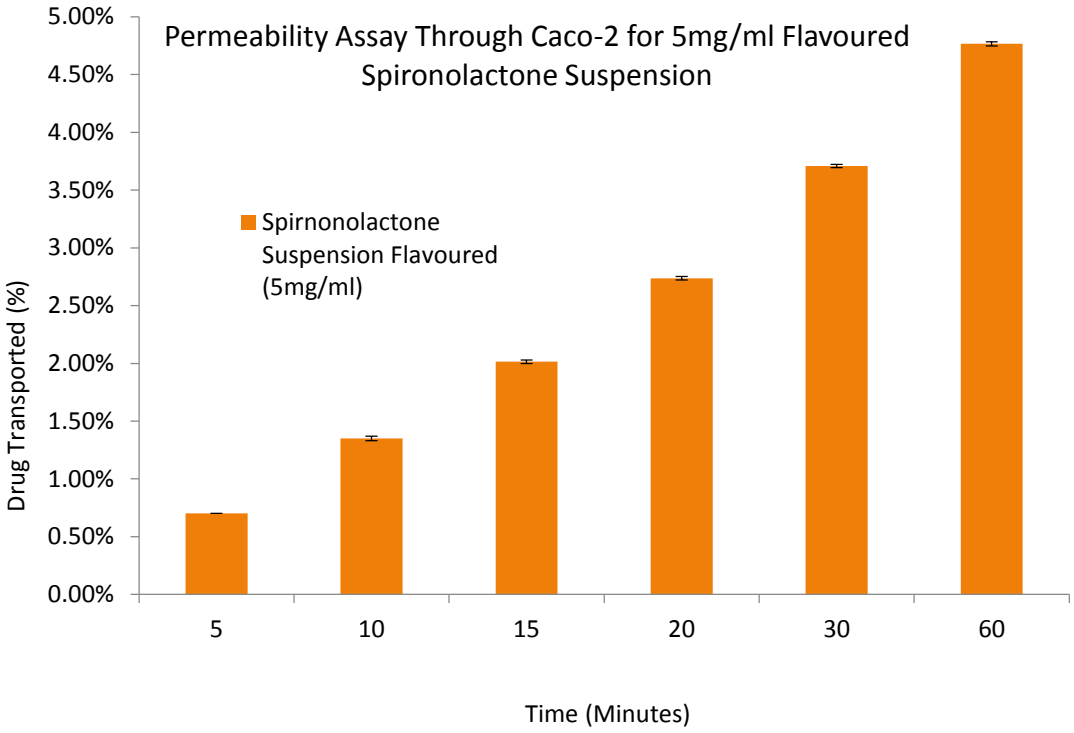

Figure 108 - Percentage of drug which had been transported from the apical compartment of the transwell to the basolateral compartment of the transwell as a factor of time for the flavoured Spironolactone suspension (5mg/ml). Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The data generated from the production of the transport vs time charts allowed for the calculation of $P_{app.}$ Applying the P_{app} equation to the data collected from the permeability assay carried out on the Ramipril and Lisinopril Solutions produced the P_{app} values detailed in figures 98 to 108.

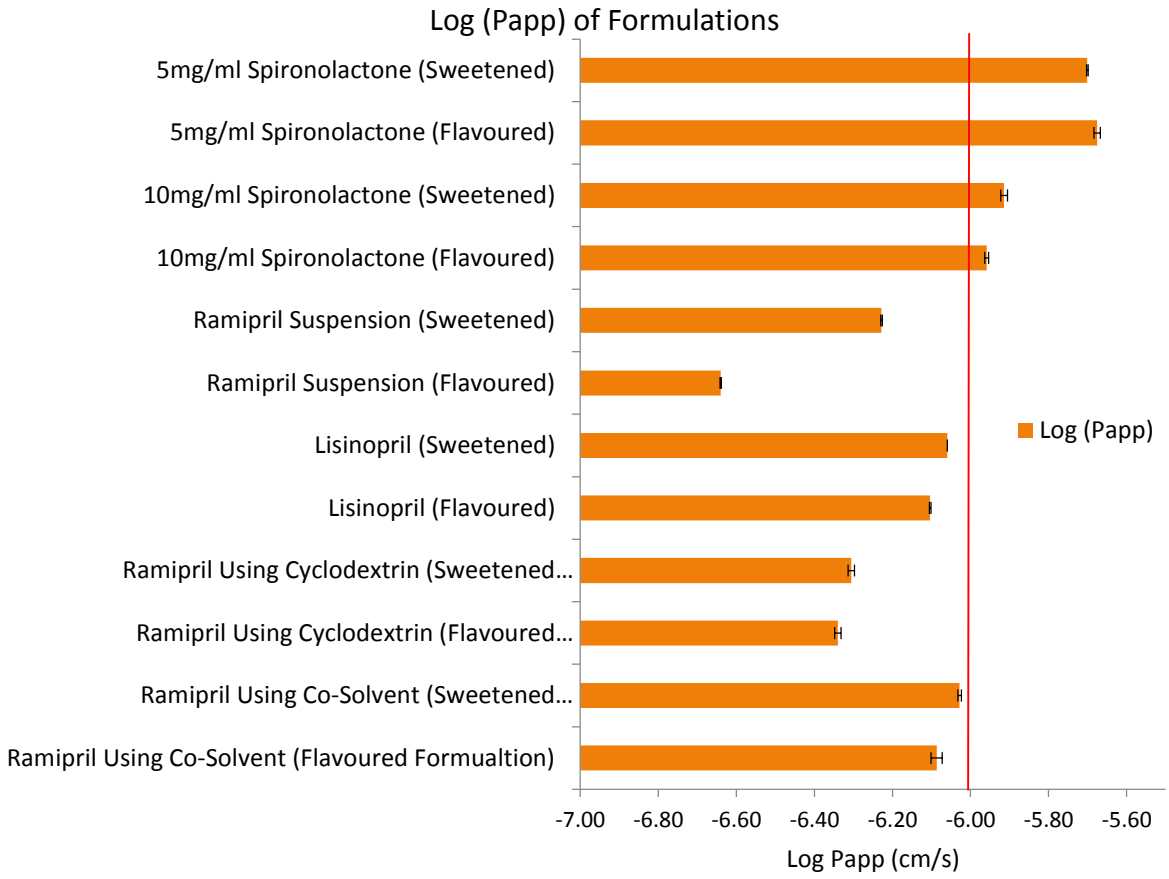

Figure 109 – Apparent permeability values for Lisinopril, Ramipril and Spironolactone formulations. It is generally accepted that completely absorbed drugs have Papp >1x10^{-6}cm/s. (LogP$_{app}$ > -6) and incompletely or poorly absorbed drugs have Papp <1x10^{-6}cm/s. (LogP$_{app}$ < -6). This threshold is indicated by the red line. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

In previous investigations into P_{app} the permeability coefficients ranged from approximately 5x10^{-8} to 5x10^{-5}. The permeability coefficients for the Ramipril, Lisinopril and Spironolactone formulations are in keeping with these findings. The permeability coefficient or apparent permeability of a molecule (P_{app}) is considered to be a reliable indicator for the expected *in vivo* drug absorption (Fraction Absorbed (fa)). It is generally accepted that completely absorbed drugs have Papp >1x10^{-6}cm/s. (LogP$_{app}$ > -6) and incompletely or poorly absorbed drugs have Papp <1x10^{-6}cm/s. (LogP$_{app}$ < -6). This threshold is indicated by the red line in figure 98. It is known that Spironolactone is fully absorbed *in vivo*, this is confirmed with a Log (Papp) greater than -6.00 for all of the

formulations. In addition, it is known that Ramipril and Lisinopril have 50-60% and 20-30% *in vivo* absorption respectively. By comparing Log (Papp) to expected absorption in humans (%) (Figure 110) it is seen that the results observed *in vitro* is a good indication of the anticipated *in vivo* absorption. In addition the comparison of our data with that from existing literature indicates that drugs used in our assay fit the expected profile.

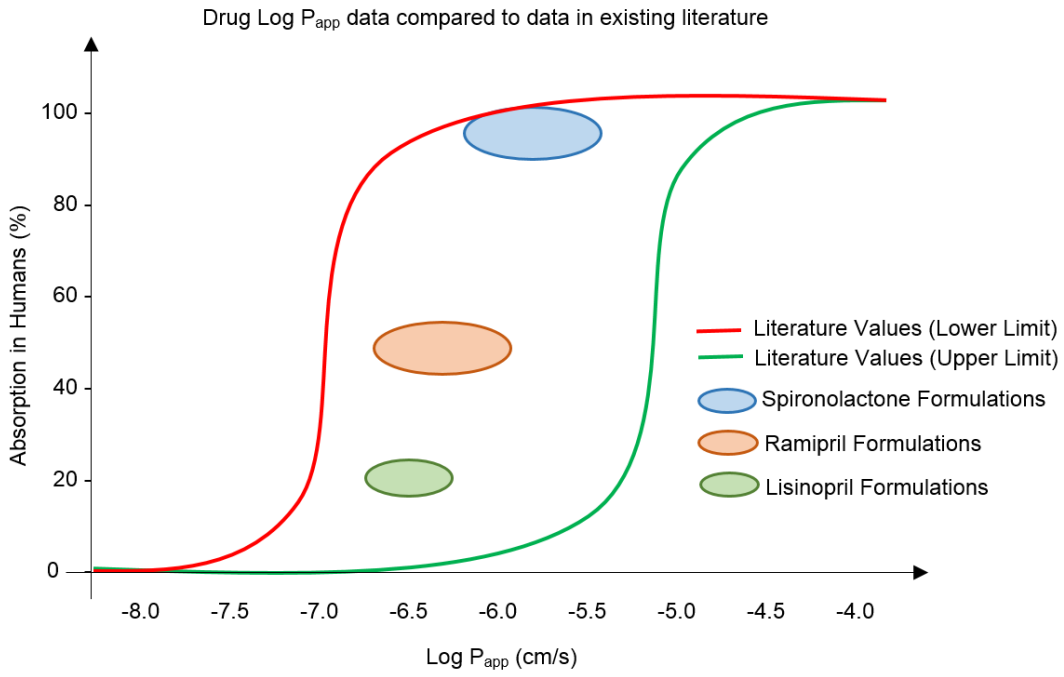

Figure 110 – Comparison of P_{app} data to predicted human absorption and also data from existing literature.

5.4.4 *In Vivo* Drug Absorption

The plasma concentration-time profile for the formulations developed and examined for *in vivo* absorption is shown below (Figure 111). Spironolactone showed the highest plasma concentrations for the duration of the analysis with the Lisinopril solution showing the next highest and the Ramipril formulations showing the lowest plasma concentrations.

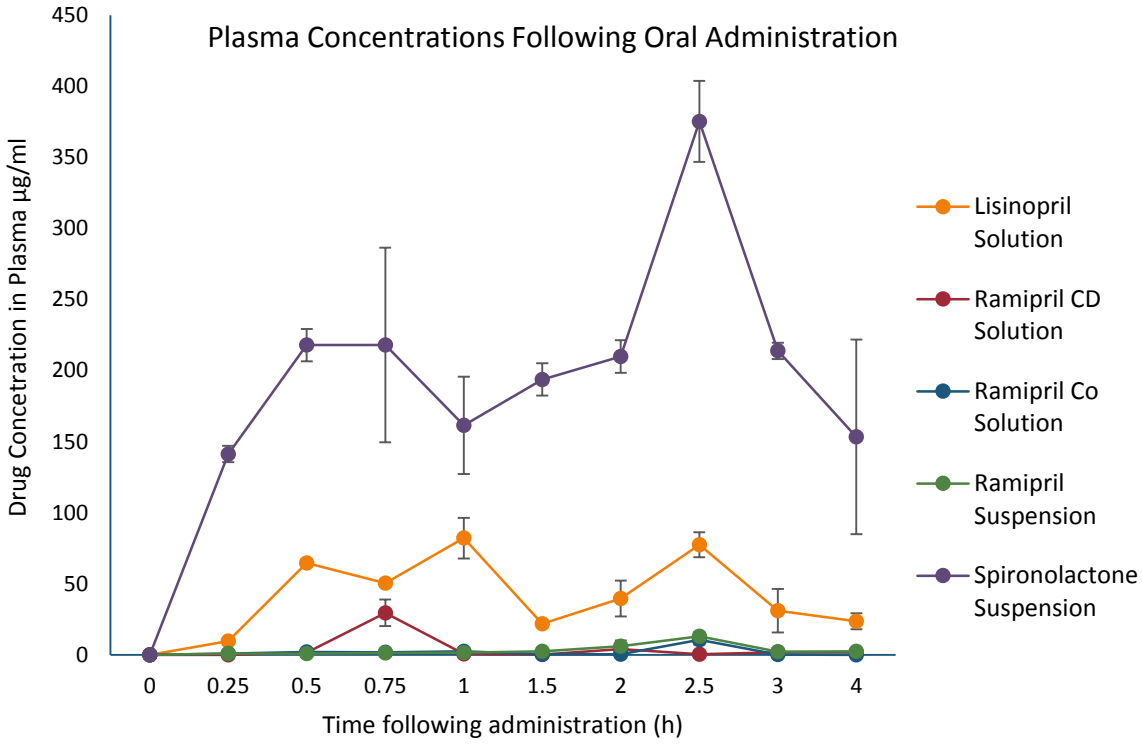

Figure 111 - Plasma Concentrations Following Oral Administration. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

From the plasma concentration-time profile, values for AUC, C_{MAX} and T_{MAX} were determined and are shown below in table 42.

Table 42 – In vivo results for AUC, C_{MAX} and T_{MAX}.

Formulation	AUC (ug*h/mL)	C_{MAX} (ug/ml)	T_{MAX} (h)
Lisinopril Solution	200.6525467	82.08	1
Ramipril Cyclodextrin Solution	15.07348068	29.58	0.75
Ramipril Co-solvent Solution	7.945549365	10.48	2.5
Ramipril Suspension	18.54717314	13.04	2.5
Spironolactone Suspension	1052.801405	375.37	2.5

Lisinopril and Ramipril AUC values are within the anticipated range although is not surprising as bioavailability is highly variable between individuals for both drugs with reports showing variation as great as 6-60% for both drugs. It is surprising to see Lisinopril absorption higher than Ramipril as Based on the majority of existing literature.

Lisinopril bioavailability is reported to be on average between 20-30% and Ramipril bioavailability is reported to be on average between 50-60%. The initial concentration of formulations will have an effect due to different concentration gradients and Lisinopril was dosed at 10mg/kg, Ramipril was dosed at 5mg/kg and Spironolactone was dosed at 40mg/kg. From this it would be reasonable to expect to see higher Lisinopril peak plasma concentrations than Ramipril. The findings reported in table 42 reflect this with Lisinopril C_{MAX} being 82.08µg/ml and Ramipril showing a C_{MAX} range of 10.48 µg/ml for the Ramipril solution produced using a co-solvent, 3.84µg/ml for the Ramipril Suspension and 29.58µg/ml for the Ramipril solution produced using cyclodextrin as a solubiliser.

The findings for C_{MAX} and T_{MAX} reveal some interesting points, for the Ramipril solution produced using a co-solvent, the Ramipril suspension and the Spironolactone suspension the occurrence of T_{MAX} 2.5h after administration is in keeping with reported literature. The Ramipril solution produced using cyclodextrin however shows a much earlier T_{MAX} occurring only 0.75h following administration. As the only formulation component which is unique to the Ramipril solution produced using cyclodextrins as a solubiliser is the HP-β-CD it is possible that this excipient is responsible for the earlier T_{MAX} and higher C_{MAX}. There is evidence that HP-β-CD decreases GI transit time in rats (Pestel *et al* 2006). As Ramipril is absorbed mainly in the Ileum, this would result in the formulation reaching the site of absorption more rapidly. Ordinarily, a decrease in intestinal residence time would see a decrease in absorption due to a shorter absorption window however cyclodextrins have the ability to improve drug absorption and this has been reported extensively. It is generally recognized that cyclodextrins act as true carriers by keeping the hydrophobic drug molecules in solution and deliver them to the surface of the biological membrane. The relatively lipophilic membrane has low affinity for the hydrophilic cyclodextrin molecules and therefore they remain in the aqueous membrane exterior, e.g. the aqueous vehicle system or GI fluid. Conventional

penetration enhancers, such as alcohols and fatty acids, disrupt the lipid layers of the biological barrier however, cyclodextrins, on the other hand, act as penetration enhancers by increasing drug availability at the surface of the biological barrier.

Another interesting factor which has presented is that of secondary maxima in plasma concentrations. The appearance of secondary maxima in plasma concentration-time curves following oral administration has been studied extensively and this is observed for the Lisinopril solution plasma concentration-time profile and less so in the Spironolactone suspension plasma concentration-time profile. As reported by Takamatsu *et al* 2002, double peaks have also been observed with other drugs, including flurbiprofen, penicillamine, aspirin, furosemide and acetaminophen. The reason for this occurrence cannot be confirmed however there are several reported hypotheses including region-dependent variation in absorption, enterohepatic recirculation, variable gastric emptying and intestinal transit rates and intestinal bacterial reconversion of biliary metabolite which have been proposed to account for these observations (Takamatsu *et al* 2002).

5.4.5 *In Vitro – In Vivo* Comparison

The findings from the *in vitro* and *in vivo* drug absorption studies were compared to identify a correlation between the results. For this the Log conversion of the AUC from the *in vivo* experiments was plotted against the Log P_{app} values calculated following the *in vitro* drug transport studies (Figure 112).

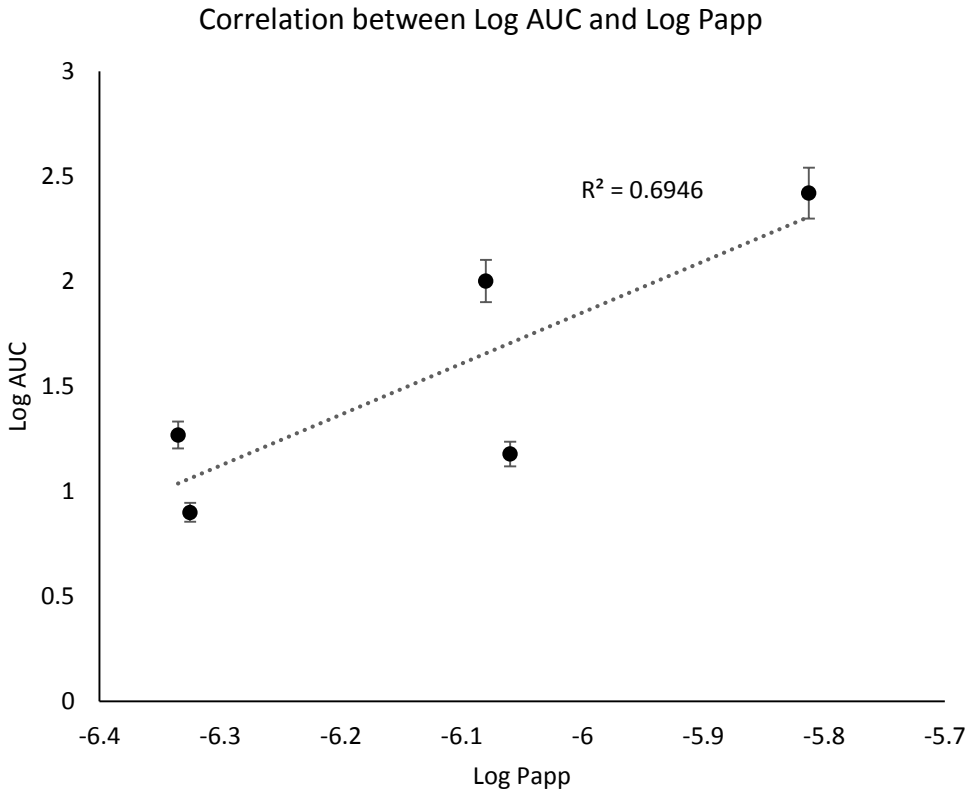

Figure 112 – For each of the formulations, the Log conversion of the AUC from the in vivo experiments was plotted against the Log P_{app} values calculated following the in vitro drug transport studies. This returned an R^2 value of 0.6946 indicating a poor correlation between the in vitro and in vivo findings. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

The correlation for the comparison of the *in vitro* and *in vivo* data sets was found to be $R^2 = 0.6946$. The R^2 value is comparable to those seen elsewhere in reported literature for *in vitro in vivo* correlations. The R^2 value would perhaps be expected to be some distance from the perfect linearity of $R^2 = 1.0$ as the comparison uses limited data points (n=5). There is also a known high degree of variation in the *in vivo* absorption of

the ACE inhibitor drugs Lisinopril and Ramipril. The next stage in the investigation examines the gene expression levels of intestinal transporters to see if there is a greater correlation between *in vitro* gene expression and *in vivo* drug absorption.

6 INVESTIGATION INTO THE POTENTIAL APPLICATION OF MICROARRAYS TO DEVELOP AN *IN VITRO – IN VIVO* CORRELATION SCREENING TOOLBOX IN THE ASSESSMENT OF LIQUID RAMIPRIL, LISINOPRIL AND SPIRONOLACTONE FORMULATIONS

6.1 CHAPTER AIMS AND OBJECTIVES

- As in chapter 5, Caco-2 cells grown on transwell inserts are used to perform *in vitro* drug transport studies for the formulations developed in chapters 2, 3 and 4.
- Extract RNA from cell samples
- Analyse RNA samples using microarray technology to identify genes which exhibit expression changes of 2 fold or more.
- Compare genomic data to the *in vitro* and *in vivo* findings to investigate the application of microarrays in the production of an *in vitro – in vivo* correlation screening toolbox.

6.2 INTRODUCTION

Microarrays are a powerful tool utilised in genomics allowing high throughput analysis of mRNA abundance. They have found application in many areas of drug discovery and development including the comparative assessment of normal and diseased state tissues, transcription and/or expression profiling, side effect profiling, pharmacogenomics and the identification of biomarkers (Russell *et al* 2013). They consist of a solid support onto which hybridisation probes are immobilised in a grid array and they are capable of producing huge amounts of data in the magnitude of tens of thousands of data points for every sample analysed. Although this can generate a wealth of information the data analysis process is crucial if anything meaningful is to be deciphered about the system being examined. To do this computational statistics are employed to generate gene lists which highlight genes of interest. The use of online databases such as KEGG is then used for pathway analysis amongst other things. In order to produce a manageable data set from the highly complex data generated from microarray scans, statistical approaches such as hierarchical cluster analysis (HCA),

principal component analysis (PCA) and statistical analysis of microarray (SAM) algorithms are used.

This chapter investigates the application of microarrays examining expressional changes following drug transport to potentially provide a tool for the prediction of intestinal drug absorption in humans. Work published recently has investigated changes in gene expression of the intestinal transporters following exposure of Caco-2 cells to pharmaceutical formulations (Khan *et al* 2010 and Khan *et al* 2011). For this the model drugs paracetamol and indomethacin were used as they are absorbed by non-carrier mediated and carrier mediated pathways respectively. Previous studies investigating transporter expression levels targeted individual transporters, many of which focusing on multidrug resistance protein 1 (MRP1), also known as permeability glycoprotein (P-gp). Khan *et al* reported for the first time that there was a wide range of genes for which expression was altered following indomethacin transport across Caco-2 monolayers. These included 25 genes in addition to MRP1 (Khan *et al* 2010). Findings for paracetamol also indicated modification in gene expression levels following transport studies (Khan *et al* 2011 and Ketterer *et al* 1983).

Lisinopril, Ramipril and Spironolactone formulations developed in house and for which *in vitro* and *in vivo* permeability data has been gathered are used as model formulations with a focus on the genes which code for the transporters ABCB1 and SLC15A1 for Lisinopril, SLC15A1 and SLC15A2 for Ramipril and ABCB1 (Ilka et al. 2008), ABCC2 and SLCO1A2 for Spironolactone (Cui et al. 2009; Kanai R. Bao, Y. Wolkoff, A. W. Schuster, V. L. 1996). Lisinopril is known to be an inhibitor of ABCB1 and a substrate for SLC15A1, Ramipril is known to be a substrate for SLC15A1 and SLC15A2. Spironolactone is and inhibitor of ABCB1 and SLCO1A2 and also an inducer of ABCC2.

