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Abstract

Ramipril is used mainly for the treatment of hypertension and to reduce incidence of fatality following heart attacks in patients who develop indications of congestive heart failure. In the paediatric population it is used most commonly for the treatment of heart failure, hypertension in type 1 diabetes and diabetic nephropathy. Due to the lack of a suitable liquid formulation, the current study evaluates the development of a range of oral liquid formulations of ramipril along with their in vitro and in vivo absorption studies. Three different formulation development approaches were studied: solubilisation using acetic acid as a co-solvent, complexation with hydroxypropyl-β-cyclodextrin (HP-β-CD) and suspension development using xanthan gum. Systematic optimisation of formulation parameters for the different strategies resulted in the development of products stable for twelve months at long term stability conditions. In vivo evaluation showed $C_{MAX}$ of 10.48 µg/mL for co-solvent, 13.04µg/ml for the suspension and 29.58µg/mL for the cyclodextrin based ramipril solution. Interestingly, both ramipril solution (co-solvent) and the suspension showed a $T_{MAX}$ of 2.5h, however, cyclodextrin based ramipril produced $T_{MAX}$ at 0.75h following administration. The results presented in this study provide translatable products for oral liquid ramipril which offer preferential paediatric use over existing alternatives.
Introduction

Ramipril is used mainly in the treatment of mild to severe hypertension as well as to reduce the incidence of fatality following heart attacks in patients who develop indications of congestive heart failure. It may also be used to slow the progression of kidney disease in patients who suffer from high blood pressure in conditions such as diabetes, microalbuminuria (albumin in the urine) or nephropathy. Once administered, maximum absorption is approximately 50-60% through solute transporters in the small intestine. In the paediatric population, ramipril is used most commonly for the treatment of heart failure, hypertension in children with type 1 diabetes and also in the treatment of diabetic nephropathy.

Solid oral dosage forms are the preferred method of administration for the general population. These however are often difficult or impossible for paediatric patients to swallow safely. The preferred dosage forms for children include oral solutions, syrups, suspensions, and drops due to their wider acceptability and dose flexibility. Additionally, liquid forms of medication may also be administered by oral feeding tube when required. Dose volume is a critical factor to consider during formulation development as the typical target dose volumes for paediatric liquid formulations are ≤5ml for children younger than 5 years and ≤10ml for children 5 years and older.

During formulation, the physiochemical properties of the drug governs the route of formulation with solubility, pKa, and palatability being primary concerns. Careful excipient selection is imperative when targeting paediatrics to achieve the desired formulation effect whilst still accommodating the highly sensitive nature of the patient segment. The European Medicines Agency (EMEA) acknowledges that there may be no single formulation which meets all requirements for all paediatric age groups but dosage forms containing minimal number and quantities of excipients which offer dose flexibility and cheaper cost of production are preferred.
Ramipril is commercially available as tablets, capsules and oral solution. However, the oral liquid is not prescribed for paediatric population owing to the reported toxicity implications associated with the use of high concentration of propylene glycol (E1520) within the formulation (EMEA/CHMP/PEG/194810/2005). (11) Research into the production of an age appropriate dosage form targeting paediatrics, was considered a priority as communicated in the revised priority list for studies into off-patent paediatric medicinal products (EMA/480197/2010).(10)

The current study aims to develop oral liquid formulations of ramipril which offers target pharmaceutical product profile (TPPP) comprising of small dose volume (not more than 5 mL to deliver 5mg, which is the common dose in children), long term stability, reduced dosing frequency (related to dose volume), flexibility for dose manipulation and evidence of biopharmaceutical performance. Three different strategies which included two solution based and one suspension based formulation approaches that meet the TPPP were investigated. Solubilisation of ramipril was studied using acetic acid as a co-solvent and through complexation with hydroxypropyl-β-cyclodextrin (HP-β-CD) whereas suspension was formulated using hydrophilic suspending agent.