6.3 Materials and Methods

6.3.1 Cell Culture

Caco-2 cells (passage 45) (A kind gift from Dr A. Collett and Dr Daniel Patten at the University of Huddersfield) were seeded at a density of $1.3x10^5$ cells/cm^2 onto permeability supports (Appelton Woods Ltd). Cells were cultured in an incubator (Sanyo) 37°C in a humidified 5% CO_2/95% air atmosphere. Media was changed every 2-3 days over a three week period. During this time TEER measurements were taken following each media change using an EVOM – Epithelial Voltohmmeter (World Precision Instruments Ltd) to develop a profile for tight junction formation and development and to ensure monolayer integrity. Additionally, TEER was monitored to ensure that monolayer integrity had been maintained for the duration of the investigation.

6.3.2 Drug Transport Assay

For the flux of Ramipril, Lisinopril and Spironolactone across Caco-2 monolayers, culture media was removed and the monolayers washed with HBSS before the monolayers were incubated at 37°C for 30 minutes with 2.5ml of HBSS in the basolateral compartment and 1.5ml of HBSS in the apical compartment. After 30 minutes the HBSS was removed from the apical compartment and replaced with 1.5ml of the formulation to be tested. Experiments were halted and cell samples prepared for RNA extraction at 0, 20 and 60 minutes. At each time point, ethanol/phenol (95:5) replaced HBSS and transwells were refrigerated for 24 hours. At which point cells were removed from the surface of the transwells carefully using a cell scraper. These cells were then transferred into centrifuge tubes and centrifuged at 2000rpm at 4°C for 10 minutes. Cell pellets were then stored at -80°C prior to RNA extraction.

6.3.3 RNA Extraction

RNA extraction for Caco-2 was performed using an RNeasy kit (Qiagen) following manufacturers guidelines. The extracted RNA was then quantified using a Nanodrop ND-1000 UV-VIS spectrophotometer (Thermoscientific, Wilmington, DE) (Table 43).

Table 43 – RNA Quantification

Formulation and Time Point	RNA Abundance (ng/ul)	260/280	260/230
Spironolactone Suspension (20min)	155.0	1.99	0.84
Spironolactone Suspension (60min)	271.2	2.06	1.73
Ramipril Suspension (20min)	250.0	2.04	0.61
Ramipril Suspension (60min)	403.3	2.01	1.50
Ramipril Solution (Cyclodextrin) (20min)	169.2	2.05	1.22
Ramipril Solution (Cyclodextrin) (60min)	161.2	2.06	0.75
Ramipril Solution (Cosolvent) (20min)	124.2	2.05	0.56
Ramipril Solution (Cosolvent) (60min)	228.4	2.04	1.50
Lisinopril Solution (20min)	382.5	2.03	1.48
Lisinopril Solution (60min)	331.5	2.01	1.34
Control (A)	210.2	2.06	1.11
Control (B)	222.6	2.04	1.11

6.3.4 Microarray Assay

The microarray assay was performed following the directions for Agilent Technologies' one-colour microarray-based gene expression analysis low input quick amp labelling kit. In short, 1.5µl of 50ng total RNA was mixed with 2µl of spike mix (dilution 4), cDNA master mix (Agilent Technologies, Santa Clara, CA) was used to prepare cDNA for all samples ahead of labelling with cyanine 3-CTP (Cy3) in the labelling reaction. The labelled and amplified cRNA was then purified using RNeasy mini spin columns (Qiagen) and quantified using Nanodrop ND-1000 UV-VIS spectrophotometer (Thermoscientific, Wilmington, DE). cRNA yield and specific activity were calculated (Table 44).

Table 44 – cRNA yield and specific activity.

Sample	Abs@260nm	pmol/ul	ng/ul	260/280	Yield (ug)	Specific Activity
Spiro Suspension (20min)	0.031	2.10	296.10	2.47	8.88	7.09
Spiro Suspension (60min)	0.043	2.80	427.30	2.37	12.82	6.55
Ramipril Susp (20min)	0.008	0.60	50.00	2.27	1.50	12.00
Ramipril Susp (60min)	0.021	1.40	63.60	1.69	1.91	22.01
Ramipril Solution (CD) (20min)	0.015	1.00	270.00	1.94	8.10	3.70
Ramipril Solution (CD) (60min)	0.030	2.00	111.80	2.23	3.35	17.89
Ramipril Solution (Co) (20min)	0.009	0.60	86.60	2.21	2.60	6.93
Ramipril Solution (Co) (60min)	0.004	0.30	62.70	2.13	1.88	4.78
Lisinopril Solution (20min)	0.005	0.30	40.00	2.36	1.20	7.50
Lisinopril Solution (60min)	0.004	0.20	36.40	2.36	1.09	5.49
Control (A)	0.006	0.40	46.50	2.36	1.40	8.60
Control (B)	0.007	0.40	41.70	2.22	1.25	9.59

All samples were then hybridised to Agilent 4x44K whole genome arrays for 17 hours at 65°C in a hybridisation oven (Sheldon manufacturer, Corneilus, OR). Following the hybridisation stage slides were washed using the gene expression wash buffer kit (Agilent Technologies, Santa Clara, CA) and acetonitrile.

Scanning was carried out at the University of Birmingham with the help of staff in the functional genomics, proteomics and metabolomics facility where an Agilent Scanner (Agilent Technologies, Santa Clara, CA) ran 20 bit scans at a resolution of 50n.

6.3.5 Data Processing

Feature Extraction software (V10.7, Santa Clara, CA) was implemented to examine the quality of the 16-bit TIFF images obtained by microarray scanning. They were assessed on grid alignment, signal quantification and overall slide quality at the

University of Birmingham with the help of staff in the functional genomics, proteomics and metabolomics facility. Data normalisation was carried out at the University of Wolverhampton with the help of Dr. David Huen and Dr. Ayesha Rahman.

6.3.6 Data Clustering and Filtering

Data clustering was performed using TMEV software (version TM4, WA, USA). The samples were clustered according to the similarities seen in the gene expression patterns using hierarchical clustering algorithm (HCA). The mean values for the level of gene expression was used to perform comparisons between data sets. For data reduction, TMEV was also used to perform principal component analysis (PCA) which was implemented to illustrate the main degree of variability in the multidimensional data set. Statistical analysis was carried out using significance analysis of microarrays (SAM) to identify statistically significant genes which demonstrated either a 2 fold up regulation in expression or a 2 fold down regulation in gene expression. Delta values were selected so as that the median false discovery rate (FDR) was lower than 5%. These gene lists were then exported to EXCEL where the gene tables of SLC and ABC genes were prepared for data entry into KEGG http://www.genome.jp/kegg/. Here pathways in which the genes of interest were involved were identified and the component affected by the gene of interest in each pathway was highlighted in red.

6.4 Results and Discussion

Investigation into gene expression changes of Caco-2 cells following drug transport studies were intended to identify any link between the predicted drug permeability and the expression of the genes which code for the intestinal transporters for which Lisinopril, Ramipril and Spironolactone are known to interact. Namely ABCB1 and SLC15A1 for Lisinopril, SLC15A1 and SLC15A2 for Ramipril and ABCB1, ABCC2 and SLCO1A2 for Spironolactone. Rather than look at genetic expression levels of only the genes for the specific transporters with which the drugs interact it was decided to investigate the transporter super families to which these transporters belong, these being the ABC and SLC super families. In order to do this drug transport studies were carried out using transwell arrangements and the genetic profiles of the subsequently harvested cells were examined. The expression patterns for Caco-2 cells in their basal state were used as a control and compared to cells which had been exposed to Lisinopril, Ramipril and Spironolactone. Following RNA extraction, over 40,000 cRNA were hybridised to their corresponding microarray probe implementing the one colour microarray based gene expression analysis protocol from Agilent Technologies. Scanning the microarray slides produced TIFF files, and example of which is shown in figure 113 which were then analysed for their grid alignment, signal quantification and overall slide quality.

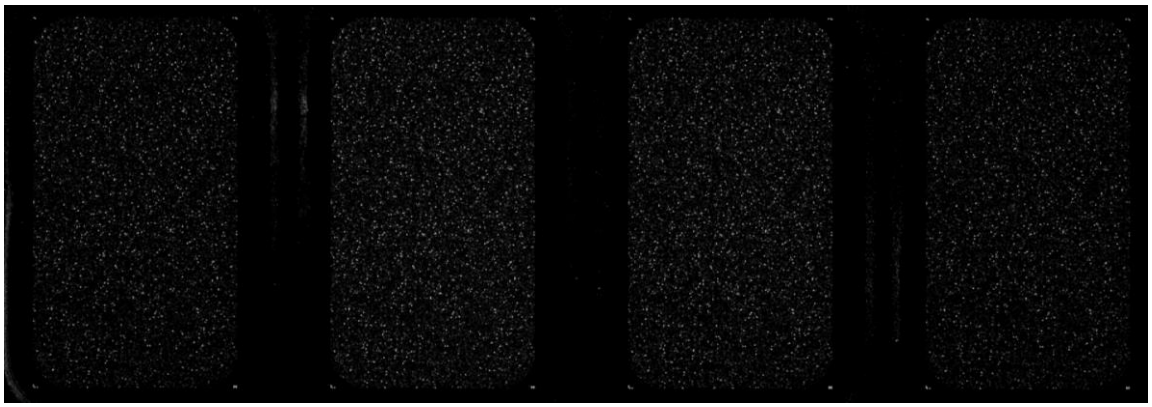

Figure 113 – TIFF image produced following microarray slide scanning.

6.4.1 TIFF Image analysis

Spot centroids are located in the corner of each array and are used to confirm that the image is correctly centred and that the image is of suitable quality. An example of which is shown in figure 114. For all of the arrays carried out the centroids were located correctly.

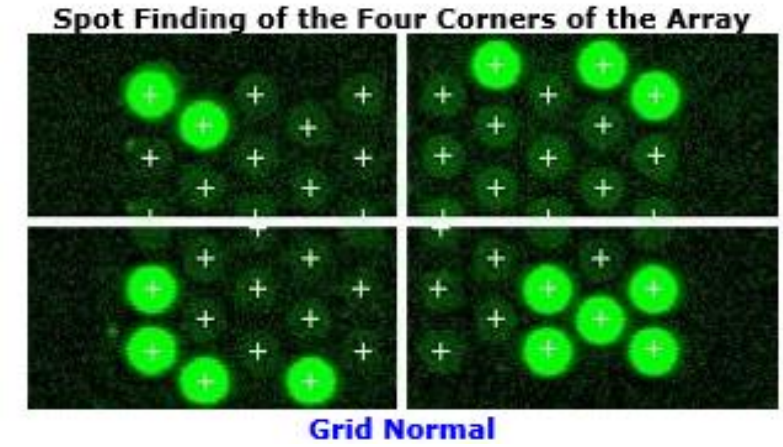

Figure 114 - Spot centroids are located in the corner of each array and are used to confirm that the image is correctly centred and that the image is of suitable quality.

The feature extraction software also assesses the signal quality of the array through the production of a histogram of signals. Again an example is shown in figure 115. This histogram plots the number of points in intensity bins against the logarithm of the processed signal.

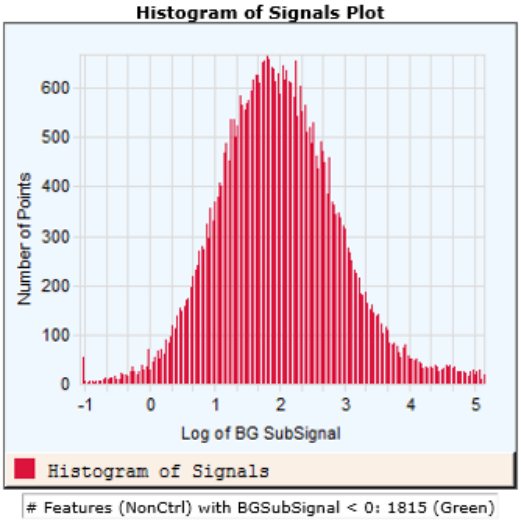

Figure 115 - The feature extraction software assesses the signal quality of the array through the production of a histogram of signals.

Once again the signal quality of all of the arrays was acceptable and this is determined by the normal distribution of the data in the histogram and the production of a symmetrical chart. The Log values of the processed signal were also confirmed to show good linearity with the Log values of the corresponding concentrations of the SpikeIns (Figure 116) for all of the arrays used as seen in the example.

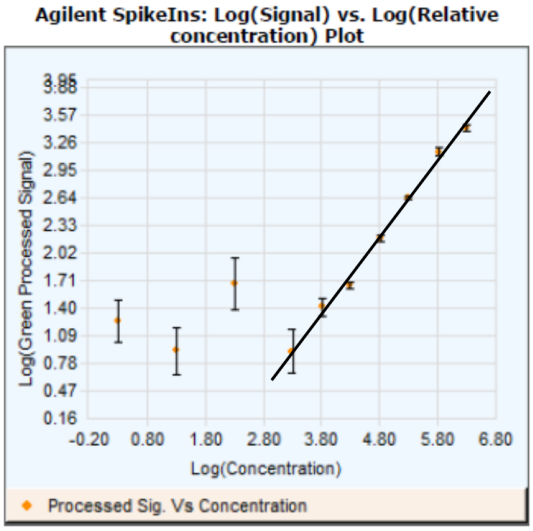

Figure 116 - Log values of the processed signal show good linearity with the Log values of the corresponding concentrations of the SpikeIns.

The median value for the processed signal and the median value for the background subtracted signal (mean signal – BG) for the whole array produced in all cases a horizontal line with sufficiently minimal variation about this line. There are multiple reference probes across the array to allow for reproducibility to be confirmed across the length and breadth of the array. A low median coefficient of variation in the signal level from these probes confirms this. An example of which is shown below (Figure 117).

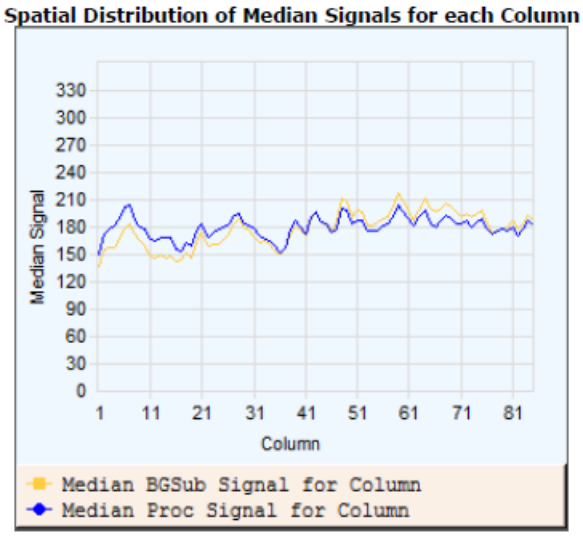

Figure 117 - Multiple reference probes across the array to allow for reproducibility to be confirmed across the length and breadth of the array. A low median coefficient of variation in the signal level from these probes confirms this

Following verification that the TIFF images produced from the microarray scanning were suitable, data normalisation was performed prior to data analysis.

6.4.2 Microarray Data Analysis

Due to the magnitude and complexity of the data generated by microarray experiments, the use of programs designed specifically to apply statistical techniques to microarray data are used to identify trends in the data. One such program is TMEV (TMEV software version TM4, WA, USA) which was used to perform HCA, PCA and SAM analysis on the microarray data. Statistically significant genes from the super

families SLC and ABC with a fold change in expression levels greater than 2 were then fed into the KEGG database to carry out pathway analysis.

6.4.3 Hierarchical Clustering Algorithm (HCA)

There are multiple computational, statistical approaches broadly classified into supervised and unsupervised learning techniques. HCA is an example of unsupervised learning and in this instance clustered samples are grouped based on the similarity in the gene expression profiles. The algorithm measures distance between rows in the data for the various samples and then combines the samples for which the distance between the genes is the shortest. Control samples were compared to formulation samples at each time point using HCA analysis (Figure 118).

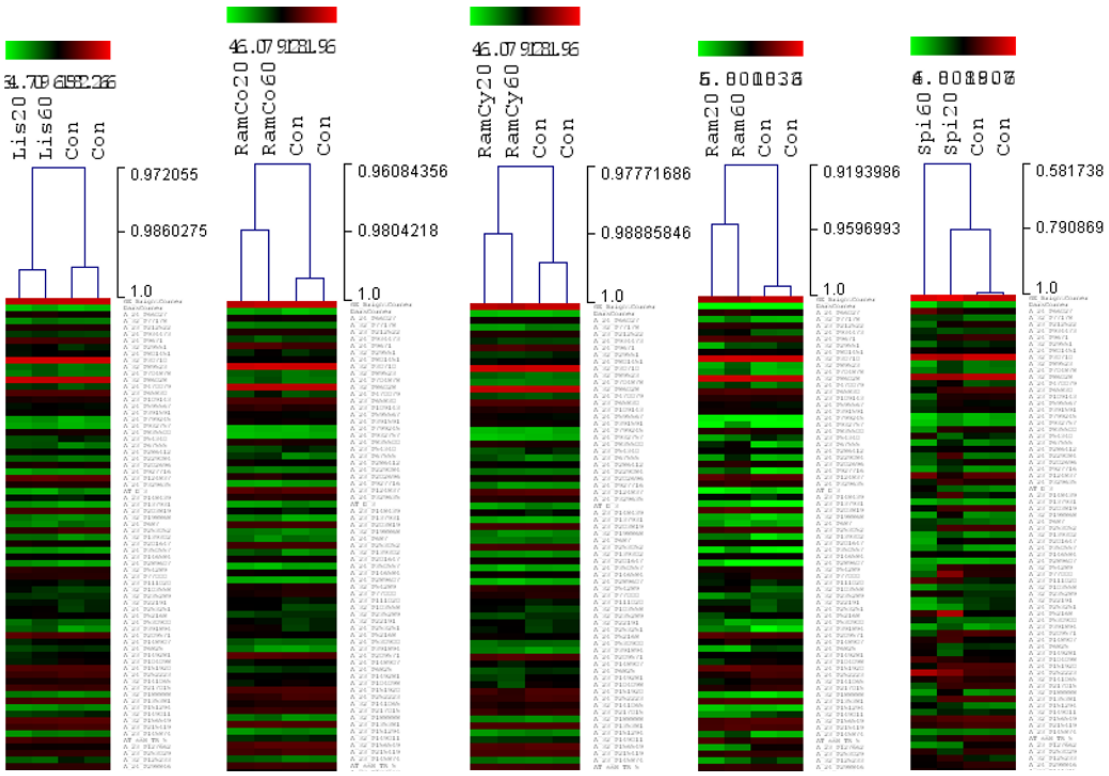

Figure 118 - Control samples were compared to formulation samples at each time point using HCA analysis.

The HCA clusters showed in all cases that gene expression levels in samples which had undergone treatment with the drug formulations had been altered from that of the control samples. Further HCA clustering comparing the formulations to each other indicated that the Spironolactone suspension elicited the greatest change in gene expression followed by the Ramipril suspension. The Ramipril solutions showed a smaller effect than the Ramipril suspension but a larger effect than the Lisinopril solution (Figure 119).

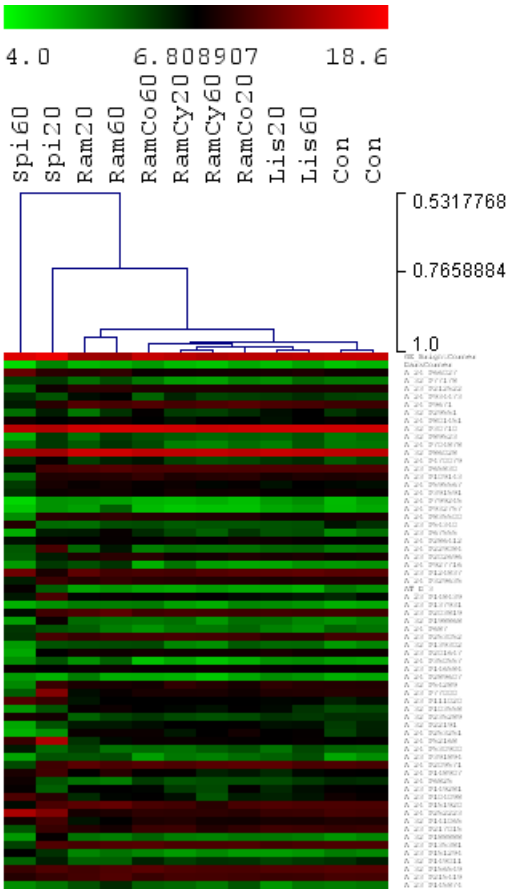

Figure 119 - HCA clustering comparing the formulations to each other indicated that the Spironolactone suspension elicited the greatest change in gene expression followed by the Ramipril suspension. The Ramipril solutions showed a smaller effect than the Ramipril.

6.4.4 Principal Component Analysis (PCA).

Using TMEV, PCA was carried out on the microarray data for all of the samples, EVD generated 12 principal components (Table 45) and from these it was observed that 89.105% of the variance in the data set and therefore genetic expression was accounted for in components 1, 2 and 3. Components 4 – 12 were discounted to aid data visualisation.

Table 45 - PCA was carried out on the microarray data for all of the samples, EVD generated 12 principal components.

Eigen Values		
Principal Component 1	5.666	62.80%
Principal Component 2	1.59	17.63%
Principal Component 3	0.782	8.67%
Principal Component 4	0.268	2.98%
Principal Component 5	0.192	2.12%
Principal Component 6	0.184	2.04%
Principal Component 7	0.12	1.33%
Principal Component 8	0.087	0.96%
Principal Component 9	0.059	0.65%
Principal Component 10	0.038	0.42%
Principal Component 11	0.025	0.28%
Principal Component 12	0.009	0.10%
First 2 components: 80.432 %		
First 3 components: 89.105 %		

First and Second PCA (Figure 120) illustrated that the Spironolactone suspension had by far the greatest effect on the gene expression levels. A greater degree of change was seen at 60 minutes than was observed at the 20 minute time point with variance seen along both components 1 and 2. The ACE inhibitor formulations were tightly grouped and showed only a small degree of variance along components 1 and 2 in comparison to the Spironolactone.

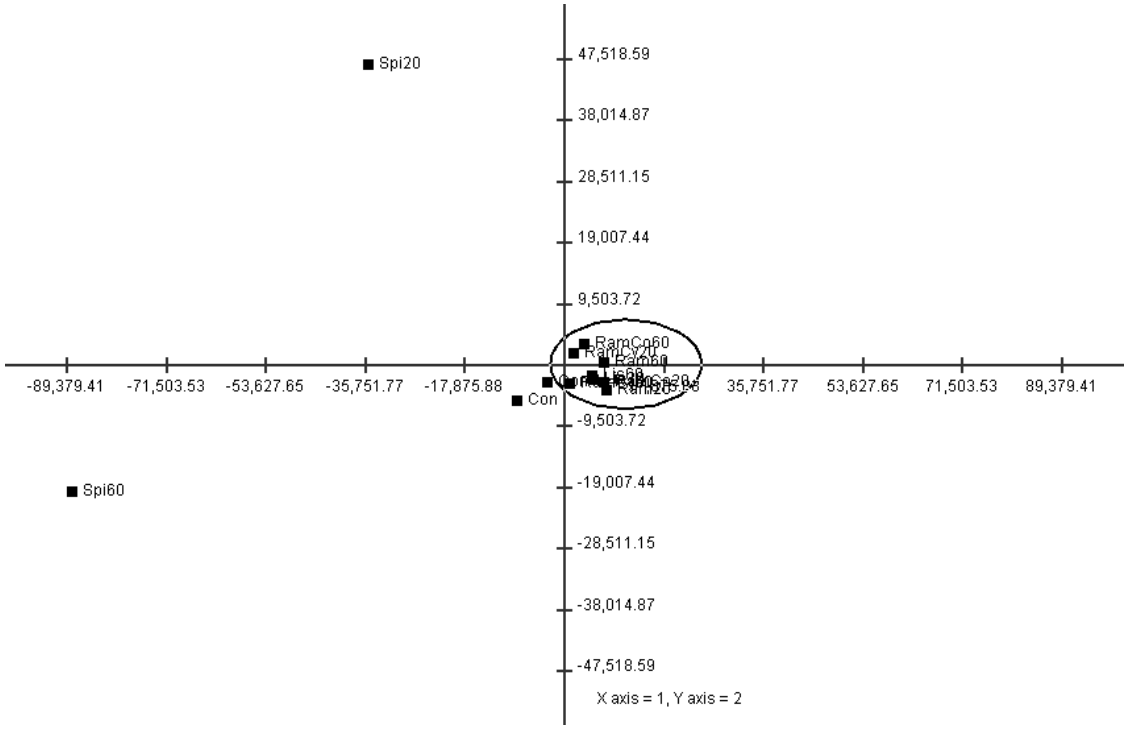

Figure 120 - First and Second PCA - Spironolactone suspension had by far the greatest effect on the gene expression levels in the samples with a greater degree of change seen at the 60 minutes than was observed at the 20 minute time point with variance seen along both components 1 and 2. The ACE inhibitor formulations were tightly grouped (circled area) and showed only a small degree of variance along components 1 and 2 in comparison to the Spironolactone

First and Third PCA (Figure 121) again illustrates the large degree of variance seen in the gene expression for the Spironolactone formulation however in this instance the degree of variance along the third component illustrates the variation in effect on gene expression between the Ramipril suspension and the other ACE inhibitor formulations. Once again the magnitude in the variation at the 60 minute time point is greater than that for the 20 minute time point showing that the overall changes in gene expression have increased with time.

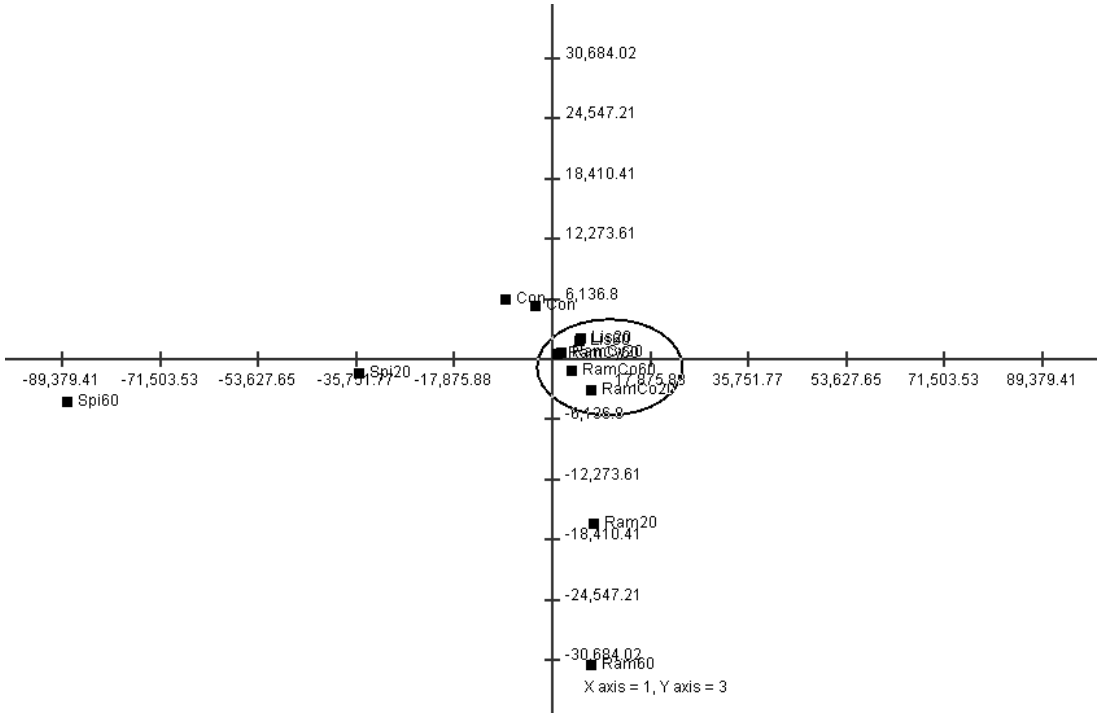

Figure 121 - First and Third PCA – A large degree of variance is seen in the gene expression for the Spironolactone formulation however in this instance the degree of variance along the third component illustrates the variation in effect on gene expression between the Ramipril suspension and the other ACE inhibitor formulations (circled area). Once again the magnitude in the variation at the 60 minute time point is greater than that for the 20 minute time point showing that the overall changes in gene expression have increased with time

Further PCA was carried out comparing individual formulations to the control samples in order to more clearly identify differences in gene expression levels at the time points tested.

6.4.5 Lisinopril Solution PCA

EDV generated 4 principal components for PCA of samples relating to the Lisinopril solution of which 87.839% of the variance was described in components 1 and 2 (Table 46). Components 3 and 4 were therefore discounted to further simplify data analysis.

Table 46 - EDV generated 4 principal components for PCA of samples relating to the Lisinopril solution

Eigen Values		
Principal Component 1	0.274	72.54%
Principal Component 2	0.058	15.30%
Principal Component 3	0.033	8.82%
Principal Component 4	0.013	3.35%
First 2 components: 87.839 %		
First 3 components: 96.654 %		

First and Second PCA (Figure 122) illustrates that there is variance in the gene expression between the samples exposed to the Lisinopril solution at both time points and the control samples along principal component 1 however there is only a very small degree of variation in the gene expression levels at the 20 minute and 60 minute time points in both principal components 1 and 2.

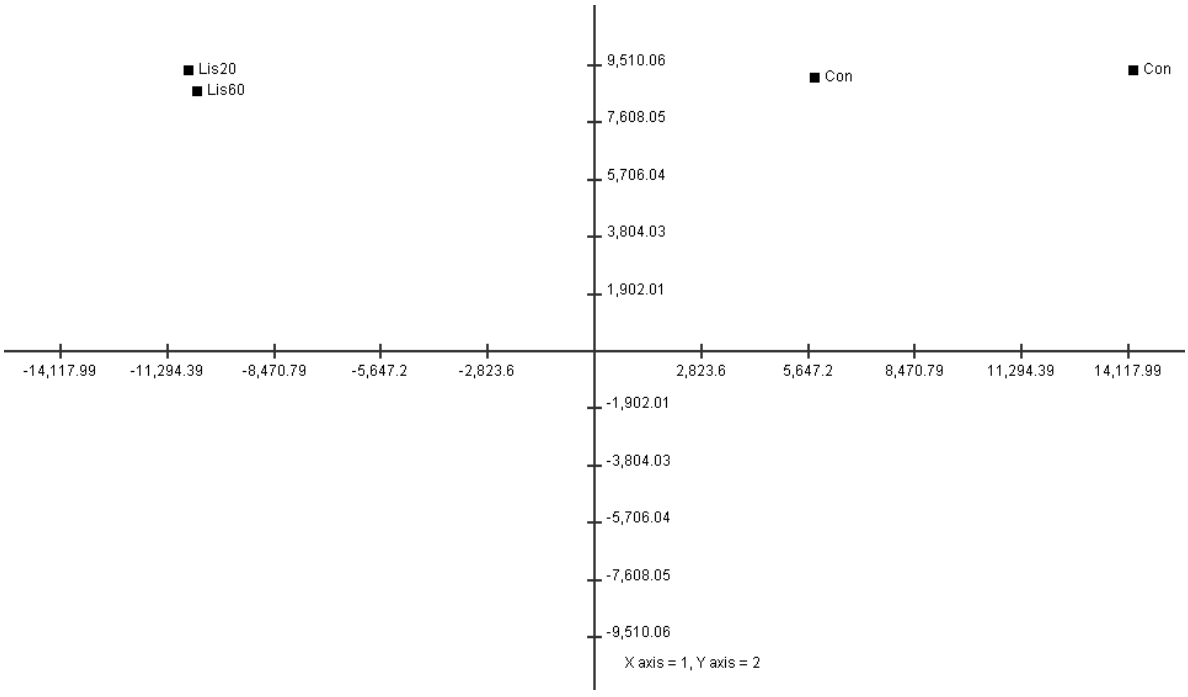

Figure 122 - First and Second PCA - There is variance in the gene expression between the samples exposed to the Lisinopril solution at both time points and the control samples along principal component 1 however there is only a very small degree of variation in the gene expression levels at the 20 minute and 60 minute time points in both principal components 1 and 2.

6.4.6 Ramipril Co-solvent Solution PCA

EDV generated 4 principal components for PCA of samples relating to the Ramipril solution produced using a co-solvent to achieve solubilisation of which 81.033% of the variance was described in components 1 and 2 (Table 47). Components 3 and 4 were therefore discounted to further simplify data analysis.

Table 47 - EDV generated 4 principal components for PCA of samples relating to the Ramipril solution produced using a co-solvent.

Eigen Values		
Principal Component 1	0.35	62.62%
Principal Component 2	0.103	18.42%
Principal Component 3	0.087	15.58%
Principal Component 4	0.019	3.39%
First 2 components: 81.033 %		
First 3 components: 96.614 %		

As with the Lisinopril solution there is a clear difference in the gene expression between the control samples and the samples collected relating to the Ramipril co-solvent solution permeability experiments in the First and Second PCA plot (Figure 123). There is a large degree in change across component 1 for the samples exposed to the formulation compared with the control samples, there is however only a limited difference along component 1 for the 20 minute and 60 minute time points. Unlike the Lisinopril formulation however there is a clear difference between the time points when principal component 2 is considered.

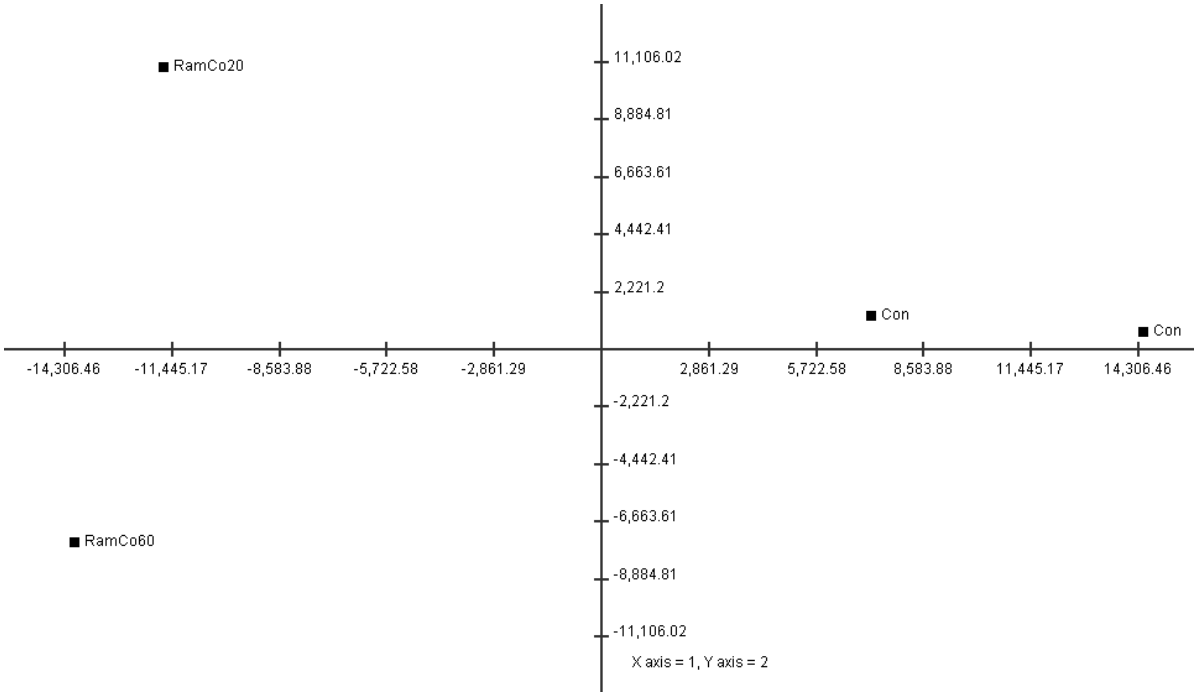

Figure 123 - First and Second PCA - There is a large degree in change across component 1 for the samples exposed to the formulation compared with the control samples, there is however only a limited difference along component 1 for the 20 minute and 60 minute time points. There is also a clear difference between the time points when principal component 2 is considered

6.4.7 Ramipril Cyclodextrin Solution PCA

EDV generated 4 principal components for PCA of samples relating to the Ramipril solution produced using cyclodextrin to achieve solubilisation of which 78.885% of the variance was described in components 1 and 2 (Table 48). Components 3 and 4 were therefore discounted to further simplify data analysis.

Table 48 - EDV generated 4 principal components for PCA of samples relating to the Ramipril solution produced using cyclodextrin.

Eigen Values		
Principal Component 1	0.202	62.77%
Principal Component 2	0.052	16.11%
Principal Component 3	0.048	14.96%
Principal Component 4	0.02	6.15%
First 2 components: 78.885 %		
First 3 components: 93.846 %		

As with the Lisinopril solution and the Ramipril solution produced using a co-solvent there is a clear difference in the gene expression between the control samples and the samples collected during Ramipril Cyclodextrin Solution permeability experiments in the First and Second PCA plot (Figure 124). There is a large degree in change across component 1 for the samples exposed to the formulation compared with the control samples, there is in this instance limited difference along component 1 for the 20 minute and 60 minute time points. Unlike previous observations however the 20 minute time point exhibits a greater variation from the control samples. There is also once again, as with the Ramipril solution produced using a co-solvent, clear difference between the time points when principal component 2 is considered.

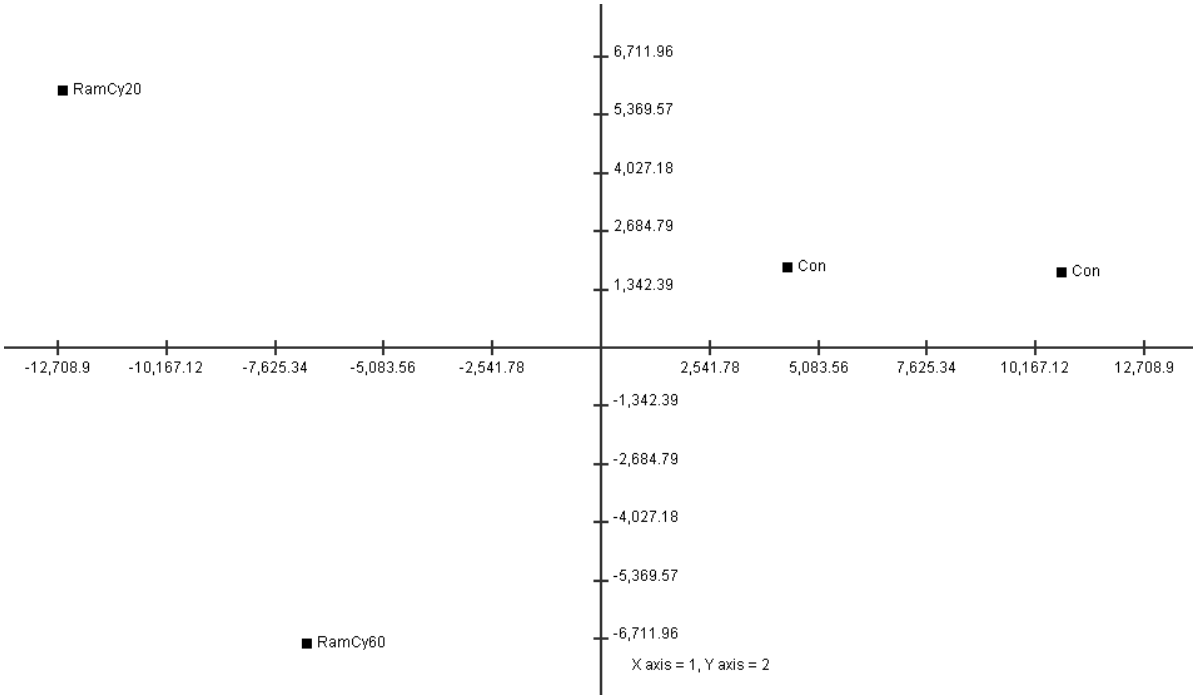

Figure 124 - First and Second PCA - There is a large degree in change across component 1 for the samples exposed to the formulation compared with the control samples, there is in this instance limited difference along component 1 for the 20 minute and 60 minute time points. The 20 minute time point exhibits a greater variation from the control samples. There is clear difference between time points when principal component 2 is considered.

6.4.8 Ramipril Suspension PCA

EDV generated 4 principal components for PCA of samples relating to the Ramipril suspension of which 82.902% of the variance was described in components 1 and 2 (Table 49). Components 3 and 4 were therefore discounted to further simplify data analysis.

Table 49 - EDV generated 4 principal components for PCA of samples relating to the Ramipril suspension.

Eigen Values		
Principal Component 1	0.766	63.46%
Principal Component 2	0.235	19.44%
Principal Component 3	0.179	14.85%
Principal Component 4	0.027	2.25%
First 2 components: 82.902 %		
First 3 components: 97.747 %		

As shown in the First and Third PCA plot displaying all of the analysed formuations (Figure 121) there is a clear difference in the gene expression levels between the control samples and the formuation time points. This is seen along component one of the First and Second PCA for the Ramipril suspension (Figure 125). The difference seen bewteen the time points again shows a greater change in the gene expression after 60 minutes when compared to the change after 20 minutes.

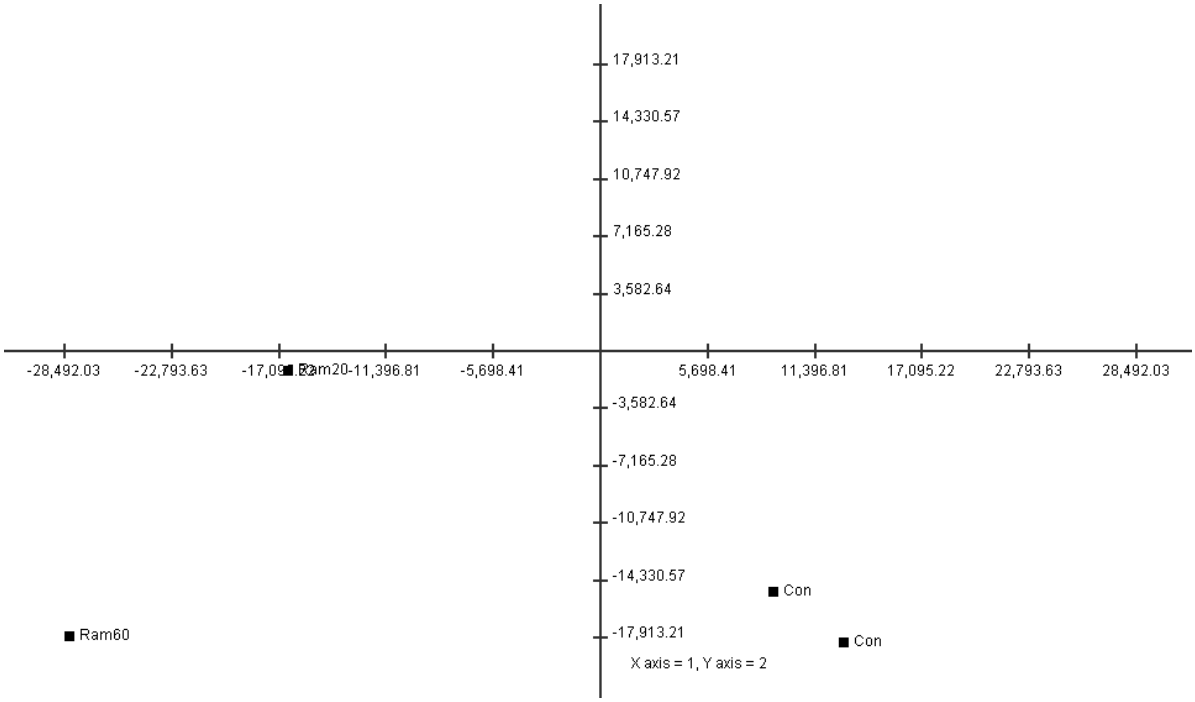

Figure 125 - First and Second PCA - There is a clear difference in the gene expression levels between the control samples and the formuation time points. This is seen along component one. The difference seen bewteen the time points again shows a greater change in the gene expression after 60 minutes when compared to the change after 20 minutes.

6.4.9 Spironolactone Suspension PCA

EDV generated 4 principal components for PCA of samples relating to the Spironolactone suspension of which 88.989% of the variance was described in components 1 and 2 (Table 50). Components 3 and 4 were therefore discounted to further simplify data analysis.

Table 50 - EDV generated 4 principal components for PCA of samples relating to the Spironolactone suspension.

Eigen Values		
Principal Component 1	3.468	63.16%
Principal Component 2	1.419	25.83%
Principal Component 3	0.579	10.55%
Principal Component 4	0.026	0.47%
First 2 components: 88.989 %		
First 3 components: 99.534 %		

As shown in the First and Second PCA plot displaying all of the analysed formuations (Figure 121) there is a clear difference in the gene expression levels between the control samples and the formuation time points. This is seen along component one and two of the First and Second PCA for the suspension (Figure 126), The difference seen bewteen the time points again shows a greater change in the gene expression after 60 minutes when compared to the change after 20 minutes.

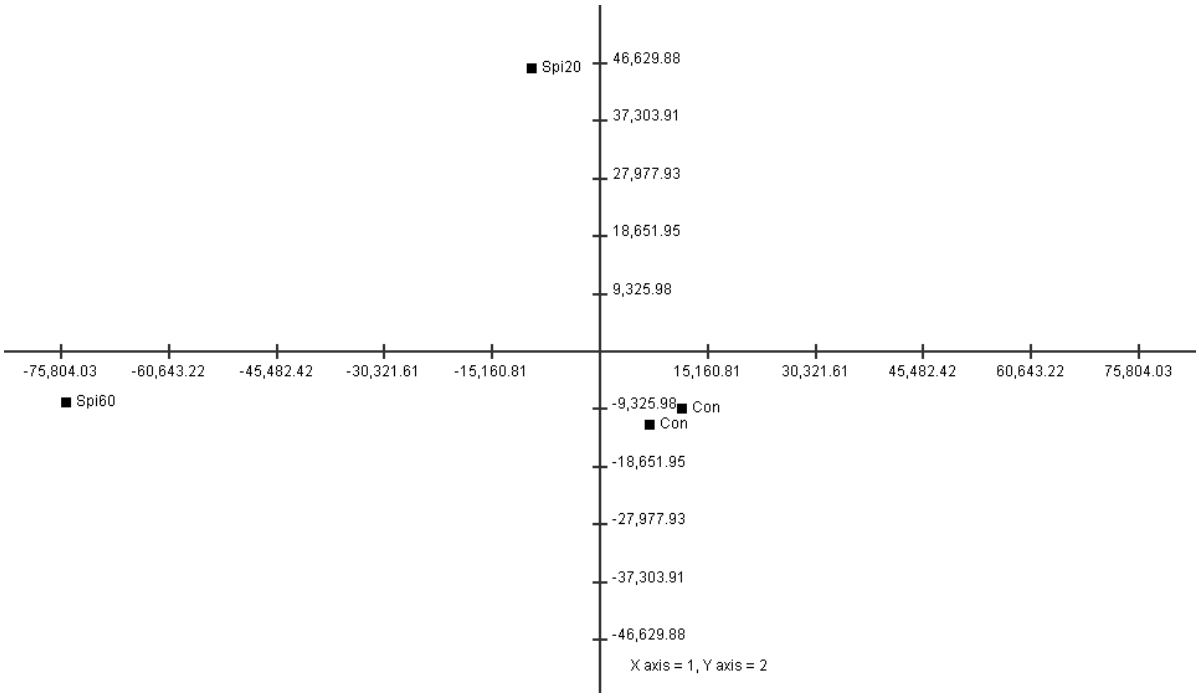

Figure 126 - First and Second PCA - there is a clear difference in the gene expression levels between the control samples and the formuation time points. This is seen along component one and two. The difference seen bewteen the time points shows a greater change in the gene expression after 60 minutes when compared to the change after 20 minutes.