Acetic acid was selected for use as a co-solvent in the solubilisation of ramipril as it dissolves the drug freely and is Generally Recognised as Safe (GRAS). Similarly, HP-β-CD (modified β-CD) was used for drug complexation as it offers improved solubility when compared to the parent molecule which would result in a linear relationship between the improvement in apparent solubility (drug) with increasing cyclodextrin concentration. Although parenteral administration of beta cyclodextrins may lead to nephrotoxicity, oral delivery is considered safe due to their low bioavailability (<1%).(12)

The formulation of oral suspension of ramipril was studied using xanthan gum as the suspending agent due to its pseudoplastic properties and solubility in water. Additionally, xanthan gum is suitable for use in a wide range of pH (between 3-12) which has benefits over hydrophobic polymers such as ethylcellulose which are sensitive to acidic conditions (as
ramipril is most stable in an acidic environment) and require heating and cooling cycles to achieve solubilisation.

Following formulation development, stability testing was carried out to ensure that the formulations achieved the long term stability requirement listed in the TPPP. Biopharmaceutical performance of the dosage forms was evaluated and included the measurement of drug permeability using cell culture models followed by in vivo assessment in rats to determine the pharmacokinetic profile. Previous work has reported a correlation of \( R^2 = 0.88 \) for drug absorption between humans and rats. (13) Similarly, drug absorption studies in the small intestine of humans and rats showed a linear correlation \( (R^2 = 0.8) \) for both passive and carrier mediated absorption pathways. (14)

**Materials**

Ramipril was purchased from Discovery Fine Chemicals (UK). Solvents for HPLC were supplied by Fisher Scientific (UK). Xantural® was purchased from Azelis (UK). All other reagents including HP-\( \beta \)-CD, NaOH, HCl, Tween 20, 40, 60 and 80, Span 20, 40, 60 and 80 and tissue culture media items were obtained from Sigma (UK) Ltd. Gibco Invitrogen (UK) supplied Hanks Balanced Salt Solution (HBSS) for tissue culture use.

**Methods**

**HPLC method for the detection of ramipril**

A Dionex GP50 Gradient Pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler was used for HPLC studies. Perchlorate Buffer and Acetonitrile (55:45) was used as the mobile phase and the stationary phase was a C18 reverse phase column (150 x 4.5mm with 5\( \mu \)m Particle Size) with a flow rate of 1mL/min at a detection wavelength of 216nm. The injection volume was 50\( \mu \)l and the run time was 10 minutes (min). Calibration standards were produced via serial dilution in methanol and acetonitrile (Diluent A) (50:50).
The standards were then diluted 1:5 in diluent B (water and acetonitrile, 45:55). The method was validated following the guidelines from International Conference on Harmonisation (ICH) (ICH Topic Q 2 (R1) 1995).(15)

**Method for particle size analysis**

Particle size for suspensions was measured using Sympatec Helos (UK) particle sizer. For the measurements of size, 100 μL of a sample was pipetted into a glass quartz cuvette (a signal of at least 15% scattering was required for an adequate reading). Three measurements were taken for each formulation and an average result obtained for size and polydispersity.

**Method for zeta potential analysis**

Zeta potential was analysed using a Brookhaven zeta potential analyser in conjunction with a BI-ZEL electrode assembly (Brookhaven Instruments). A 1:10 dilution was made of each formulation in distilled water to bring detection levels into a readable range and placed into the zetasizer cuvette. Triplicate readings were taken from three batches for each formulation and the mean value reported.

**Co-solvent approach to the solubilisation of ramipril**

Acetic acid solutions were produced at concentrations of 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, 0.04%, 0.03%, 0.02% and 0.01% v/v. Ramipril was then added to each co solvent at 0.01%w/v. The solutions were stirred for 1 hour (h) and filtered before HPLC to confirm complete solubilisation.

**Cyclodextrin Approach to the Solubilisation of Ramipril**

**Phase solubility**
Method described by Higuchi and Connors was used to investigate phase solubility of ramipril and HP-β-CD complex.(16) This method was also adapted to assay the effect of temperature and pH on solubilisation. Six concentrations of cyclodextrin were produced in distilled water ranging between 1.2mM to 3.75µM using serial dilution. These were cooled to 15°C and excess ramipril was added. Solutions were then maintained at this temperature for 1h at which point a sample was taken, filtered and prepared for HPLC. This was repeated at 30°C, 45°C, 60°C and 75°C. Similarly for pH, six concentrations of cyclodextrin were produced from 1.2mM down to 3.75µM using serial dilution. These were produced at pH 3, 5 and 7 to which excess ramipril was added. Solutions were left stirring for 1h before samples were filtered and prepared for HPLC analysis.

**Determination of ramipril – HP-β-CD binding constant and stoichiometry**

In order to identify the binding constant and stoichiometry for drug-cyclodextrin complex, known amounts of ramipril were added to 4.8x10^{-3}M solution of HP-β-CD in water at increasing increments of 1.0x10^{-3}M up to 1.0x10^{-2}M. Temperature was maintained at 25°C and pH was recorded throughout. Conductometry readings were taken using a conductometer equipped with a probe containing 2 platinum electrodes (surface 1 cm², separation distance of 1 cm) after each addition of drug. A graph of the conductance readings was produced. Stoichiometry and binding constant of the drug-cyclodextrin complex was calculated by plotting conductance against the molar ratio of cyclodextrin.

**Suspension approach to ramipril Fformulation**

**Rheology**

Ramipril suspensions containing 0.1-0.5%w/v (recommended concentration as suspending agent) of Xantural® were prepared to study flow behaviour. Suspension rheology was investigated to provide information on flow properties by measuring suspension viscosity at different shear rates using a Brookfield DV – L + Viscometer at shear rates of 0.08, 0.16, 0.32, 0.83 and 1.67 reciprocal seconds at 20°C.
Investigation into the rate and volume of sedimentation

The theoretical rate and volume of sedimentation was calculated using Stokes equation. For investigation into the actual rate and volume of sedimentation, ramipril suspensions containing 0.1-0.5% w/v Xantural® were prepared and allowed to stand undisturbed for four weeks at 20°C. Actual sedimentation was obtained from the sedimentation volume. Studies were carried out on three different batches of the formulations.

Particle size and investigation into Ostwald Ripening

Ramipril suspensions were produced in distilled water using Xantural® 180 at concentrations ranging between 0.1-0.5% w/v and particle size was measured using a Sympatec HELIOS/BF particle size analyser. The suspensions were heated and cooled repeatedly and particle size was measured after each cycle. Microscopic images were also recorded to document any crystal growth. Each cycle involved heating to 40°C for 24h followed by cooling to 4°C for 24h. Studies were carried out on three different batches of the formulations.

Analysis of zeta potential

Ramipril suspensions (0.1-0.5% w/v Xantural® 180) were analysed using a zeta plus zeta potential analyser in conjunction with a BI-ZEL electrode assembly (Brookhaven Instruments). 100µl of the formulation was added to the cuvette containing approximately 1.5ml of distilled water and zeta potential was recorded on three different batches of the same formulation. The values presented are the mean of the three measurements.

Stability analysis

The conditions for stability testing were based on the guidelines set out in the ICH Harmonisation Guidelines Q1A(R2). Stability investigations were carried out at 40°C and 75% humidity for accelerated testing and 25°C and 60% humidity for long term stability testing.
The final formulations were tested on a monthly basis and assessed quantitatively by HPLC and for pH. Formulations were also assessed qualitatively for colour changes and odour and suspensions were assessed for resuspendibility of any sediment.

Assessment of absorption *In Vitro*

**Culture of Caco-2 cells for permeability assay**

Caco-2 cells (passage 45) (A kind gift from Dr Andrew Collett and Dr Daniel Patten at the University of Huddersfield) were seeded at a density of 1.3x10⁵ cells/cm² onto permeability supports (Appleton Woods Ltd). Cells were cultured in an incubator (Sanyo) at 37°C in a humidified 5% CO₂/95% air atmosphere. Media was changed every 2-3 days over a three week period. Trans-epithelial electronic resistance (TEER) measurements were taken following each media change and immediately before and after experiments using an EVOM – Epithelial Voltohmmeter (World Precision Instruments Ltd). Minimum TEER threshold of 350Ωcm² (before and after the experiment) was set as a standard to ensure monolayer integrity.