From the observation of the PCA in conjunction with the supporting data from the HCA it is interesting that the degree of gene expression change is greatest for Spironolactone, less great for Ramipril and lowest for Lisinopril. This mirrors the *in vivo* bioavailability trend and may indicate a potential correlation between the overall change in gene expression levels and drug absorption.

6.4.10 Significance Analysis of Microarrays (SAM)

SAM was carried out on the microarray data to identify genes which show a significant change in their level of expression following transport experiments using Lisinopril, Ramipril and Spironolactone formulations. Genes with a fold difference >2 in the SLC and ABC superfamilies were tabulated and positive or negative change in expression indicated.

For the Lisinopril Solution the significant gene expression information generated from the SAM analysis (Figure 127) and the SAM chart (Figure 128) are displayed below. For this data set the delta value used was 0.39550424 and produced a median FDR of 4.83397%. A total of 213 positively significant genes and 240 negatively significant genes were identified, 40640 genes were not significant.

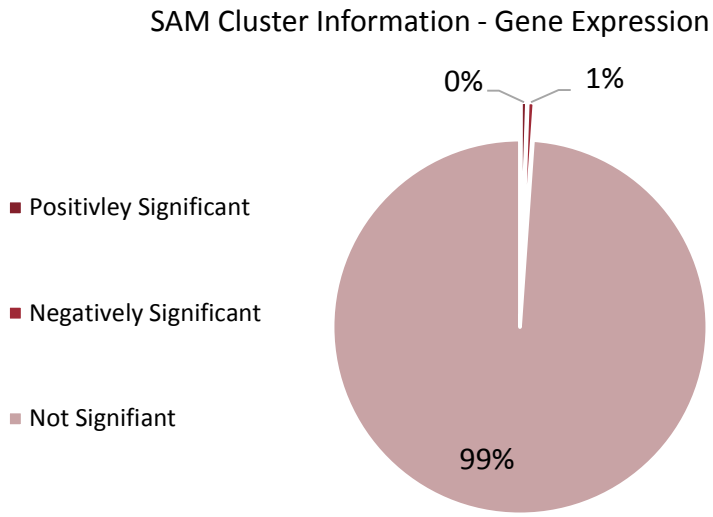

Figure 127 - SAM Cluster Information - Gene Expression - 213 positively significant genes and 240 negatively significant genes were identified, 40640 genes were not significant.

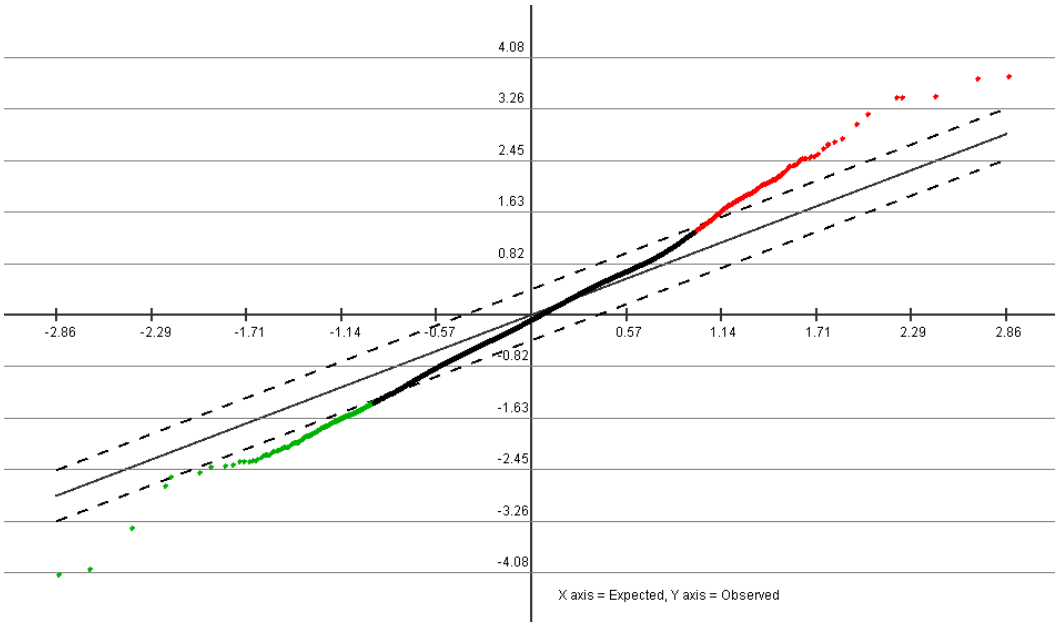

Figure 128 – SAM Chart - delta value used was 0.39550424 and produced a median FDR of 4.83397%.

The genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen were listed (Table 51) to form the data set which was subsequently used in KEGG pathway identification. For the Lisinopril solution there was no significant effect on any of the genes in the ABC family however there was 10 genes in the SLC family for which a fold change >2 was observed. For all of these genes they were up regulated.

Table 51 - Genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen.

Lisinopril Solution			
SLC	**Transporter**	**Fold Change**	**+ve or -ve**
	SLC14A1	2.0704432	+ve
	SLC16A3	3.1122882	+ve
	SLC22A7	3.7140026	+ve
	SLC43A2	2.2528124	+ve
	SLC44A2	2.258665	+ve
	SLC4A1	2.077871	+ve
	SLC4A10	2.0645945	+ve
	SLC8A1	2.1728525	+ve
	SLC8A2	2.917442	+ve
	SLC9B2	2.417506	+ve
ABC	**None**		

For the Ramipril solution produced using a co-solvent as a solubiliser, the significant gene expression information generated from the SAM analysis (Figure 129) and the SAM chart (Figure 130) are displayed below. For this data set the delta value used was 0.21017464 and produced a median FDR of 4.94209%. A total of 502 positively significant genes and 362 negatively significant genes were identified, 40229 genes were not significant.

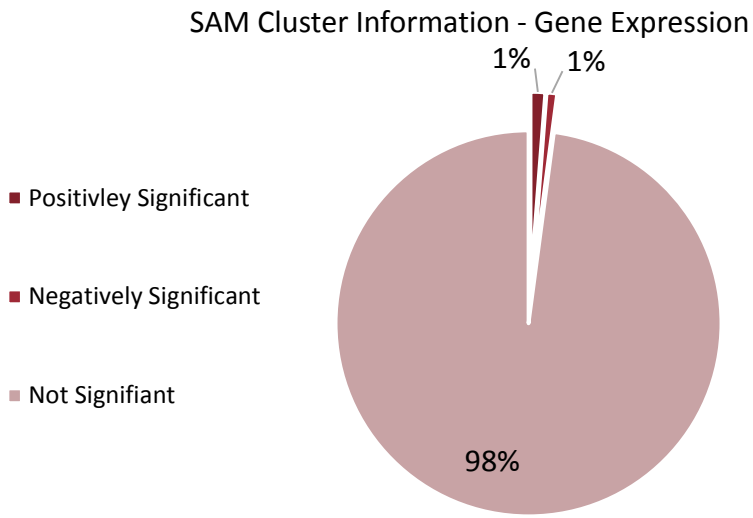

Figure 129 - SAM Cluster Information - Gene Expression - A total of 502 positively significant genes and 362 negatively significant genes were identified, 40229 genes were not significant.

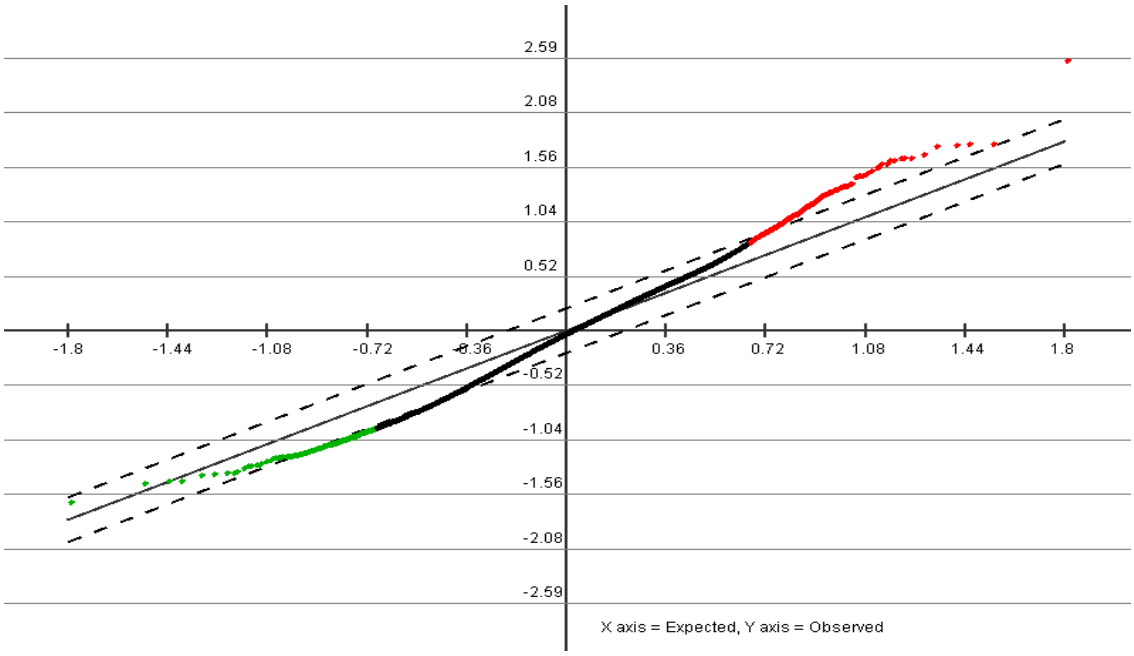

Figure 130 - SAM Chart - delta value used was 0.21017464 and produced a median FDR of 4.94209%.

The genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen were listed (Table 52) to form the data set which was subsequently used in KEGG pathway identification. For the Ramipril solution produced using a co-solvent as a solubiliser there was significant effect on one gene in the ABC family and there was 26 genes in the SLC family for which a fold change >2 was observed. For all of these genes they were up regulated.

Table 52 - Genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen.

Ramipril Co-solvent Solution			
	Transporter	**Fold Change**	**+ve or -ve**
	SLC11A1	2.0692508	+ve
	SLC16A10	2.5774577	+ve
	SLC17A7	2.4984982	+ve
	SLC22A17	2.2027316	+ve
	SLC22A23	2.3579023	+ve
	SLC23A3	3.3429158	+ve
	SLC24A2	2.1618395	+ve
	SLC24A4	2.2888455	+ve
	SLC27A1	2.43765	+ve
	SLC30A3	4.4293623	+ve
	SLC35B4	2.6245763	+ve
	SLC39A7	2.1983893	+ve
SLC	SLC4A1	2.2079337	+ve
	SLC5A2	2.004006	+ve
	SLC6A13	2.2618265	+ve
	SLC8A1	2.7300835	+ve
	SLC8A2	2.9829826	+ve
	SLC9A9	2.679537	+ve
	SLC2A4RG	2.1210656	+ve
	SLC34A2	2.93993	+ve
	SLC35A4	2.2494004	+ve
	SLC43A1	2.461622	+ve
	SLC45A2	2.1256492	+ve
	SLC4A3	2.3697224	+ve
	SLC50A1	2.2596252	+ve
	SLC9B2	2.430448	+ve
ABC	ABCF2	2.2513223	+ve

For the Ramipril solution produced using a cyclodextrin, the significant gene expression information generated from the SAM analysis (Figure 131) and the SAM

chart (Figure 132) are displayed below. For this data set the delta value used was 0.92349243 and produced a median FDR of 0.36899 %. A total of 771 positively significant genes and 149 negatively significant genes were identified, 40173 genes were not significant.

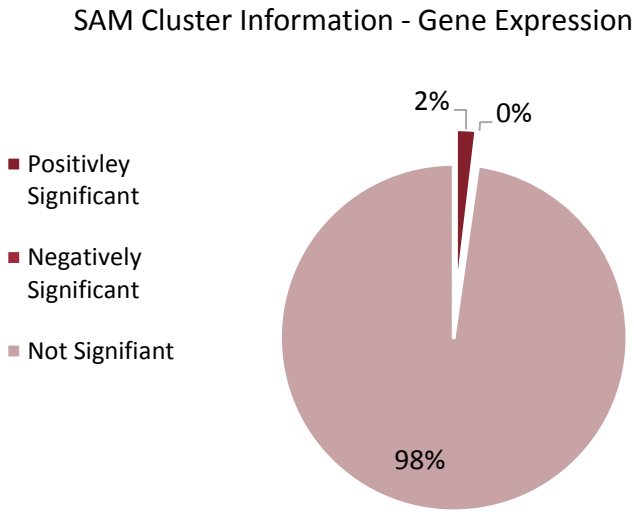

Figure 131 - SAM Cluster Information - Gene Expression - A total of 771 positively significant genes and 149 negatively significant genes were identified, 40173 genes were not significant.

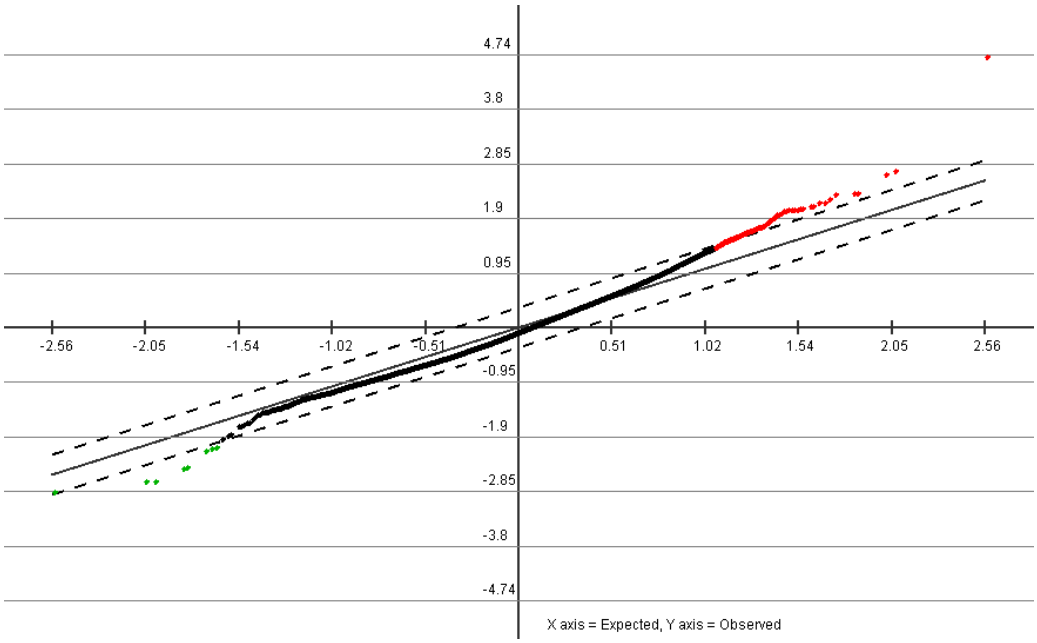

Figure 132 - SAM Chart - delta value used was 0.92349243 and produced a median FDR of 0.36899 %.

The genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen were listed (Table 53) to form the data set which was subsequently used in KEGG pathway identification. For the Ramipril solution produced using a cyclodextrin as a solubiliser there was significant effect on one gene in the ABC family and there were 10 genes in the SLC family for which a fold change >2 was observed. All of these genes were up regulated.

Table 53 - Genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen

Ramipril Cyclodextrin Solution			
	Transporter	**Fold Change**	**+ve or -ve**
SLC	SLC11A1	2.7121615	+ve
	SLC16A10	2.2996733	+ve
	SLC17A7	2.564548	+ve
	SLC29A4	2.3865407	+ve
	SLC2A4RG	2.415126	+ve
	SLC30A3	3.506687	+ve
	SLC35B4	2.9588718	+ve
	SLC9A9	3.2706385	+ve
	SLC22A17	2.29381	+ve
	SLC27A1	3.686685	+ve
ABC	ABCA2	2.5894725	+ve

For the Ramipril suspension, the significant gene expression information generated from the SAM analysis (Figure 133) and the SAM chart (Figure 134) are displayed below. For this data set the delta value used was 0.6749133 and produced a median FDR of 0.00000%. A total of 2143 positively significant genes and 293 negatively significant genes were identified, 38675 genes were not significant.

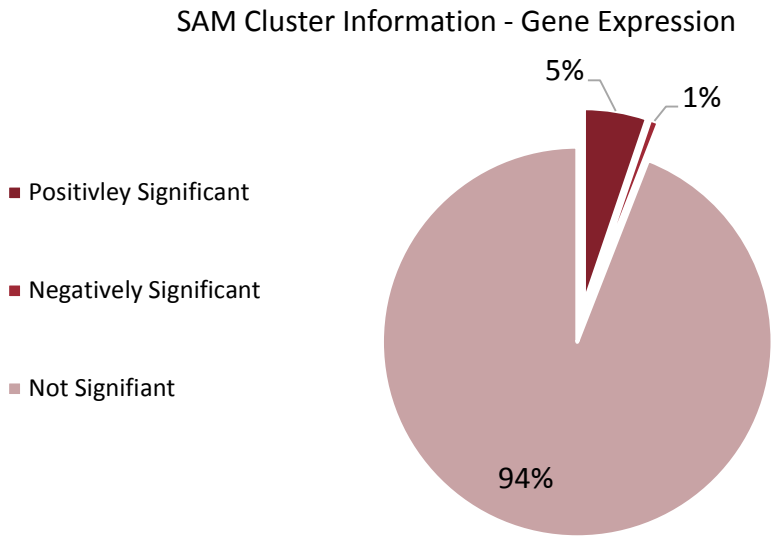

Figure 133 - SAM Cluster Information - Gene Expression - A total of 2143 positively significant genes and 293 negatively significant genes were identified, 38675 genes were not significant.

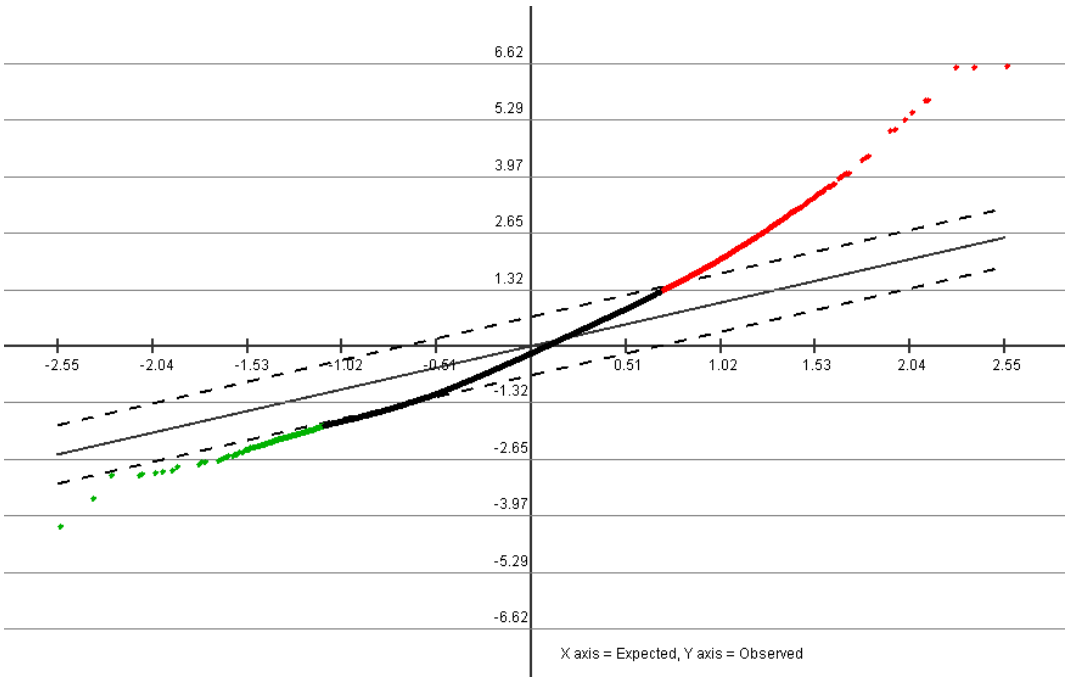

Figure 134 - SAM Chart - delta value used was 0.6749133 and produced a median FDR of 0.00000%.

The genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen were listed (Table 54) to form the data set which was subsequently used in KEGG pathway identification. For the Ramipril suspension there was significant effect on 2 genes in the ABC family and there were 29 genes in the SLC family for which a fold change >2 was observed. All of these genes were up regulated.

Table 54 - Genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen.

Ramipril Suspension			
	Transporter	**Fold Change**	**+ve or -ve**
SLC	SLC16A10	5.6771617	+ve
	SLC16A11	2.9428	+ve
	SLC17A7	5.3337197	+ve
	SLC18A2	9.033469	+ve
	SLC22A10	2.8761852	+ve
	SLC22A12	4.0482225	+ve
	SLC22A17	3.8142965	+ve
	SLC22A23	4.1997943	+ve
	SLC24A4	5.2337623	+ve
	SLC26A1	6.197696	+ve
	SLC27A1	4.6477814	+ve
	SLC2A10	3.12832	+ve
	SLC2A5	4.3415933	+ve
	SLC30A7	3.027596	+ve
	SLC35B4	3.4955595	+ve
	SLC6A6	4.3639517	+ve
	SLC6A7	2.9340563	+ve
	SLC9A9	9.54847	+ve
	SLC22A31	34.691284	+ve
	SLC23A3	6.286539	+ve
	SLC25A28	23.11727	+ve
	SLC25A39	69.7663	+ve
	SLC2A3	12.64015	+ve
	SLC30A3	6.3733015	+ve
	SLC35C1	8.036819	+ve
	SLC35C2	12.777995	+ve
	SLC38A1	13.190632	+ve
	SLC41A3	82.516136	+ve
ABC	SLC7A6OS	13.556567	+ve
	ABCA5	11.006652	+ve
	ABCC6	10.992928	+ve

For the Spironolactone suspension, the significant gene expression information generated from the SAM analysis (Figure 135) and the SAM chart (Figure 136) are displayed below. For this data set the delta value used was 0.34634054 and produced a median FDR of 1.48148%. A total of 2576 positively significant genes and 1235 negatively significant genes were identified, 37282 genes were not significant.

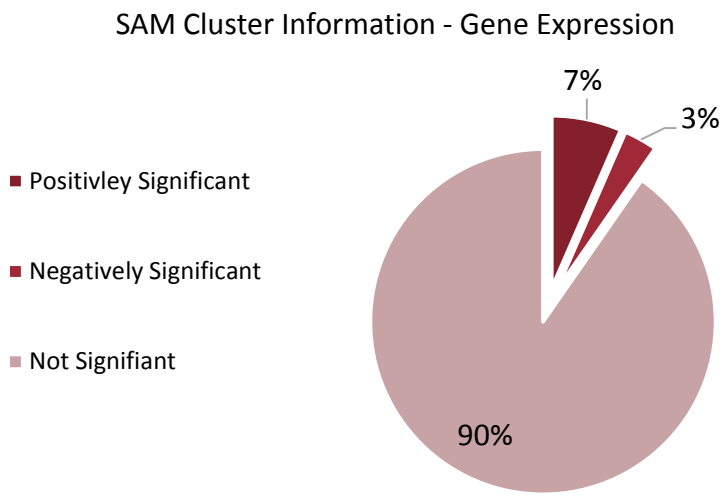

Figure 135 - SAM Cluster Information - Gene Expression - A total of 2576 positively significant genes and 1235 negatively significant genes were identified, 37282 genes were not significant.

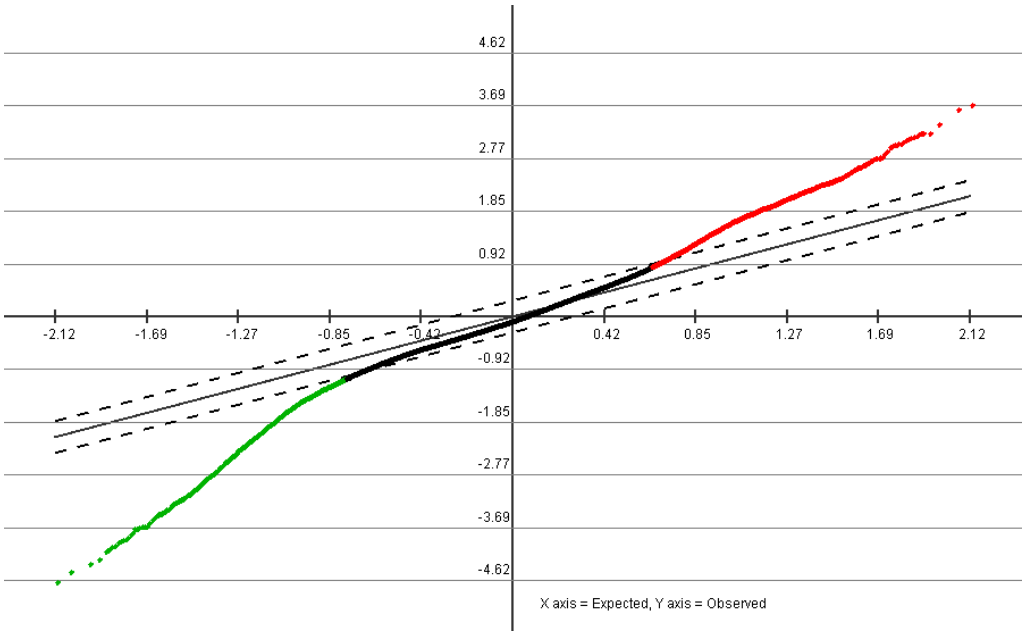

Figure 136 – SAM Chart - delta value used was 0.34634054 and produced a median FDR of 1.48148%.

The genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen were listed (Table 55) to form the data set which was subsequently used in KEGG pathway identification.

Table 55 - Genes from the SLC and ABC superfamilies for which a significant change in gene expression was seen.

Spironolactone Suspension			
	Transporter	**Fold Change**	**+ve or -ve**
SLC	SLC17A9	6.4354215	+ve
	SLC1A5	13.079359	+ve
	SLC25A1	10.693407	+ve
	SLC25A29	9.254947	+ve
	SLC25A6	10.979601	+ve
	SLC29A2	15.156823	+ve
	SLC29A4	10.382369	+ve
	SLC2A8	24.344704	+ve
	SLC31A1	16.562778	+ve
	SLC35B2	8.844945	+ve
	SLC35C2	17.574041	+ve
	SLC35D2	19.421774	+ve
	SLC35E2	16.530993	+ve
	SLC35F6	9.12032	+ve
	SLC38A5	7.1403027	+ve
	SLC39A1	17.563322	+ve
	SLC39A5	7.296592	+ve
	SLC39A7	18.911186	+ve
	SLC3A2	14.07006	+ve
	SLC6A10P	7.336151	+ve
	SLC6A8	16.636656	+ve
	SLC7A5	13.480293	+ve
	SLC7A8	8.355325	+ve
	SLC9A8	9.476612	+ve
	SLC11A2	31.62154	+ve
	SLC16A4	7.989935	+ve
	SLC1A1	19.64092	+ve
	SLC22A5	8.93393	+ve
	SLC23A3	10.974421	+ve
	SLC25A10	17.145796	+ve
	SLC25A13	9.850117	+ve
	SLC25A23	26.403795	+ve
	SLC25A24	7.24933	+ve
	SLC25A36	10.516455	+ve
	SLC25A37	13.912533	+ve
	SLC25A46	10.360896	+ve
	SLC25A5	53.59311	+ve

	SLC25A6	46.862362	+ve
	SLC26A11	16.092371	+ve
	SLC26A3	9.05577	+ve
	SLC27A1	13.418841	+ve
	SLC29A3	13.328121	+ve
	SLC2A2	7.823031	+ve
	SLC2A3	13.813991	+ve
	SLC2A4RG	14.815919	+ve
	SLC30A3	20.643759	+ve
	SLC31A2	7.2412624	+ve
	SLC35A2	8.219815	+ve
	SLC35B1	17.630474	+ve
	SLC35C1	20.138887	+ve
	SLC35D2	20.541061	+ve
	SLC35E1	15.807547	+ve
	SLC38A1	18.407154	+ve
	SLC38A6	10.197243	+ve
	SLC39A11	6.759471	+ve
	SLC39A13	9.199426	+ve
	SLC39A14	24.078133	+ve
	SLC3A1	18.950901	+ve
	SLC41A3	11.449558	+ve
	SLC43A2	39.156944	+ve
	SLC44A1	10.62931	+ve
	SLC44A2	13.139595	+ve
	SLC45A3	6.9271073	+ve
	SLC50A1	11.52964	+ve
	SLC5A11	5.9074626	+ve
	SLC5A6	20.858412	+ve
	SLC6A19	6.3885436	+ve
	SLC6A4	6.203496	+ve
	SLC7A4	8.900564	+ve
	SLC7A7	11.712494	+ve
	SLC9A3R1	6.900848	+ve
	SLCO2B1	10.498462	+ve
ABC	ABCC10	7.0169935	+ve
	ABCC8	7.0988774	+ve
	ABCF2	8.409261	+ve
	ABCB6	11.907367	+ve
	ABCB7	7.421333	+ve
	ABCC3	6.1075015	+ve
	ABCC5	9.429524	+ve
	ABCD4	16.697014	+ve
	ABCF3	13.205725	+ve

For the Spironolactone suspension there was significant effect on 9 genes in the ABC family and there were 72 genes in the SLC family for which a fold change >2 was observed. All of these genes were up regulated. Following the production of the SLC and ABC gene tables the gene names were entered into KEGG to identify the pathways affected by the changes in gene expression.

6.4.11 KEGG Pathway Analysis

KEGG pathway analysis of the significantly up regulated ABC transporter genes indicated that the genes listed (Table 56) only provided specific pathway information for the genes ABCA2, ABCC3, ABCC8 and ABCD4. ABCA2 was identified as coding for a Lysosome membrane protein, ABCC3 plays a role in bile secretion pathways, while ABCC8 was identified in pathways linked to Type II diabetes mellitus and insulin secretion pathways. Finally ABCD4 was found in Peroxisome pathways. None of the pathways were linked to intestinal absorption pathways, and as such not investigated further.

Table 56 – KEGG Pathways in which significantly up-regulated genes are active.

KEGG Pathways	
Gene	**KEGG Pathway Identification**
ABCA2	Eukaryotic Type ABC Transporter
	Lysosome Membrane Protein
ABCA5	Eukaryotic Type ABC Transporter
ABCB6	Eukaryotic Type ABC Transporter
ABCB7	Eukaryotic Type ABC Transporter
ABCC10	Eukaryotic Type ABC Transporter
ABCC3	Eukaryotic Type ABC Transporter
	Bile Secretion Pathways
ABCC5	Eukaryotic Type ABC Transporter
ABCC6	Eukaryotic Type ABC Transporter
ABCC8	Eukaryotic Type ABC Transporter
	Insulin Secretion Pathways
	Type II Diabetes Mellitus
ABCD4	Eukaryotic Type ABC Transporter
	Peroxisome Pathways
SLC16A10	Protein Digestion and Absorption
	Thyroid Hormone Signalling
SLC1A1	Protein Digestion and Absorption

SLC1A5	Glutamatergic Synapse
	Protein Digestion and Absorption
SLC22A7	Bile Secretion Pathways
SLC25A10	Proximal Tubule Bicarbonate Reclamation
SLC26A3	Mineral Absorption
	Pancreatic Secretion
SLC2A2	Maturity Onset Diabetes in the Young
	Type II Diabetes Mellitus
	Carbohydrate Digestion and Absorption
	Prolactin Signalling Pathway
	Insulin Secretion Pathways
SLC2A5	Carbohydrate Digestion and Absorption
SLC31A1	Mineral Absorption
SLC36A5	GABAergic Synapse
SLC38A1	GABAergic Synapse
	Glutamatergic Synapse
SLC3A1	Protein Digestion and Absorption
SLC3A2	Protein Digestion and Absorption
SLC45A3	MicroRNAs in Cancer
	Transcriptional Misregulation in Cancer
SLC4A1	Collecting Duct and Secretion
SLC5A6	Vitamin Digestion and Absorption
SLC6A19	Mineral Absorption
	Protein Digestion and Absorption
SLC6A4	Serotonergic Synapse
SLC7A7	Protein Digestion and Absorption
SLC7A8	Protein Digestion and Absorption

KEGG pathway analysis of the significantly up regulated SLC transporter genes returned pathway information for all of the genes listed (Table 56) however as seen with the ABC transporters many of the pathways were unrelated to intestinal absorption pathways. However, SLC16A10, SLC1A1, SLC1A5, SLC3A1, SLC3A2, SLC6A19, SLC7A7 and SLC7A8 were identified as having involvement in the intestinal absorption of proteins (Figure 137), coding for the TAT1, EAAT3, ASCT2, rBAT, 4F2hc, B^0AT1, y^+LAT1, and LAT2 transporters respectively. SLC16A10 was seen to be significantly up regulated for all of the Ramipril formulations however not for Lisinopril or Spironolactone formulations. SLC1A1, SLC1A5, SLC3A1, SLC3A2, SLC6A19, SLC7A7 and SLC7A8 were all significantly up regulated by the Spironolactone suspension only. EAAT3, ASCT2, B^0AT1 and rBAT are located in the apical membrane of intestinal epithelial cells

here EAAT3 facilitates the uptake of anionic amino acids from the intestinal lumen, ASCT2 and B^0AT1 facilitate the transport of neutral amino acids and rBAT works with $B^{0,+}$AT1 (not significantly up regulated) to facilitate the uptake of cationic amino acids. TAT1, y^+LAT1, LAT2 and 4F2hc are present in the basolateral membrane of intestinal epithelial cells and transport neutral amino acids into and out of the blood (Ramadan *et al* 2001, del Amo *et al* 2008).

There are significant expressional changes for numerous SLC transporters in the protein absorption pathway across the intestinal epithelial but not SLC15A1, the transporter for which Ramipril and Lisinopril are substrates. SLC15A1 codes for the PEPT1 transporter which is present in the apical membrane of intestinal epithelial cells and responsible for the absorption of small peptides (Daniel 2004, Buyse *et al* 2001).

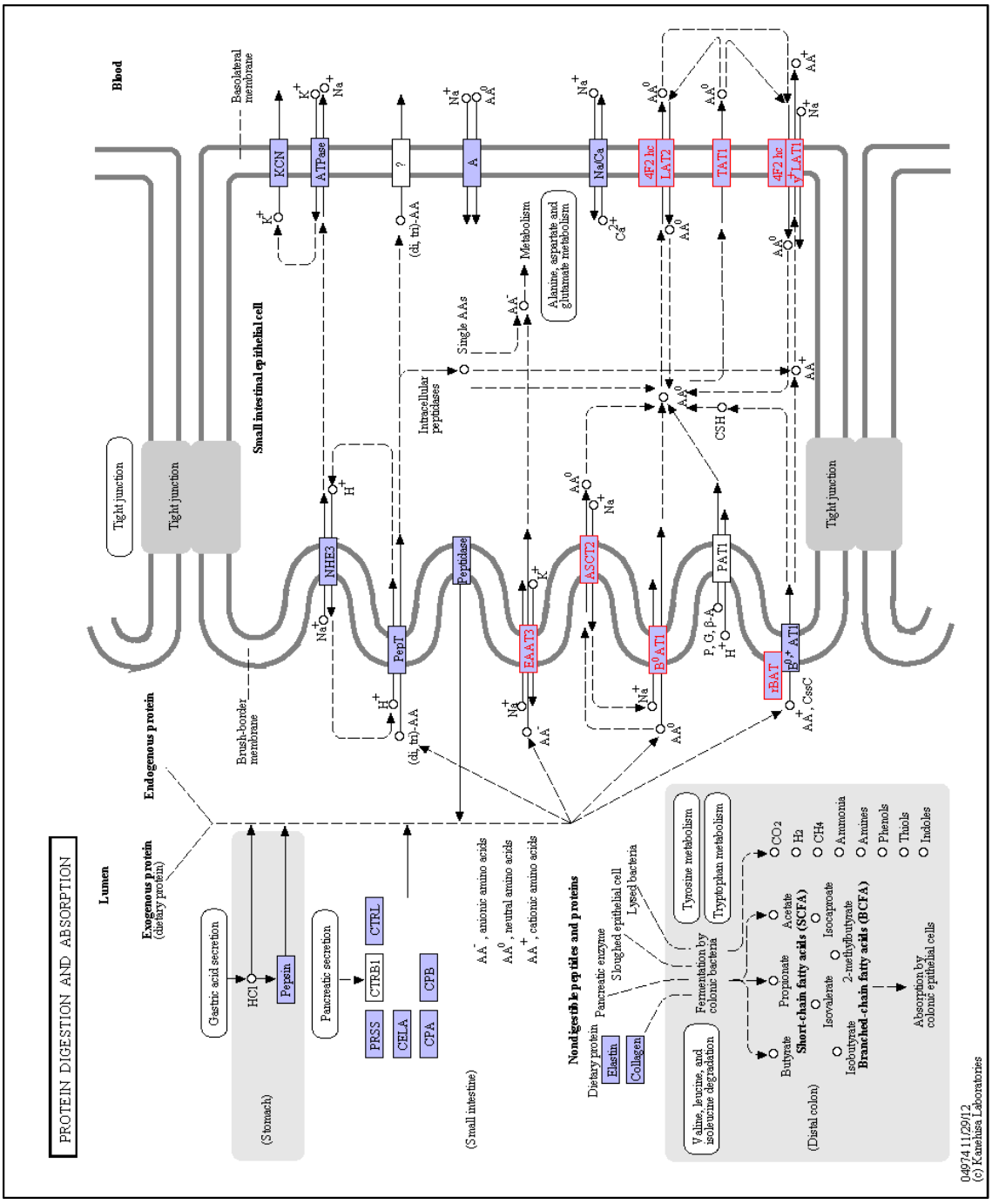

Figure 137 – KEGG pathway for protein digestion and absorption. Transporters highlighted in red are significantly up-regulated.

In addition to the protein absorption pathway, the Spironolactone formulation caused up regulation in genes which code for transporters is mineral absorption pathways (Figure 1387). SLC26A3, SLC6A19 and SLC31A1 code for the transporters DRA, B^0AT1 and CTR1 found in the apical membrane of intestinal epithelial cells (Lee *et al* 2012, Ganapathy *et al* 2009, Tennant *et al* 2002).

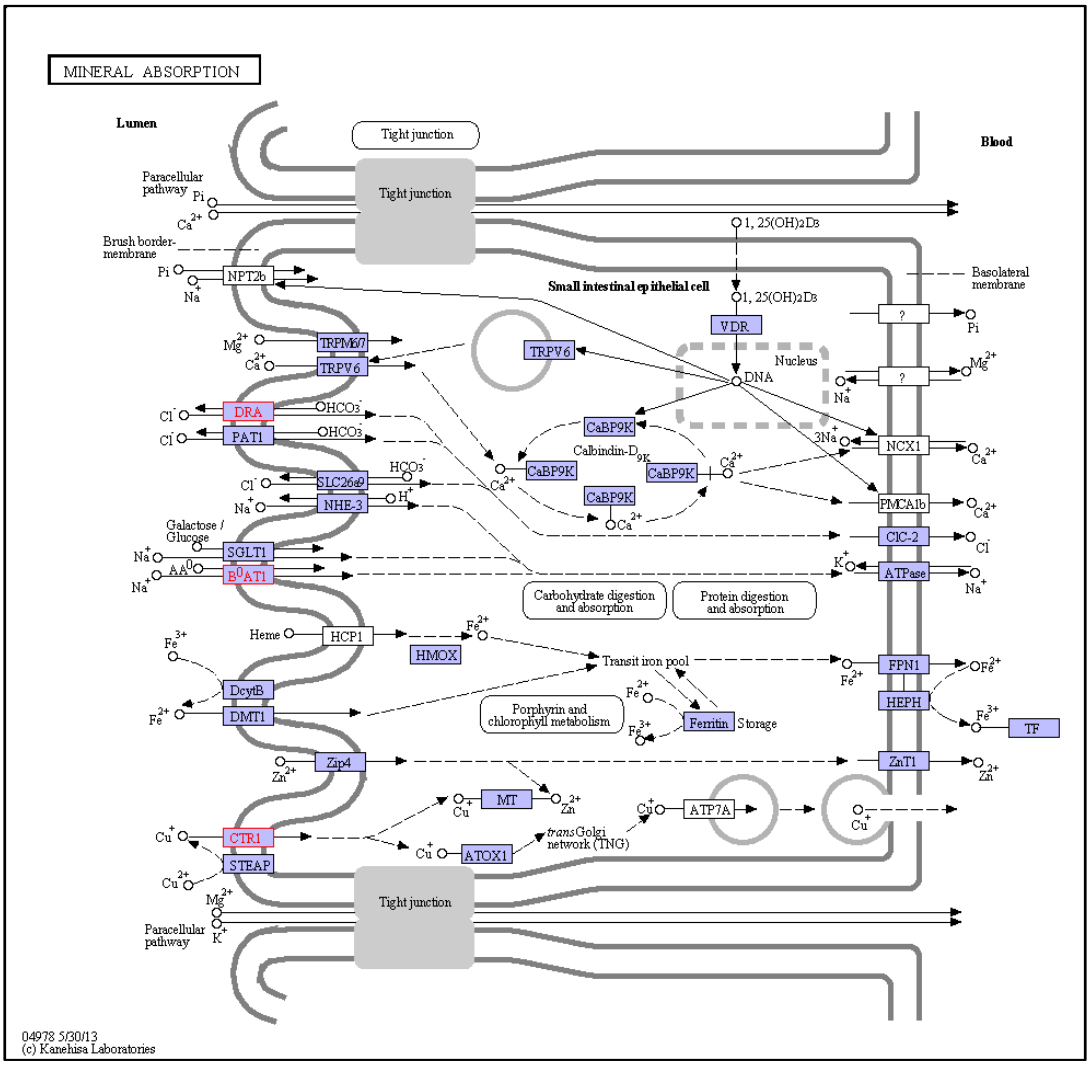

Figure 138 – KEGG pathway for mineral absorption. Transporters highlighted in red are significantly up-regulated.

The Spironolactone formulation also caused up regulation in SLC5A6, this gene codes for the protein SMVT which is again found in the apical membrane of intestinal epithelial cells (Weisz and Rodriguez-Boulan 2009) as part of the vitamin absorption pathway (Figure 139).

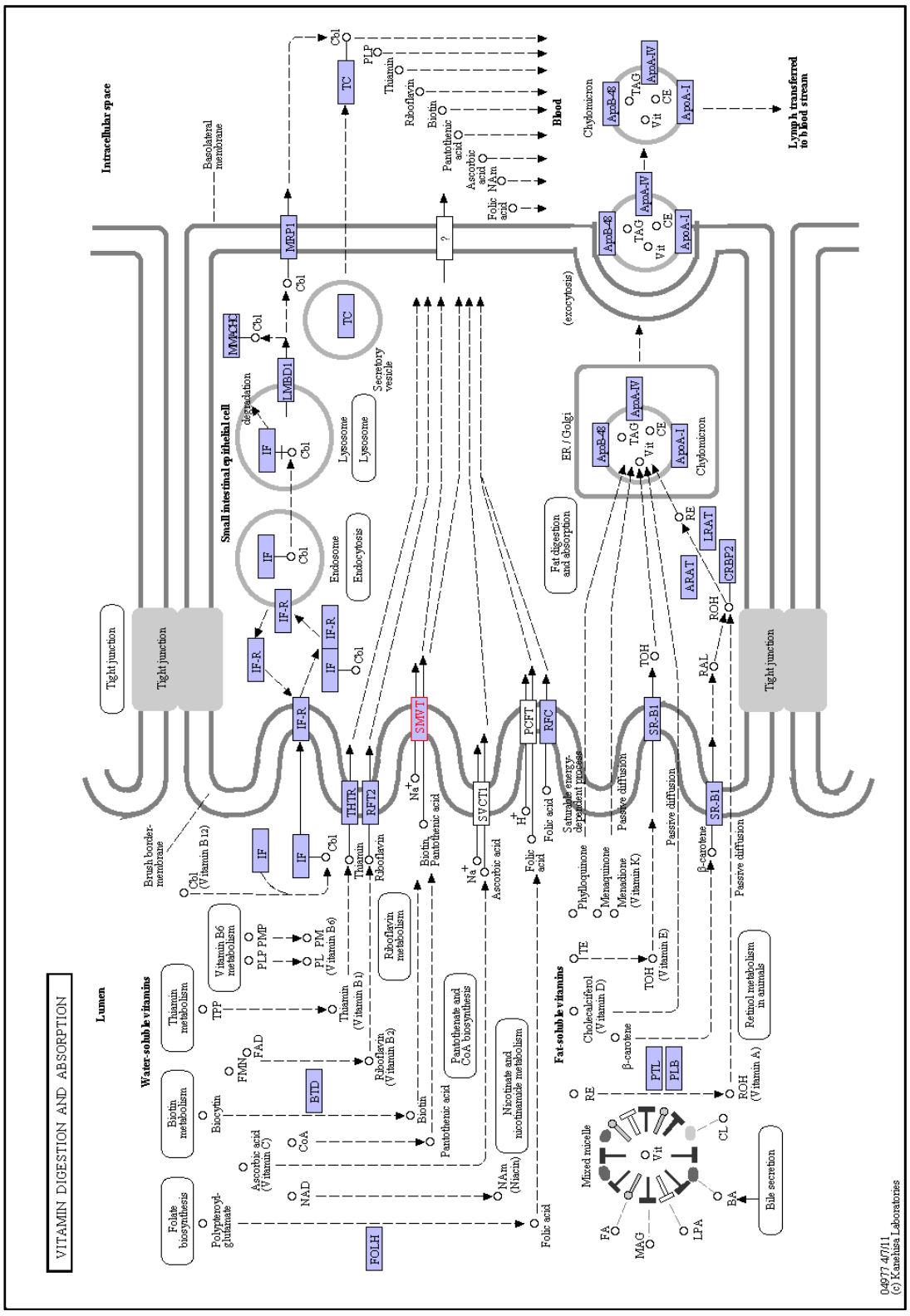

Figure 139 – KEGG pathway for Vitamin digestion and absorption. Transporters highlighted in red are significantly up-regulated.

Finally, the Spironolactone suspension caused up regulation in SLC2A2 and the Ramipril suspension caused up regulation in SLC2A5, these genes both code for transporter proteins in the carbohydrate absorption pathway (Figure 140). SLC2A2 codes for GLUT2 and SLC2A5 codes for GLUT5, these transporters are responsible for the uptake of Glucose and Fructose from the intestinal lumen and the transport of Glucose, Galactose and Fructose across the basolateral membrane of the cell into the blood (Sakar *et al* 2009, Concha *et al* 1997, Mahraoui *et al* 1994).