**Trans-epithelial flux of ramipril across Caco-2 monolayers**

Culture media was removed and the monolayers were washed and incubated (pH range 6.7 - 7.8) at 37°C for 30min with 2.5ml and 1.5ml of HBSS basolaterally and apically respectively. In each case, HBSS was then replaced in the apical compartment with 1.5ml of the optimised formulation to be tested. Monolayer containing transwells (not exposed to the drug) were used as controls. 200µl samples were then taken from the basolateral compartment at time points of 5, 10, 15, 20, 30, 60, 90, and 120min. 200µl of HBSS was added in each case to maintain volume of solution in the basolateral compartment which was accounted for in calculations. Samples were analysed via HPLC. All of the experiments were performed in triplicate and the level of transport was described as the percentage of drug arriving in the basolateral compartment. 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, $P_{app}$ was calculated.\cite{18}

**Genomic profiling of Caco-2 following in vitro absorption studies**

Investigation into gene expression changes of Caco-2 cells following drug transport studies were intended to identify link between the predicted drug permeability and the expression of the genes which code for the intestinal transporters of ramipril. Microarray assay was performed on RNA samples collected from Caco-2 cells following trans-epithelial flux experiments on ramipril solutions developed using co-solvency and cyclodextrin approaches in addition to the suspension formulation. These assays were carried out 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). All samples were then hybridised to Agilent 4x44K whole genome arrays for 17(h) 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 using Agilent Scanner (Agilent Technologies, Santa Clara, CA) at a resolution of 50n. Feature Extraction software (V10.7, Santa Clara, CA) was implemented to examine the quality of the 16-bit TIFF images obtained by microarray scanning.

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 when compared to their expression levels in control populations. 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 the Kyoto Encyclopaedia of Genes and Genomes (KEGG) http://www.genome.jp/kegg/. (19)

**Assessment of absorption in vivo.**

Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK) and was carried out in a designated establishment. Wistar rats with a body weight of approximately 250g-300g were used. Prepared formulations were loaded into a 1ml syringe and administered via oral gavage. Dosing was calculated depending upon the weight of each individual animal (5mg/kg) and formulations were diluted in water. Following dosing, an additional 1ml of H₂O was administered to rinse in the formulations. Blood samples (45µl) were taken via tail bleeds at 15min, 30min, 45min, 1h, 1.5h, 2h, 2.5h, 3h and 4h time points following administration. Plasma was extracted from blood samples by centrifugation at 2800 x g for 10min. The plasma samples were then diluted 1:5 in mobile phase and analysed using HPLC.

**Results and Discussion**

The first phase of product development was focused on excipient selection and process optimisation. One of the main considerations was to ensure that the formulations were developed with minimal number of excipients at their lowest possible concentration. The first
approach towards the development of oral liquid solution was studied using acetic acid as a co-solvent to enhance the apparent solubility of ramipril.

**Co-solvent approach to the solubilisation of ramipril**

Initial investigations were focussed on solubility profiling of ramipril following the addition of a range of different concentrations of acetic acid in water. Acetic acid-water co solvents were prepared in the concentration range between 0.01 – 4% v/v of acetic acid and the solubility of ramipril was assessed using HPLC. The various co solvent mixtures were also assessed for their organoleptic properties such as colour and smell as well as their final pH. All of the co-solvent solutions achieved complete solubilisation of ramipril to form colourless solutions. The pH of the solutions became less acidic as the acetic acid concentration decreased. For instance, the pH of the lowest acetic acid concentration (0.01% v/v) was 3.8 and the highest acetic acid concentration (4.0% v/v) was 2.4. For all of the solutions, the smell of acetic acid was negligible.

Ramipril was solubilised utilising acetic acid and distilled water as a co-solvent system even at acetic acid concentrations as low as 0.01% (v/v). This lends itself to use in the production of an oral liquid dosage form as the low levels of co-solvent are ideal. This is of particular importance when targeting paediatrics and especially so with acetic acid owing to unpleasant taste and smell being potentially significant factors. Additionally, low concentrations of acetic acid minimises requirements for taste and odour masking. The concentration of acetic acid solution deemed most suitable for use in the solubilisation of ramipril was 0.02% (v/v). This was the lowest concentration at which instantaneous solubilisation was achieved at the same time yielding a suitable pH (3.7), colour (clear) and odour (odourless).