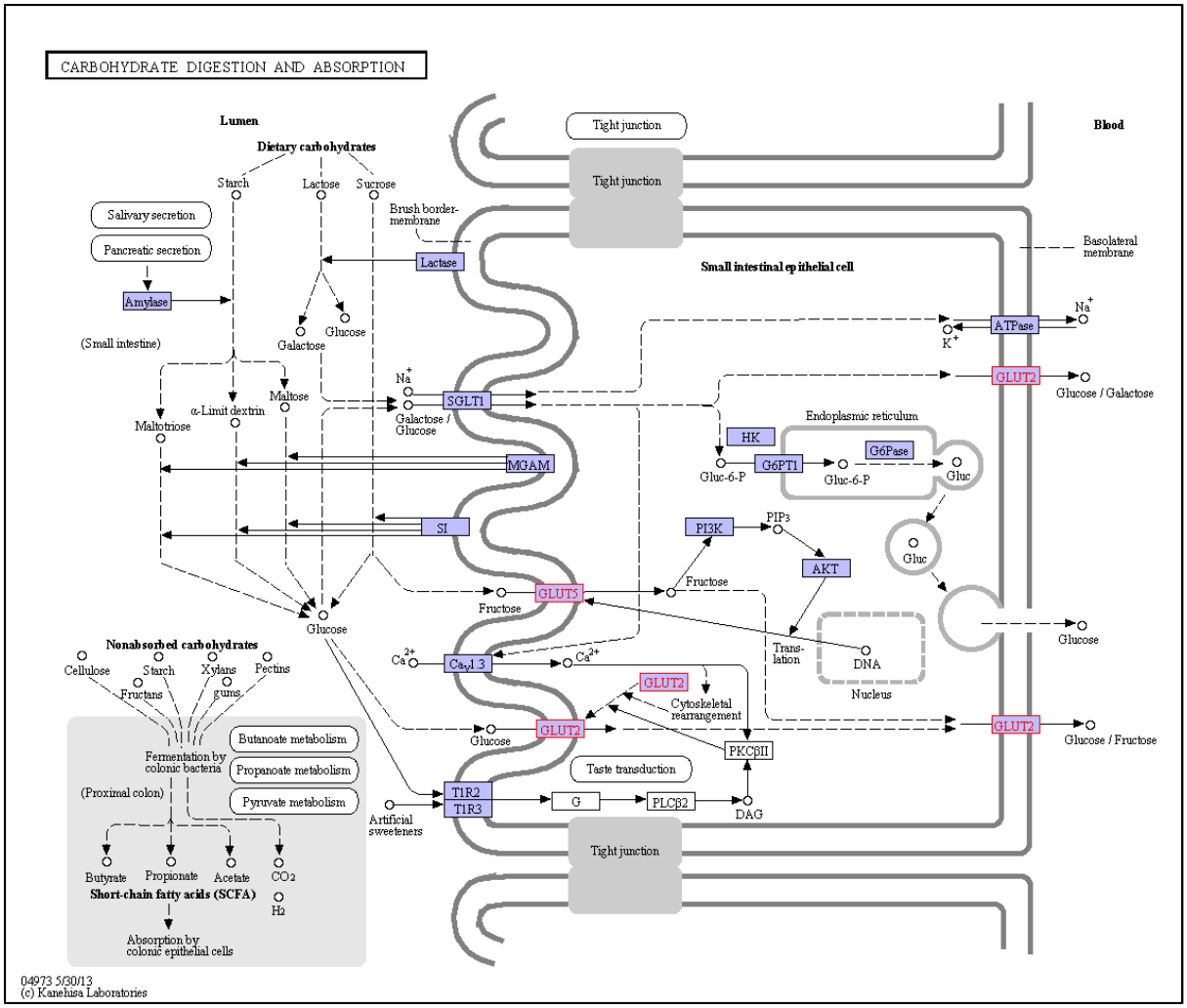

Figure 140 - KEGG pathway for Carbohydrate digestion and absorption. Transporters highlighted in red are significantly up-regulated.

The pathways and proteins, for which the SLC and ABC genes displayed significant modification in gene expression are not clearly linked to the formulations used in the investigations. The changes in expression levels could have resulted following interaction with the API in the formulations, however the excipients present could have also had an effect. This would account for up regulation in genes coding for transporter proteins which would not have necessarily been anticipated as a result of interaction with the API alone. For example, the sweetening agent Xylitol and the suspending agent Xanatural 180 present in the Ramipril and Spironolactone suspensions could potentially contribute to the changes in gene expression linked to the carbohydrate absorption pathway (Levin 1994). Likewise the Spironolactone suspension contains sodium benzoate and sodium metabisulphate which could attribute to the up regulation of genes coding for proteins present in the mineral absorption pathway (Wood and Han 1998). This lends strength to the recently developing ideology that excipients potentially possess a degree of biological activity and are not in actual fact truly inactive ingredients (Osman *et al* 2013, Kalinkova 1999).

6.4.12 Application of microarray to develop an *in vitro* and *in vivo* correlation screening toolbox.

Following the analysis of the microarray data it was identified that the shortlisted genes of interest, namely ABCB1 (Inhibitor) and SLC15A1 (Substrate) for Lisinopril, SLC15A1 (Substrate) and SLC15A2 (Substrate) for Ramipril and ABCB1 (Inhibitor), ABCC2 (Inducer) and SLCO1A2 (Inhibitor) for Spironolactone were all found to show non-significant changes in their expression levels following transport studies using the Lisinopril, Ramipril and Spironolactone formulations. There were however numerous SLC and ABC genes for which the expression had changed significantly.

Due to the variety of genes expressed and the lack of common genes affected by all formulations in the data presented, a means by which human drug absorption can be predicted by measuring gene expression levels of a single gene or a family of genes

is impossible to derive. There is however indication that examination of genome wide expression changes in the total number of genes showing significant change (Figure 141) correlates to the predicted absorption in humans. Plotting the number of genes with significantly altered expression levels for each drug against the percentage absorption in humans (Figure 142) provides a linear correlation with an R^2 value of 0.9934.

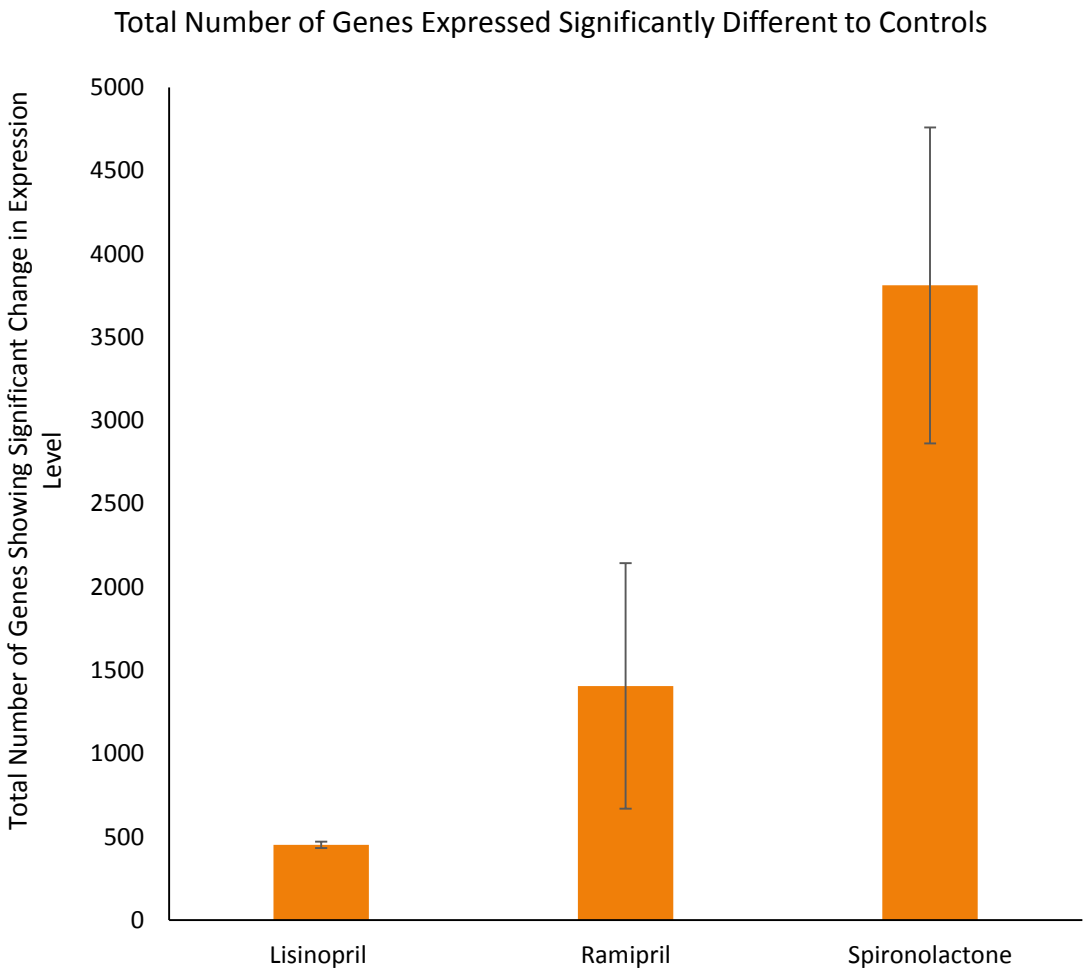

Figure 141 - Total number of genes expressed at a significantly altered level in comparison to controls for all lisinopril, ramipril and spironolactone formulations. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

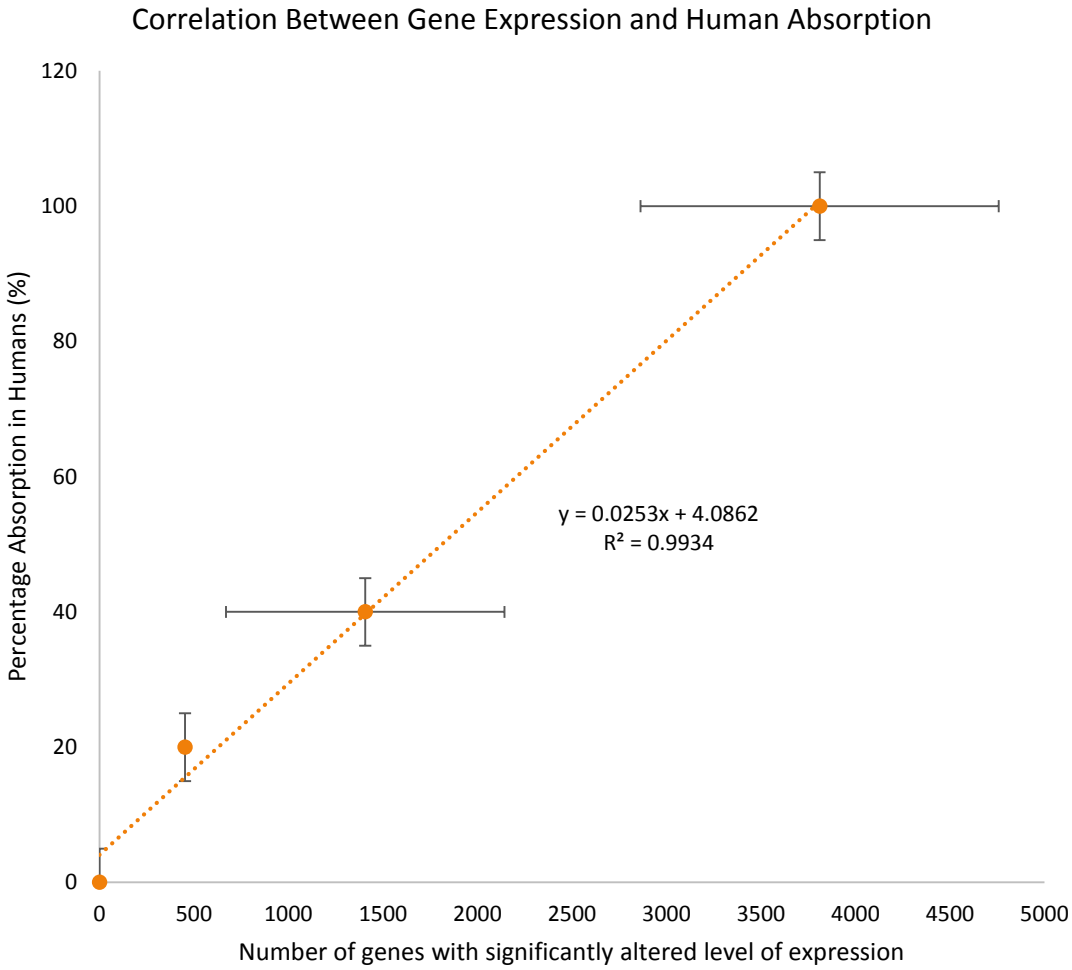

Figure 142 - Correlation between gene expression and human absorption. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

It must be stressed however that this correlation is based on minimal amounts of data and for any trend between human drug absorption and the level of genome wide gene expression changes to be considered as a model for the prediction of human drug absorption many more formulations would need to be analysed investigating a range of dosage forms, dosage concentrations and degree of human absorption.

7 DEVELOPMENT OF AN *IN VITRO* TASTE ASSESSMENT MODEL - ESTABLISHMENT OF LONG-TERM FUNCTIONAL CULTURES OF BOVINE TASTE RECEPTOR CELLS (BTRCS): ISOLATION, CULTURE AND CHARACTERISATION.

7.1 Chapter Aims and Objectives

- Isolate and culture bovine taste receptor cells (BTRCs) to provide a platform for the production of an *in vitro* taste assessment model.
- Optimise culture method.
- Characterise cultured BTRCs cells to confirm that the cultured cells are taste receptor cells.
- Investigate *in vitro* functionality of BTRCs in response to model tastants.

7.2 Introduction

Investigations into taste receptor cell (TRC's) function has been done using explant cultures from rodents (Mbiene 1997), semi-intact taste buds in tissue slices (Caicedo 2000) and primary cultures established from freshly isolated TRC's (Ruiz *et al* 2001, Ookura 2002, Qin 2008). The lack of progress in this area is quite likely due to the limitations of primary culture, most notably limited passage number typically below P5, which have been encountered by many of the groups who have attempted primary culture of TRC's. One group in particular has reported more promising results and have successfully established long term cultures of both Rat (Ozdener 2006) and Human (Ozdener 2011, Ozdener 2013) TRC's. Both of these cell lines are useful in their own right however there are refinements which can be made to significantly reduce both the cost and complexity of establishing and maintaining the cultures while at the same time maintaining and adding to the reported advantages which each of these cultures provide.

TRC's are found in the taste buds which are clustered within structures known as taste papillae. These taste bud containing papillae exist in three distinct forms which are located in specific areas on the tongue depending upon the type of papillae. The three types of papillae which contain taste buds are; fungiform, circumvallate and foliate. Fungiform papillae are located on the anterior portion of the tongue while circumvallate and foliate papillae are located in posterior areas of the tongue (Figure 143). The taste

buds which are contained within the papillae are constructed of four different cell types (Figure 144).

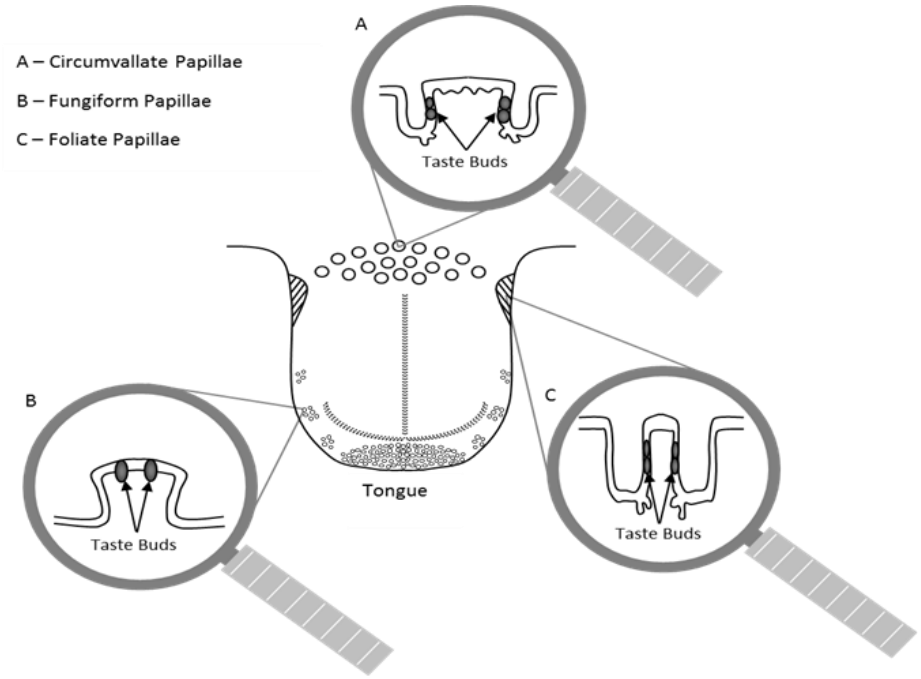

Figure 143 - Location of the three types of papillae which contain taste buds (fungiform, circumvallate and foliate). Fungiform papillae are located on the anterior portion of the tongue while circumvallate and foliate fungiform are located on the posterior portion of the tongue

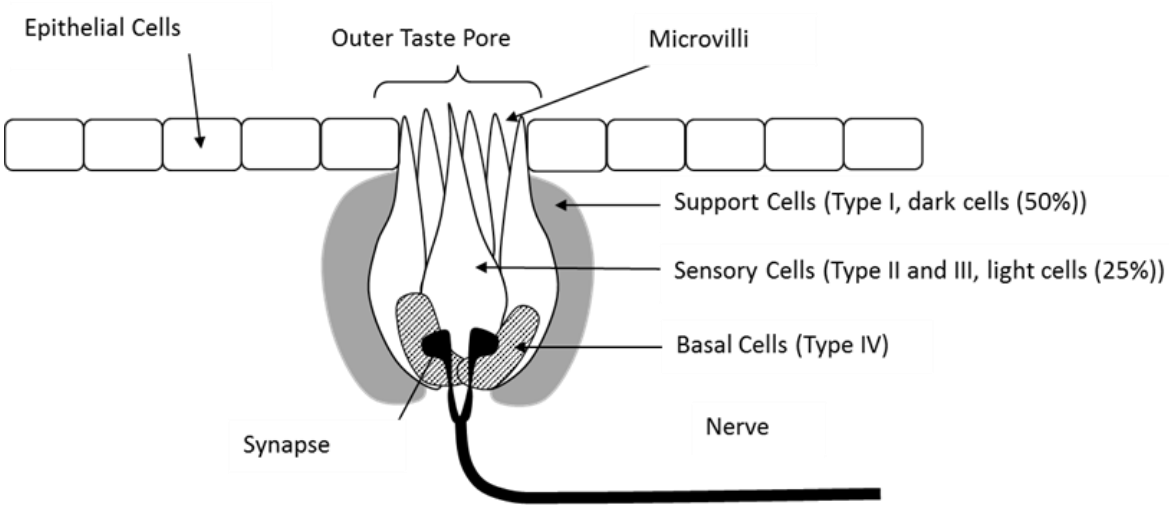

Figure 144 - Taste bud structure illustrating cell arrangement and distribution. Taste buds are contained within the papillae are constructed of four different cell types. The support cells (Type 1 cells, or dark cells), account for 50% of the cells present in a taste bud. The sensory cells (Types 2 &3 or light cells), account for around 25% of the cells present in a taste bud. Basal cells (Type 4 cells) account for the remaining 25% of the cells.

The support cells (Type 1 cells, or dark cells), account for 50% of the cells present in a taste bud. The sensory cells (Types 2 &3 or light cells), account for around 25% of the cells present in a taste bud. Basal cells (Type 4 cells) account for the remaining 25% of the cells. Of the sensory cells, type 2 cells are thought to be responsible for the detection of bitter, sweet and umami tastants through transduction pathways involving proteins such as Gustducin and Phospholipase C (PLC) -β_2, which are used as reliable indicators of Type 2 TRC presence in TRC cultures. Likewise, neural cell adhesion molecule (NCAM) is a reliable indicator of Type 3 sensory cells. These are thought to be the cells responsible for the detection of acid taste while the cell type responsible for the detection of salty taste is yet to be identified.

Following initial attempts which failed to deliver a suitable a bovine taste receptor cell culture (BTRC-CR1,2,3 and 4's), this report details the establishment and characterization of a long-term primary culture of bovine taste receptor cells (BTRC-CR5s), taking the first step towards the development of a high-throughput, low cost, easy to use means of assaying the taste of oral pharmaceutical preparations throughout the pharmaceutical formulation process.

7.3 MATERIALS AND METHODS

7.3.1 Preparation of Culture Media

The media for the culture of BTRC-CR5s consists of two parts mixed 1:5 as described by Ozdener *et al* 2011.

7.3.2 Preparation of Pronase and Elastase Solution

The enzyme solution used in the enzymatic digestion of the bovine fungiform papillae consists of 26mM Sodium Bicarbonate, 2.5mM Sodium Dihydrogen Phosphate, 20mM Glucose, 65mM Sodium Chloride, 20mM Potassium Chloride and 1mM Ethylenediaminetetraacetic acid made up in sterile ultrapure water supplemented with 1.5mg/ml Pronase E (Sigma) and 1mg/ml elastase (Sigma).

7.3.3 Preparation of Calcium free Ringer's Solution

Calcium free ringers solution was prepared by dissolving NaCl (14.5%w/v), KCl (0.0373%w/v), $MgCl_2$ (0.0203%w/v), EDTA (2mM), Na-pyruvate (0.0110%w/v), and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Na (0.476%w/v) in sterile ultrapure water.

7.3.4 Isolation of BTRCs and Establishment of BTRC-CR5 culture

Bovine tongues were collected fresh from the slaughter house (C.H. Rowley LTD) and transported to the lab on ice in a cool box. Cells were isolated following standard techniques used in primary culture. 48 hours after seeding the culture medium was changed and the plates returned to the incubator. The media was then changed once every 7 days until the cells reached confluence.

7.3.5 First Passage (P1) of BTRC-CR5s

Following three weeks in culture the cells reached confluence and were removed from the surface of the wells in the 24 well plates AND split into T25 flasks. Media was

then changed 48 hours after seeding and then every 7 days until confluent. Upon reaching confluence the cells from T25 flasks were passaged split into T75 flasks (P2).

7.3.6 Routine Maintenance of BTRC-CR5s

Once flasks reach confluence, tissue culture media is removed and cells are washed twice with HBSS. A 1:5 dilution of trypsin with HBSS is then added and the cells incubated at 37°C for 5-8 minutes. Following gentle tapping to dislodge any remaining cells, culture media is added to prevent any further action from the trypsin. The resulting cell suspension is adjusted to contain $6x10^4$ cells/ml before 2ml is added to each T75 continuation flask. If the flask is not required to reach confluence quickly then this concentration can be reduced and flasks cells can be cultured for 4 weeks before reaching confluence. This is then made up to a volume of 25ml with culture media. The remaining cell suspension is then used to seed any additional plastic-ware or glass-ware as required for experimental procedures.

7.3.7 Culture of Cells on Coverslips

A cell suspension with a cell concentration of $6x10^4$ cells/ml was produced following the passage of a confluent T75 flask and cover slips were seeded. Media was changed after 48 hours and then every 7 days, or the day before intended use, until the coverslips were deemed suitable for use.

7.3.8 Senescence test for BTRC-CR5s

To test for senescence a senescence cells histochemical staining kit (Sigma) was used. BTRC-CR5s from each passage were grown to confluence in six well plate plates. Once confluent the test was performed as per the manufacturer's instructions. Imaging was performed using a light microscope at x10 magnification.

7.3.9 Cryogenic Preservation of BTRC-CR5s

Cells were removed from a confluent T75 (P2) flask and Biofreeze was added to produce a cell suspension, this was then added to cryo vials (Gibco Invitrogen)) before they were transferred to a -18°C freezer in an insulated container for 60 minutes. After this time the insulated container was transferred to a -80°C freezer overnight. The following day the vials were removed from the -80°C freezer and transferred into liquid nitrogen.

7.3.10 Revival of Cryogenically Preserved BTRC-CR5s

Three vials of banked stock BTRC-CR5s were removed from liquid nitrogen storage conditions following 1 month in storage. The cell suspensions were defrosted in a 37°C incubator following which the contents of each vial was transferred to separate T25 flasks. 5ml of tissue culture media was added and the flasks returned to the incubator at 37°C in a humidified 5% CO_2 atmosphere. The media in the flask was changed after 48 hours and then at 7 day intervals. Once confluent the cells were transferred to T75 flasks and from here the cells were treated as described for the routine maintenance of BTRC-CR5s.

7.3.11 Intracellular Ca^{2+} and cAMP Response to Taste Stimuli

Taste stimuli, 2mM Denatonium, 1mM Sucralose, 250ppm Acesulfame-K and 3mM Monopotassium Glutamate, were dissolved in calcium free ringers solution.

For calcium assays, BTRC-CR5s were grown on coverslips for 4 days following seeding to produce a relatively sparse covering of cells. Tissue culture media was removed from the wells of the six well plate and replaced with modified culture media which contained Pluronic F127 (1%w/v) and Fluo-4 (14mM). The cells were then incubated for 2 hours at 37°C to allow for the uptake of Fluo-4 into the BTRC-CR5s. During experiments solutions for test were perfused over coverslips mounted onto the

stage and images were taken every 5 seconds using a fluorescence microscope. Calcium assays were carried out with the help of Dr Rhein Parri.

cAMP assays were carried out with the help of Professor David Poyner at Aston University. BTRC-CR5s were cultured in 24 well plates and each well treated with various concentrations of the model tastants for 30 minutes. Experiments were then immediately terminated by removing tastant solutions and adding 100% ethanol. cAMP concentration analysis then followed.

7.3.12 Confocal Identification of TRC markers Gustducin and PLC-β2

BTRC-CR5s were grown on coverslips for 4 days following seeding to produce a relatively sparse covering of cells. Tissue culture media was removed from the wells of the six well plate and replaced with 4% Paraformaldehyde (PFA) in PBS. The plate was then incubated for 10 minutes at room temperature. Following this incubation the PFA in PBS was removed and a blocking buffer added followed by a further incubation of 60 minutes at room temperature. The blocking buffer consisted of normal goat serum (3% v/v), bovine serum albumin (3% w/v) and Triton X-100 (0.3% v/v) made up in PBS. The primary antibody solutions, Rabbit polyclonal $G_{\alpha gust}$ (I-20) and Rabbit polyclonal PLC-β2 (Q-15) were both diluted 1:400 in blocking buffer. After the 60 minutes, the blocking buffer was removed, the primary antibody solutions were added to their respective wells and the plate was incubated at 4°C overnight. The following day the secondary antibodies Goat anti-rabbit IgG Alexa 633 and Goat anti-rabbit IgG Alexa 488 were both diluted 1:400 in fresh blocking buffer. Primary antibodies were removed, the cells were washed with PBS and then the secondary antibody solutions were added. The plate was incubated for 30 minutes at room temperature before the secondary antibody solutions were removed and the coverslips were washed thoroughly with PBS and then ultrapure water. The coverslips were allowed to dry before being mounted to the microscope slides using Vectashield hardset mounting medium. The slides were left undisturbed for 15 minutes to allow the mounding media to harden and then either imaged immediately or

stored at 4°C until they were imaged. All slides were imaged within 24 hours of mounting using a Leica TCS SP5 II confocal imaging system. An Argon laser at 488nm and a HeNe laser at 633nm were used in conjunction with a HCX PL APO CS 40X oil immersion lens and the numerical aperture set to 1.25.

7.4 Results and Discussion

7.4.1 Establishment of BTRC-CR5 culture.

The aim of this investigation was to isolate and culture TRC's from a bovine tongue. The first stage in the isolation procedure was to harvest the fungiform papillae from the apical region of the tongues. Fungiform papillae were selected in favour of foliate and circumvallate as their structure has clear boundaries and allows for the easy removal of only the papillae (Figure 145).

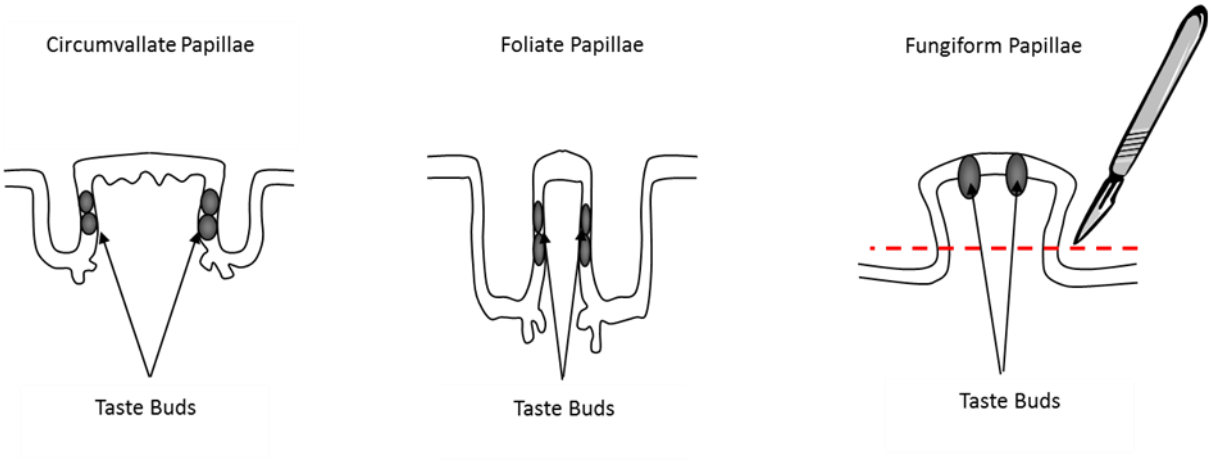

Figure 145 - Illustration demonstrating how the raised structure of fungiform papillae has clear boundaries and allows for the easy removal of only the papillae without the risk of taking excess tissue.

It was important to harvest as cleanly as possible just the fungiform papillae and not surrounding tissue in order to maximise the percentage of desired cells entering the culture process. It was observed that the fungiform papillae on the bovine tongues were significant and obvious structures with a diameter of between 1-3mm (Figure 146). The number of fungiform papillae found on the bovine tongues used (n=7) ranged from 37 to 58 with an mean number of 46. Thanks to this we found that only one tongue is required to yield enough taste cells to initiate up to 24 primary culture lines.

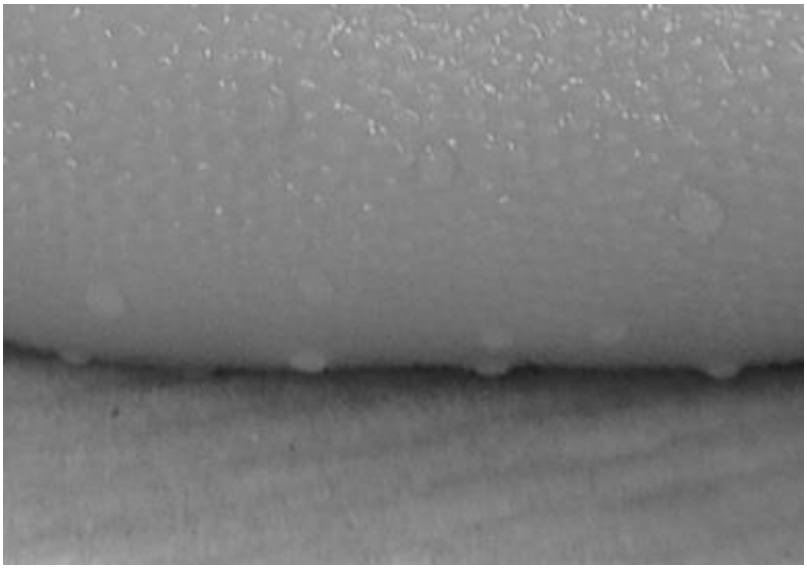

Figure 146 - Close up image of an area of the anterior region of a bovine tongue. The raised structures which can be clearly seen are the fungiform papillae which are the target for extraction during the dissection procedure.

Following the removal of the fungiform papillae, half were treated with a Pronase/Elastase solution for 30 minutes following existing protocol (Ozdener e*t al* 2011). The other half were not subjected to the enzymatic digestion stage. Both methods produced suspensions of fungiform which were then cultured. It was observed that initially and up to 24 hours following the seeding of the plates there was a very heterogeneous mix of cells with a very low degree of adhesion (Figure 147a, 148a). Following the first media change which occurred two days after the initial seeding, (Figure 147b, 148b) the cells appeared much more homogeneous with a significant number of adhered cells displaying the expected morphology of TRC's. There were still some cells present which did not display the morphology expected of TRC's. Following the next media change (Figure 147c, 148c), 7 days following the initial seeding the TRC's had proliferated and the number of non-TRC's had decreased indicating that the culture conditions were not suited to non-taste cells and as such over time the culture will contain only the desired TRC's. This was seen after the change of media 14 days following seeding (Figure 147d, 148d) which contained a greatly increased number of TRC's and

no cells which were not of the expected morphology. The cells were grown to confluence which was observed by day 21 (Figure 147e, 148e). It was also interesting to see that both sets of cells, one which had undergone enzymatic treatment and the other which had not, when cultured in parallel behaved in an identical manner with similar cell numbers at each time point, identical morphology throughout and reaching confluence at the same time (Figure 147a-e, 148a-e). This indicates that the enzymatic digestion step is superfluous and removing it from the protocol would shorten the process, reduce cost and also eradicate any potentially significant damage, however unlikely, that the enzymes may be inflict upon the cells.

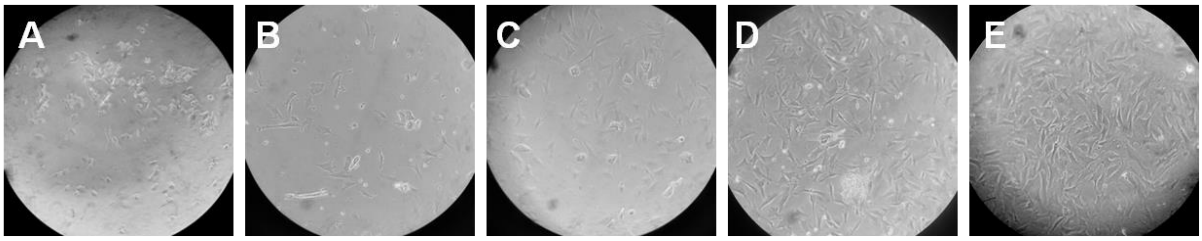

Figure 147- Culture of BTRC-CR5s achieved using enzymatic digestion during the isolation stage. Time points following seeding at 24h (A), 2 days (B), 7 days (C), 14 days (D) and 21 days (E).

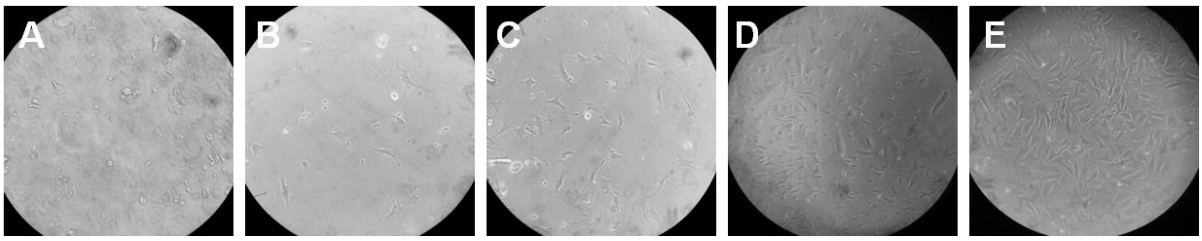

Figure 148 – Culture of BTRC-CR5s achieved using mechanical disruption during the isolation stage. Time points following seeding at 24h (A), 2 days (B), 7 days (C), 14 days (D) and 21 days (E).

7.4.2 Maintenance of BTRC-CR5 culture.

Following three weeks in culture the primary BTRC-CR5s reached confluence and were removed from the surface of the wells in the 24 well plates and seeded into T25 flasks, cells were seeded into both untreated and collagen coated flasks to investigate the need for collagen. It was found that the cells proliferated at a similar rate to that seen in the cells harvested fresh from the bovine tongue (Figure 149a-e, 150a-e) with confluence being reached after around 21 days. It was also evident that collagen coating was not necessary for the adhesion of the BTRC-CR5s to the plastic ware.

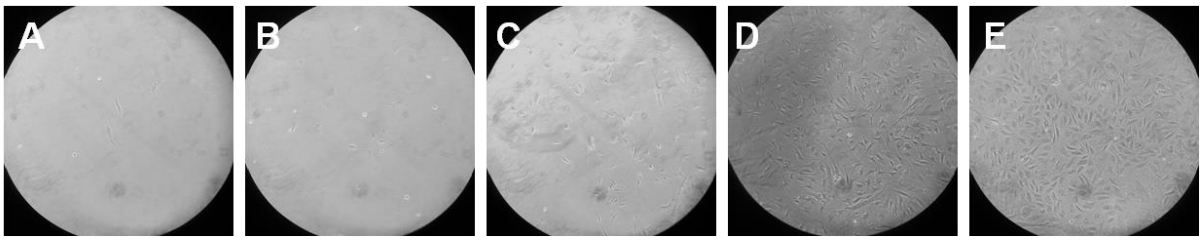

Figure 149 - Culture of BTRC-CR5s in collagen coated T25 flask. Time points following seeding at 24h (A), 2 days (B), 7 days (C), 14 days (D) and 21 days (E).

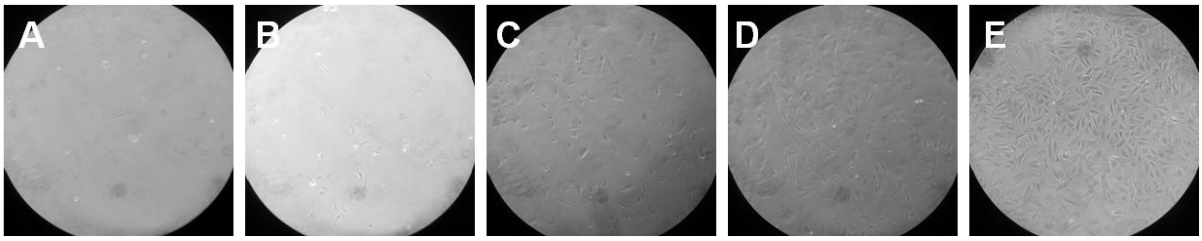

Figure 150 - Culture of BTRC-CR5s in an uncoated T25 flask. Time points following seeding at 24h (A), 2 days (B), 7 days (C), 14 days (D) and 21 days (E).

Once the cells were confluent in the T25 flasks they were then split to produce a cell suspension containing $6x10^4$ cells, from which 2ml was used to seed each T75 flasks to upscale and generate sufficient numbers of cells for experimentation. At this concentration, T75 flasks reached confluence within 7 days (Figure 151). It is possible to reduce the seeding concentration and in this way extend the time it takes for cells to

reach confluence. In this way the cells have been maintained in culture for 8 months from the initial isolation. The cells were maintained for up to 8 passages before unacceptable levels of senescence and loss of cell morphology was observed (Figure 152).

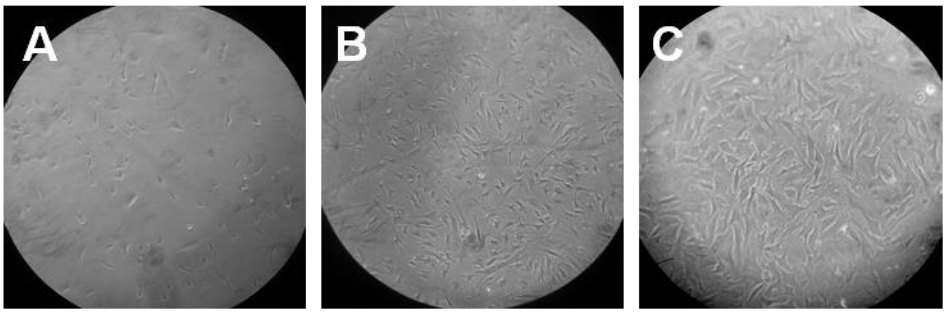

Figure 151 - Culture of BTRC-CR5s in a T75 flask. Time points following seeding at 2 days (A), 4 days (B), 7 days (C).

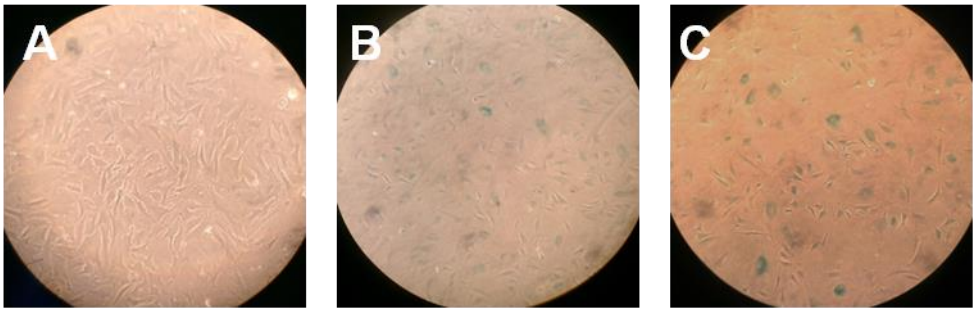

Figure 152 - Confluent cell cultures following senescence testing, blue staining indicates senescence. Early passages represented by 10a show no signs of senescence. Later passages P8 (10b) and P9 (10c) show the onset of senescence and extensive senescence respectively. At this point cells would be considered unsuitable for use in assay.

As reaching senescence is an inevitable limitation of primary cell cultures the potential to establish a stock of frozen cells is of great benefit, reducing how regularly isolation of cells from fresh tongues must be carried out. Cells were frozen down at passages 2 and 3 in bulk to provide a large stock of early passage cells. To ensure successful cryogenic preservation sample vials have been revived (n=3 from each

batch) (Figure 153). The revived cells have been tested in the same manner as the initial primary cultures and have performed in the same way for every assay showing that the cells have been successfully preserved.

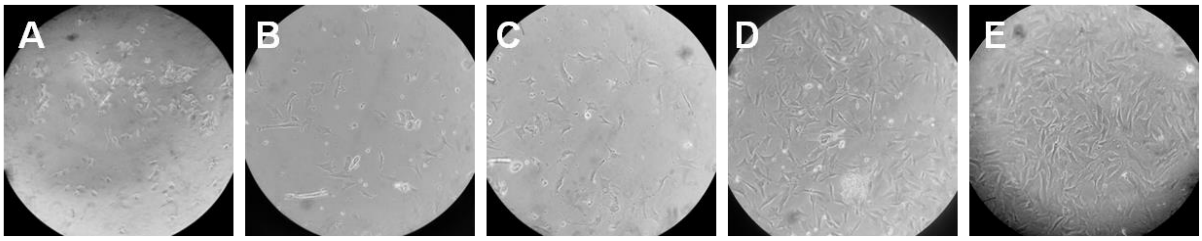

Figure 153 – Revived culture of BTRC-CR5s in an uncoated T25 flask. Time points following seeding at 24h (A), 2 days (B), 7 days (C), 14 days (D) and 21 days (E).

7.4.3 Intracellular Ca^{2+} and cAMP Responses to Taste Stimuli

An increase in intracellular calcium is the one of the final stages in the signalling cascade that leads to the release of neurotransmitter following the stimulation of TRC's with a relevant tastant. We tested cells at early (P2), middle (P4) and late (P7) passages to provide firstly evidence that the cells which were being cultured were TRC's and in addition that the cellular response was not altered by passage number. Cells were grown on coverslips and then exposed to the model tastants, Denatonium, Sucralose, Acesulfame-K and Monopotassium glutamate. These are tastants which have been used previously in the assay of intracellular calcium response of TRC's to tastants (Ozdener *et al* 2006, Ozdener *et al* 2011) and as such will allow us to draw comparisons between our findings and the results reported previously in the literature. The cultured cells displayed intracellular calcium increases to all of the tastants (Figure 154). Our results indicated that increases in passage number had no effect on the intracellular calcium response of BTRC-CR5s to the model tastants. The highest frequency of response was to 2mM Denatonium with on average 17% of cells eliciting a response. Sucralose and Acesulfame-K showed on average 5% and 8% of cells eliciting a response respectively and Monopotassium glutamate elicited a response in on average 3% of cells. All of the

responses were seen within 150 seconds of the tastants being added and there was no problems stemming from autofluorescence of photo bleaching.

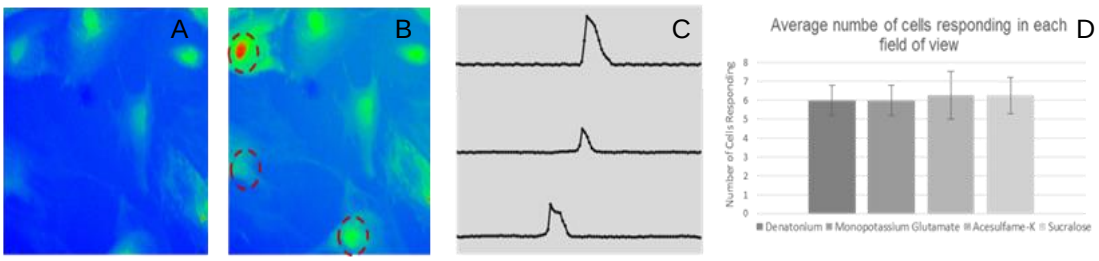

Figure 154 – Example images of fluorescence before stimulation (A) and after stimulation (B), Example traces showing intracellular calcium levels (C) and average number of BTRCs responding per field of view when exposed to the model tastants Denatonium, Sucralose, Acesulfame-K and Monopotassium Glutamate (D).

As with intracellular Ca^{2+}, intracellular cAMP is another important marker in taste transduction cascades with decreases in cAMP being observed following stimulation of BTRCs with model tastants (Table 57). The analytical process involved in the measurement of cAMP provides the benefit that repeatable quantitative results can be collected. In addition, as with intracellular calcium concentrations, intracellular cAMP flux is dependent upon stimuli concentration. As it stands, cAMP measurement allows for the concentration of known tastant to be calculated following the production of a calibration chart using standards of know concentrations.

Table 57 - Intracellular cAMP concentration changes in BTRCs which have been stimulated with the model tastants Denatonium (D), Monopotassium Glutamate (MPG), Acesulfame-K (A) and Sucralose (S).

Tastant	Max response	Log EC50
Denatonium	68.50 ±11.37	-4.944±0.4428
Monopotassium Glutamate	99.37±10.07	-4.449±0.2619
Acesulfame-K	97.49±8.29	-4.403±0.2706
Sucralose	79.53±12.86	-3.881±0.4302

The term half maximal effective concentration (EC50) refers to the concentration of tastant which induces a response halfway between the baseline and maximum after a specified exposure time. It is indicative as a measure of tastants potency.

7.4.4 Confocal Identification of TRC markers Gustducin and PLC-β2

Confocal microscopy was used to examine the cultured BTRC-CR5s for proteins which are considered to be reliable markers for the presence of TRC's. The proteins Gustducin and PLC-β_2 which are proteins involved in the transduction of bitter, sweet and umami taste and are markers for Type 2 sensory cells. We tested cells at early (P2), middle (P4) and late (P7) passages. From the observations it was clear that the cells expressed both of the proteins of interest and determined that 56% of the examined cells exhibited Gustducin (Figure 155) and around 38% of the examined cells were found to exhibit PLC-β_2 (Figure 156). Testing for non-specific binding was performed and found not to be present, validating the results collected. It was found that the effect of increasing passage number had no effect on the level of proteins detected and the detection of the proteins added more evidence to confirm that the cells cultured were definitely TRC's.