The final formulation composition and process consisted of addition of 1mg/ml of ramipril into 0.02% v/v acetic acid - water cosolvent mixture under stirring to ensure complete solubilisation and homogeneity. This was followed by the addition of other excipients in the following order:
ascorbic (0.1mg/ml; antioxidant), xylitol (200mg/ml; sweetener), parabens (propyl -0.1mg/ml, butyl – 0.06mg/ml; preservative) and finally orange flavour (0.1%v/v) (Table 1).

**Cyclodextrin approach to solubilising ramipril**

Cyclodextrin based liquid dosage forms were investigated as they provide dual advantage including enhancement of apparent drug solubility as well as taste masking through drug encapsulation.

**Phase Solubility**

Phase solubility profile of ramipril in the presence of various concentrations HP-β-CD was studied to identify the stoichiometry during drug-complex formation. The results showed that the phase solubility profile for ramipril complexed with HP-β-CD was that of the $A_L$ type (Figure 1) which demonstrates a linear relationship between the increase in drug solubilisation with the increase in cyclodextrin concentration. 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. Using the gradient from the equation for the line it can be estimated that there was a 1:1 molar binding ratio between ramipril and cyclodextrin molecules. In the current study, as the target dose of ramipril in 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).

Drug solubility following complexation with cyclodextrin has been show to vary upon modification of the pH of a system. This possibly occurs as a result of alteration to the complexation constant with solubility increases resulting in an increase in complexation constant.(20) As shown in Figure 2, the optimum pH for the solubilisation of ramipril using HP-β-CD was 3 with both pH 5 and 7 showing reduced solubilisation. These differences in solubilisation at different pH values could possibly be due to the existence of either ionised or un ionised form of the drug. The concentration of ionised form of ramipril is higher at pH close to neutral and previous research has shown that the degree of complex formation for HP-β-
CD is lowered for ionised molecules when compared with their uncharged form. The negative charge present following ionisation of ramipril could result in repulsive forces between cyclodextrin and drug molecules and between drug molecules leading to a reduction in complexation.

Following the evaluation of drug-cyclodextrin molar ratio and the impact of pH on the resultant complex, the production of the formulation consisted of the preparation of cyclodextrin solution by dissolving 3.51mg/mL of HP-β-CD in distilled water. The next stage included the addition of 1mg/ml ramipril under stirring until it was completely dissolved. Following this, the other excipients were added in the following order: ascorbic (0.1mg/ml; antioxidant), xylitol (200mg/ml; sweetener), parabens (propyl - 0.1mg/ml, butyl – 0.06mg/ml; preservative) and finally orange flavour (0.1%v/v) (Table 1). The pH of the final formulation was pH 3.75.

Suspension formulation development

The development of a suspension requires selection and optimisation of a suitable concentration of suspending agent along with the assessment of sedimentation rate and re-suspendibility of the formulation.

Suspension Rheology, rate and volume of sedimentation

The first sets of investigations included determination of flow properties of the suspending medium using shear rate response to changes in shear stress which was evaluated using a viscometer. Five different suspensions were produced using different
concentrations of Xantural® ranging between 0.1-0.5%w/v (recommended concentration range as suspending agent) and analysed at five different shear stresses at 20°C. The results (Figure 3) showed that Xantural® 180 displayed pseudoplastic properties which provide desirable flow properties with the largest change in apparent viscosity being observed for the highest concentration of Xantural® (0.5% v/v). These studies were followed up by assessing the theoretical rate of sedimentation for ramipril in Xantural® using Stokes’ equation and experiments to confirm the rate and volume of sedimentation. Suspensions with the different concentrations of Xantural® were prepared and inspected at various time points over a four week period. The results showed (data not included) that there was no sedimentation observed at any of the time points for the entire range of concentrations investigated and the zeta potential was within +/-25mV range. Similarly, particles in a suspension can also give rise to ostwald ripening or crystal growth. 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. Particle size measurements were performed after heating and cooling cycles to investigate Ostwald ripening. The findings from these studies (data not included) showed that there was no increase in particle size when subjected to temperature changes between 4 and 40°C.