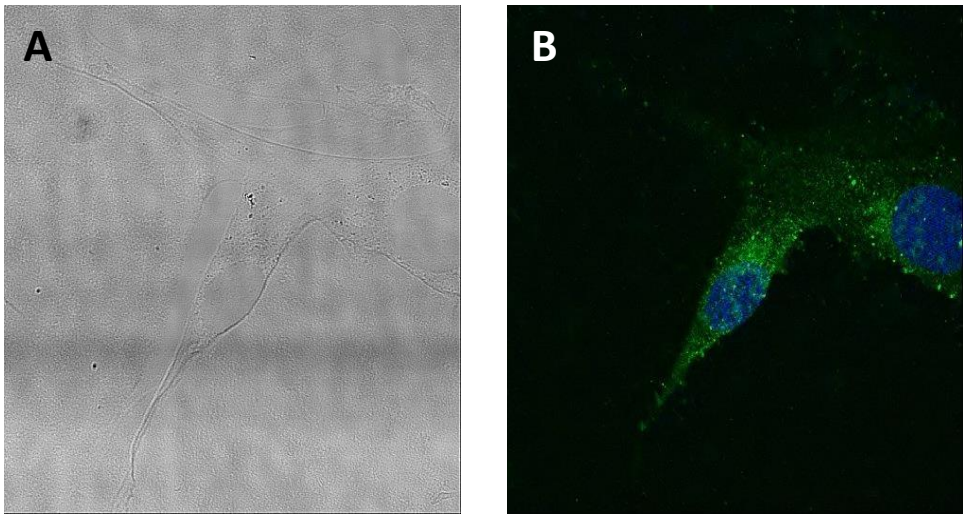

Figure 155 - Phase contrast (A) and Confocal (B) imaging of BTRC-CR5s expressing the taste marker Gustducin

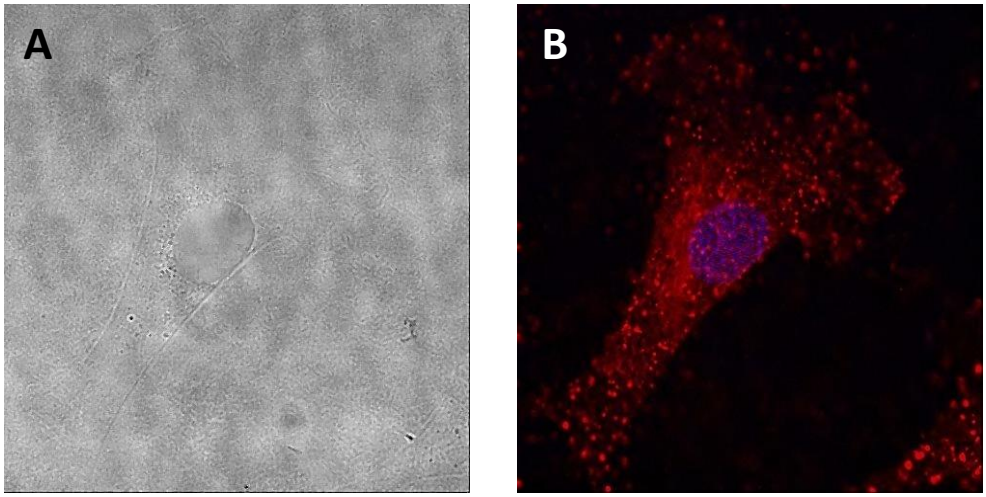

Figure 156 - Phase contrast (A) and Confocal (B) imaging of BTRC-CR5s expressing the taste marker PLC- β_2

Work carried out by Ruiz-Avila *et al* 2000 utilised taste papillae harvested from a bovine source to develop a method to screen for high-potency bitterness inhibitors, in their paper they identified the presence of the taste specific G-protein Gustducin. This protein is known to be present in the TRC's of many species including fish, amphibians, rodents, cattle and humans (Ruiz *et al* 2001). As such, before commencing this work there was evidence that cells derived from the bovine tongue would contain relevant taste apparatus and a culture of these cells would be beneficial. The cell line could also serve as a useful model in endocrinological and neurobiological investigations (Dasgupta *et al* 1990) in addition to the broad application of a taste assay which is of primary concern. Relatively little attention has been devoted to the bovine tongue and no reported attempt to culture TRC's from a bovine source has been found in searches of existing literature. One of the immediately apparent observations is the size of a bovine tongue, this means that bovine tongue provides a plentiful source of taste papillae, this contrasts to work which has been previously carried on rodents (Ozdener *et al* 2006) and humans (Ozdener *et al* 2011). This carries the advantage that relatively few organs are required to generate significant numbers of TRC's and culture lines. Ozdener *et al* 2011 reported that from each human subject, a maximum of 8 fungiform papillae were harvested and also mention that an oral surgeon was required for the collection of the

papillae. The bovine tongue, on average yielded 46 papillae (37 minimum) and the isolation of the papillae structures was a simple procedure which even a novice at dissection procedures could easily achieve. The method for isolation and culture uses the taste papillae from one tongue to seed each well in a 24 well plate and from each well there are enough cells to initiate a T25 flask meaning that each tongue has the potential to initiate a total of 24 cultures. In addition to this the use of a bovine source means that ethical issues which may be associated with maintaining lab animals or using human subjects are avoided. Finally costs are kept low with each tongue costing less than £5.

BTRC-CR5s have been successfully cultured up to passage 7 and flasks seeded at lower densities have permitted the cell line to be active for a total of 8 months. At passage 8 changes in cell morphology were seen and by passage 9 the cell morphology was changed significantly indicating senescent cells. Senescence is an inevitable eventuality with all primary cell cultures and it was identified through the use of a histochemical staining kit that senescence did not present until passage 8 and was not widely prevalent in the cultures until passage 9. In both cases the cells were deemed unfit for use in assays. The cells in passages 2-7 maintained their morphology and performed consistently in intracellular calcium level investigations and also confocal microscopy examinations.

The method of culture described in this paper is based upon methods presented elsewhere in literature for the culture of rat and human TRC's (Ozdener et al 2006, Ozdener et al 2011) with modifications implemented to simplify the procedure, minimising time required and financial expenditure without jeopardising quality of the cultures produced.

The method of culture described removes the need to collagen coat all plastic and glass ware, as cells have been shown to grow equally well without the need for coating. This has the benefit of reducing the cost if pre-coated plastic or glassware was

intended for use. Where plastic and glassware would be self-coated time required to coat plastic and glassware would be saved and in addition the eradication of this requirement in the method would avoid any inconsistencies which may result from potential variables in the coating such as thickness, dryness and collagen concentration.

During the isolation procedure in all of the previously reported TRC culture methods there is an enzymatic digestion stage. In the culture of BTRC-CR5s this has been shown to be superfluous with comparable cultures of BTRC-CR5s having been developed both when implementing and bypassing this stage. This is of benefit as the implementation of an enzymatic digestion procedure can cause damage to cells. For example trypsin and Pronase provide excellent disaggregation properties however can damage cells. Trypsin is known to be too aggressive for mild enzyme digestions as it removes cell-cell and cell-extracellular matrix connections too aggressively (Kaeffer 2002). Enzymatic digestion requires careful optimisation to avoid problems associated with yield and viability. Low yield low viability (LYLV), low yield high viability (LYHV), high yield low viability (HYLV) and high yield high viability (HYHV) are all possible outcomes. If the enzymes used are too digestive and used at too great a concentration or if incubation time is incorrect then this can lead to LYLV and HYLV returns. Similarly too low of an enzyme concentration can lead to LYHV, only through trial and error is it possible to ensure HYHV.

One further stage implemented in our culture has been the cryopreservation of the BTRC-CR5s in a liquid nitrogen cell bank. Freezing TRC's for storage has been reported before however only at -80°C (Ozdener et al 2011) and not below -130°C, the temperature at which liquid water will no longer exist and where the only physical states which exist are crystalline or glassy. -196°C is the temperature of liquid nitrogen and at this point there is insufficient energy for thermal reactions to occur hence such a high efficiency for cell preservation. Contrary to popular belief it is not the extremely low temperatures which are a threat to cell preservation but the transition periods during

freezing and thawing procedures when the cells are in the region of -15°C to -60°C. Although some cells will remain viable over reasonably long periods of time at -80°C there is evidence in the literature stating that some cells will remain viable as briefly as 3-4 months (Mishra *et al* 2010). This is due to the fact that the biological events in cells stored at -80°C have only been slowed and so cellular damage, and/or changes can still occur. In addition the cells may be unstable due to traces of unfrozen solution still existing (Mazur 1984). In contrast cells stored in liquid nitrogen have been shown to remain viable for decades. Storing at -196°C prevents all thermally driven chemical reactions meaning that so long as they are preserved correctly the cells will remain viable indefinitely (Mazur 1984). From the cultures which have been established, BTRC-CR5s have been successfully cryogenically preserved at -196°C and revived at various time intervals to ensure continued cell viability. The revived cells have been shown to behave in an identical way to cells which had not been subjected to the cryogenic storage procedures when tested for responses to taste stimuli and during examination through confocal microscopy. Currently it is estimated that there are enough viable BTRC-CR5s frozen to maintain the current rate of work for the next 15 years.

In the culture of primary cells has the initial hurdle of starting with a heterogeneous cell mix from which the target cells need to be selected for. It is the culture media which provides this selection and it was observed than non-taste cells did not proliferate during culture. In order to confirm the cells cultured as TRC's, Immunofluorescence confocal microscopy was implemented. Immunofluorescence confocal microscopy is a widely used technique in identifying the presence of a protein of interest in a sample of cells or tissue (Hegde *et al* 2002, Schwab *et al* 2013, Wang *et al* 2013, Woods *et al* 2013, Yang *et al* 2013). In the instance of this investigation, the proteins of interest were gustducin and PLC-β_2, both proteins mentioned are considered to be reliable markers for the presence of Type 2 TRC's and are involved in taste transduction signalling cascades. The extent of cells expressing gustducin and PLC-β_2

in BTRC-CR5 cultures was found to be 56% and 38% respectively and these are numbers reflected in the data presented in existing literature for the TRC's of rats and humans (Ozdener *et al* 2006, Ozdener *et al* 2011).

Isolating and culturing cells is only one part in the challenge of developing a representative cell model, the cultured cells need to maintain their *in vivo* functionality if they are to produce an accurate model. Cells in culture often behave differently in culture to how they would in their real world environment, this is at times due to the loss of the synergistic effect of surrounding cells. In order to illustrate that the BTRC-CR5s cultured still respond in a manner which is suitably representative of their *in vivo* activity, *in vitro* measures of TRC response to taste stimuli was performed. To do this intracellular calcium levels were examined following the exposure of BTRC-CR5 cultures to model taste stimuli. Increases in intracellular calcium levels are common to all of the taste transduction cascades and is the precursor to neurotransmitter release (Gilbertson et al 2000).

It has been illustrated that TRC's cells will elicit a spike in intracellular concentration in response to exposure of the TRC's to model taste stimuli for the bitter, sweet and umami taste groups (Ozdener *et al* 2006, Ozdener *et al* 2011). BTRC-CR5s which have been exposed to the same model tastants returned responses which were in keeping with the data reported previously in the literature for rat and human cells. This is important in not only confirming that the cultured cells have retained the relevant mechanistic characteristics but also that there is similarity in the performance of cells gained from a bovine source when compared to cells which have been gained from a human source. Observing responses through increases in intracellular calcium at this stage is incapable of differentiating between tastant type, i.e. bitter or sweet, this is not to say that measurements of intracellular calcium may not play a part in the final model for taste assessment. It has been observed (data not shown) and previously reported (Ozdener *et al* 2006) that the degree of calcium increase is affected by the concentration

of tastant and therefore indicative of the tastant strength. As such there is a chance that measurement of intracellular calcium may play a role in the final *in vitro* taste assay.

In conclusion a method for the long term culture of BTRC-CR5 taste receptor cells harvested from a bovine tongue has been developed. The method of culture has been simplified to reduce time and cost of the process when compared to existing methods for the culture of taste receptor cells, eradicating the need for collagen coating of plastic and glassware and the removal of an enzymatic digestion stage in the isolation procedure. The cells have been maintained in culture up to passage 7 before signs of senescence were first observed and conformation of cell type and activity has been achieved through confocal microscopy and the measurement of intracellular calcium level responses to model tastants. This has been the successful first step into the development of an *in vitro* taste assay which would have far reaching application.

8 CLOSING COMMENTARY

The objectives of the work carried out in project can be categorized into three main areas of research;

1. Reformulation of the angiotensin converting enzyme (ACE) inhibitors lisinopril and ramipril and potassium-sparing diuretic spironolactone to produce oral liquid formulations better targeting the paediatric patient segment.
2. Using the model formulations produced in the first stage of the project, an *in vitro – in vivo* correlation between changes in gene expression following *in vitro* transport studies and *in vivo* absorption will be investigated using microarray technology.
3. During the production of any oral formulation targeting any patient segment the palatability of the formulation is an important consideration. It became apparent during the formulation development stages of the project that the available analytical techniques for taste assessment are inadequate. As such initial stages into the development of an *in vitro* cell based taste assessment method merited investigation.

It is recognized globally that the drug products currently available for treating the paediatric population, which suitably meet the needs of the patient segment are lacking in their coverage. In order to improve on this situation bodies such as the FDA and the EMA have devised legislation to guide the research and development of paediatric medicines through priority listings. By selecting and reformulating off patent medications as oral liquids in cases where oral liquids are not already available the paediatric population have been more appropriately targeted.

Angiotensin converting enzyme (ACE) inhibitors lisinopril and ramipril were selected from EMA/480197/2010 and the potassium-sparing diuretic spironolactone was selected from the NHS specials list for November 2011 drug tariff with the view of

producing oral liquid formulations providing dosage forms targeting paediatrics. Lisinopril, ramipril and spironolactone were chosen for their interaction with transporter proteins in the small intestine. Previous research illustrated that gene expression of intestinal membrane transporters changes in response to drug transport. It was therefore considered worthwhile investigating the relationship between the expressional changes seen and the extent of drug absorption as the development of an *in vitro – in vivo* correlation would be of benefit to a number of fields, potentially aiding in the areas of drug discovery and development as well as potentially reducing the need for animal based drug absorption studies.

Formulation limitations such as poor solubility or pH sensitivity needed consideration. Lisinopril was formulated without extensive development as drug and excipients were water soluble. Ramipril and spironolactone are both insoluble in water and strategies combating this were employed. Characterisation, stability profiling and permeability assessment were then performed following formulation development.

As detailed, for the Lisinopril solution, the API and all excipients are soluble in water the production of the formulation is simple. Following excipient selection the constituent parts for Lisinopril solutions are as follows;

- Lisinopril (2mg/ml)
- Citric Acid (3mg/ml)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (30% w/v in sweetened formulation) (10% w/v in flavoured formulation)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

Where Ramipril is concerned, the poor aqueous solubility of the drug is a limitation in the production of an oral liquid dosage form and to combat this, three formulation strategies were employed. Two of these involved the production of Ramipril

solutions, one using a co-solvency approach of acetic acid and water to dissolve the API and the other utilising complexation of the drug with HP-β-CD. The final formulation of the Ramipril solution produced using acetic acid as a co-solvent is as follows;

- Ramipril (1mg/ml)
- Acetic Acid (0.02% v/v)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (20%)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

For production of the formulation, a 0.02% v/v acetic acid solution was produced. Into this Ramipril was added and under stirring allowed to fully dissolve before any further excipients were added. With Ramipril dissolved in the acetic acid solution the other excipients were added with flavouring being added as the final excipient. The development of a Ramipril solution using complexation with HP-β-CD, arrived at a final formulation as follows;

- Ramipril (1mg/ml)
- HP-β-CD (3.51mg/ml)
- Ascorbic Acid (0.1mg/ml)
- Propyl Paraben (0.1mg/ml)
- Butyl Paraben (0.06mg/ml)
- Xylitol (20%w/v)
- Orange Flavour (0.1% v/v) (Flavoured formulation only)

For production of the formulation a cyclodextrin solution was first produced by dissolving 3.51mg/ml in distilled water. With this produced the next stage was to add the Ramipril until it was completely dissolved. Following this the other excipients are added until fully dissolved with flavour being added last.

The remaining Ramipril formulation approach was that of a suspension. Due to the inherent stability concerns that are associated with suspensions additional excipients are necessary. Hydrophilic polymers are includeed as suspending agents and have the effect of increasing the viscosity of the liquid vehicle. This prolongs suspension of particles but can in the same way make it difficult to re-suspend particles which sediment. To get the best results from the addition of hydrophilic polymers, a suspensions resulting flow profile was required to be pseudoplastic or shear thinning and thixotropic. This is useful as the relatively high viscosity under low shear conditions e.g. during storage slows the sedimentation process and the relatively low viscosity under high shear conditions e.g. shaking or pouring, allows for the easy re-dispersion of any sediment and easy dispensation of the medication. Xanatural 180 is a hydrophilic polymer which provides such rheology and is utilised in the suspensions produced in this project. The suspension was further optimised through investigations into the rate and volume of sedimentation, Ostwald ripening, zeta potential and wettability. The result was an elegant formulation with the desired physical stability. The final composition of the Ramipril suspensions are as follows;

Flavoured Suspension:

- Ramipril (1mg/ml)
- Xanatural 180 (0.1% w/v)
- Sodium Metabisulphate (1.0% w/v)
- Sodium Benzoate (0.5% w/v)
- Xylitol (40% w/v)
- Strawberry Concentrate (0.2%v/v) (Flavoured formulation only)

The suspensions were produced by first producing a 0.1% Xanatural 180 solution. Into this Ramipril was then added slowly during vigorous stirring and evenly distributed

throughout the vehicle. The remaining excipients are then added to the formulation with the strawberry concentrate being added as the last ingredient.

Spironolactone dosages were too high for solubilisation techniques to be effective so suspensions were developed following the same methodology as used in the Ramipril formulations. There were slight differences in that a buffer system was needed to control formulation pH for the maximum stability of the sensitive drug. Additionally, a precisely balanced surfactant and suspending agent mix was needed to produce a pseudoplastic liquid vehicle in which the drug did not clump together during formulation production. The resulting vehicle provided excellent physical stability. From the investigations carried out Xanatural 180 and Pluronic F127 are essential components in the liquid vehicle. Based on knowledge gained in the Ramipril and Lisinopril formulations, the other excipients selected for the formulation were Sodium Metabisulphate and Sodium benzoate to act as an antioxidant and preservative respectively. Flavouring would be present in the form of Xylitol as a sweetener and strawberry flavour included in a flavoured variety.

The final ingredients for the formulation are as follows;

- Spironolactone (10mg/ml or 5mg/ml)
- Xanatural 180 (0.4%w/v)
- Pluronic F127 (1.0%w/v)
- Sodium Metabisulphate (1.0%w/v)
- Sodium Benzoate (0.5%w/v)
- Xylitol (40%w/v)
- Strawberry Flavour (0.2%) (Flavoured Formulation Only)

Following production of the formulations they were entered into stability testing conditions as laid out in ICH guidelines. According to the parameters outlined by the ICH

harmonised tripartite guideline stability testing of new drug substances and products Q1A(R2**)**, “significant change” for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation product’s exceeding its acceptance criterion;
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., colour, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH; or
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

The Lisinopril formulations tested under accelerated conditions showed significant decrease in the drug assayed in comparison to the day zero analysis. The formulations in long term storage conditions did not show any significant decrease in the drug assayed even after 12 months in storage. This suggests that the degradation seen under accelerated conditions is a result of the elevated temperature. It is known that Lisinopril diketopiperazine (DKP) degradate is the primary degradation compound for Lisinopril. Degradation occurs through intramolecular condensation and sees the formation of a second amide bond. This reaction is known to occur when Lisinopril experiences conditions of elevated temperature, as such the advice for storage of the medication would have to emphasise storage in a cool location (<25°C). The humidity of the storage conditions is not a large issue as the medication will be contained within bottles impervious to moisture. With regards to excursions outside of the storage conditions such as transport during distribution, degradation due to temperature should

not be a concern so long as excursions are short in duration and infrequent. This is as degradation was only seen to be significant after >1 month and the temperature at which degradation was seen is higher than most environmental temperatures the medication would experience.

The Ramipril formulations tested under accelerated conditions showed significant decrease in the drug assayed in comparison to the day zero analysis. This was seen in all formulations regardless of the method of formulation. The formulations in long term storage conditions did not show any significant decrease in the drug assayed even after 12 months in storage. This suggests that the degradation seen under accelerated conditions is a result of the elevated temperature. It is known that Ramipril diketopiperazine (DKP) degradate is the primary degradation compound for Ramipril. Degradation occurs through intramolecular condensation and sees the formation of a second amide bond.

This reaction is known to occur with Ramipril as was seen with Lisinopril when the drug experiences conditions of elevated temperature. The pH of the formulation in both accelerated and long term conditions did not change beyond the acceptance levels for the formulation. In addition, the appearance and odour of the formulations remained acceptable for the duration of the stability testing and in both long term and accelerated conditions. The advice for storage of the medication would have to emphasise storage in a cool location (<25°C), the humidity of the storage conditions is not a large issue as the medication will be contained within glass bottles impervious to moisture. With regards to excursions outside of the storage conditions such as transport during distribution, degradation due to temperature should not be a concern so long as excursions are short in duration and infrequent. This is as degradation was only considered significant after >1 month and the temperature at which degradation was seen is higher than most environmental temperatures the medication would experience.

The Spironolactone formulations tested under all conditions showed no significant decrease in the drug assayed in comparison to the day zero analysis. This was seen in all formulation types and at all dosages. In addition, the appearance and odour of the formulations remained acceptable for the duration of the stability testing and in both long term and accelerated conditions. Sedimentation did not occur in formulations stored in long term storage conditions and some degree of sedimentation was observed in formulations stored in accelerated storage conditions. The reason behind the sedimentation seen is most likely the increased temperature decreasing the viscosity of the vehicle and thus allowing particles to gravitate more easily. Caking did not occur however and the suspension was returned to a homogenous state easily and with minimal agitation meaning that the physical stability of the formulations in all conditions was maintained for the duration of testing. The advice for storage of the medication would have to advise against storage in hot conditions and directions for the procedure of re-suspension would have to be provided to ensure formulation homogeneity immediately prior to administration. With regards to excursions outside of the storage conditions such as transport during distribution, degradation and lack of physical stability should not be a concern.

Although formulations were successfully produced for Ramipril, Lisinopril and Spironolactone, the formulation process highlighted serious shortcomings in currently available techniques for taste assessment of pharmaceutical preparations resulting in early stage research into a novel *in vitro* cell based assay. A method for the long term culture of BTRC-CR5 taste receptor cells harvested from a bovine tongue has been developed. The method of culture has been simplified to reduce time and cost of the process when compared to existing methods for the culture of taste receptor cells, eradicating the need for collagen coating of plastic and glassware and the removal of an enzymatic digestion stage in the isolation procedure. The cells have been maintained in culture up to passage 7 before signs of senescence were first observed and

conformation of cell type and activity has been achieved through confocal microscopy and the measurement of intracellular calcium level responses to model tastants. This has been the successful first step into the development of an *in vitro* taste assay which would have far reaching application.

The formulations developed in the initial phase of the research were used as model formulations investigating potential microarray application in an *in vitro-in vivo* correlation for carrier mediated drug absorption. The *in vitro* and *in vivo* rate of drug absorption was first examined for correlation. The findings from the *in vitro* and *in vivo* drug absorption studies were compared to identify a correlation between the results. For this the Log conversion of the AUC from the *in vivo* experiments was plotted against the Log P_{app} values calculated following the *in vitro* drug transport studies. The correlation for the comparison of the *in vitro* and *in vivo* data sets was found to be $R^2 = 0.6946$. The R^2 value is comparable to those seen elsewhere in reported literature for *in vitro in vivo* correlations. The R^2 value would perhaps be expected to be some distance from the perfect linearity of $R^2 = 1.0$ as the comparison uses limited data points (n=5). There is also a known high degree of variation in the *in vivo* absorption of the ACE inhibitor drugs Lisinopril and Ramipril. The next stage in the investigation examines the gene expression levels of intestinal transporters to see if there is a greater correlation between *in vitro* gene expression and *in vivo* drug absorption.

Caco-2 cells were subsequently assessed following transport studies for changes in genetic expression of the ATP-binding cassette and solute carrier transporter superfamilies using microarray technology. Following the analysis of the microarray data it was identified that the shortlisted genes of interest, namely ABCB1 and SLC15A1 for Lisinopril, SLC15A1 and SLC15A2 for Ramipril and ABCB1, ABCC2 and SLCO1A2 for Spironolactone were all found to show non-significant changes in their expression levels following transport studies using the Lisinopril, Ramipril and Spironolactone formulations.

There were however numerous SLC and ABC genes for which the expression had changed significantly.

Due to the variety of genes expressed and the lack of common genes affected by all formulations in the data presented, a means by which human drug absorption can be predicted by measuring gene expression levels of a single gene or a family of genes was impossible to derive. There is however indication that examination of genome wide expression changes in the total number of genes showing significant change correlates to the predicted absorption in humans. Plotting the number of genes with significantly altered expression levels for each drug against the percentage absorption in humans provides a linear correlation with an R^2 value of 0.9934. It must be stressed however that this correlation is based on minimal amounts of data and for any trend between human drug absorption and the level of genome wide gene expression changes to be considered as a model for the prediction of human drug absorption many more formulations would need to be analysed investigating a range of dosage forms, dosage concentrations and degree of human absorption.

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