The final composition and process for preparing ramipril suspensions included production of 0.1%w/v Xantural® 180 solution, into which 1mg/ml ramipril was then added slowly with vigorous stirring to ensure it is evenly distributed throughout the vehicle. This stage was followed by the addition of sodium meta bisulphate (10mg/ml; antioxidant), xylitol (400mg/ml; sweetener), sodium benzoate (5mg/ml; preservative) and strawberry flavour (0.2%v/v). The pH of the final formulation was 4.25.

For all the optimised dosage forms, it was inevitable to include lowest possible concentration of preservatives as the primary vehicle is water and supports microbial growth. Taste assessment studies including indirect methods to evaluate taste such as drug release and dissolution testing (which are generally used for taste masked drug and require validation
through direct taste assessment studies) have not been included as there are no taste related issues reported for ramipril. Indeed the inclusion of sweeteners and flavours in all the different dosage forms as well the formulation of drug complexed cyclodextrin liquid will ensure patient acceptability and compliance without any concerns for palatability. Following formulation profiling together with investigation of optimal process and excipient parameters that meet the TPPP, the next stage of evaluation consisted of stability testing and subsequent drug absorption and genomic analysis.

**Stability Analysis**

Over the course of six month for stability testing at accelerated conditions at 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. pH remained constant for the duration of the stability testing in accelerated conditions. At long term conditions at 25°C and 60% relative humidity, drug content for all of the formulations remained >95% of the starting dose and pH remained constant for the duration of the 12 month testing period. Additionally, all formulations maintained their aesthetics. Suspension formulations did not display any sedimentation throughout the study suggesting that the inherent limitations of physical instability experienced by many suspension based formulations was successfully overcome and that sedimentation was totally prevented.

Degradation seen under accelerated conditions is possibly as a result of the elevated temperature. Ramipril diketopiperazine (DKP) degradate is the primary degradation compound for ramipril. Degradation occurs through intramolecular condensation with the formation of a second amide bond.(22) The pH of the formulation in both accelerated and long term conditions did not change beyond the acceptance levels for the formulation. Additionally, the appearance and odour of the formulations remained acceptable for the duration of the stability testing in both long term and accelerated conditions. The results show that formulations do not require refrigeration but would require storage in a cool location (<25°C).
In Vitro and In Vivo Evaluation of Ramipril Formulations

Assessment of Absorption In Vitro

Permeability coefficient or apparent permeability of a molecule (P_{app}) is considered a reliable indicator for the expected in vivo drug absorption (Fraction Absorbed (fa)). It is generally accepted that completely absorbed drugs have P_{app} > 1 \times 10^{-6} \text{cm/s} (\text{LogP}_{app} > -6) and incompletely or poorly absorbed drugs have P_{app} < 1 \times 10^{-6} \text{cm/s} (\text{LogP}_{app} < -6).(23)

Following permeability experiments, samples were analysed via HPLC and the findings from which were plotted to reveal the percentage of drug that was transported from the apical to the basolateral compartment of the transwell as a factor of time. TEER values were measured before and after the permeability experiments for each transwell to ensure the integrity of the monolayer. In all cases the TEER remained above an acceptable level of 350Ω cm².

Previous investigations into P_{app} reported permeability coefficients ranging from approximately 5 \times 10^{-8} to 5 \times 10^{-5} \text{cm/s}. Permeability coefficients for ramipril formulations in the current investigations were in keeping with these findings and demonstrated limited absorption of the drug for all the different formulations (Figure 5)(23).

Genomic Assessment of Caco-2 Following In Vitro Absorption Assay

Investigation into gene expression changes of Caco-2 cells following drug transport studies were carried out to identify link between the predicted drug permeability and the expression of the genes which code for the intestinal transporter of ramipril (SLC15A1 and SLC15A2).(5) 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 ramipril formulations.

The genes from the solute carrier transporters (SLC) superfamily for which a significant change in gene expression was seen were used in KEGG pathway identification. For the
ramipril solution produced using cyclodextrin as a solubiliser there was significant effect on 10 genes in the SLC family for which a >2 fold up regulation was observed. For the ramipril co solvent solution, there was significant effect on 26 genes in the SLC family. The ramipril suspension caused significant up regulation of 29 genes in the SLC family.

KEGG pathway analysis of the significantly up regulated SLC transporter genes returned pathway information for SLC16A10 which codes for TAT1. TAT1 is present in the basolateral membrane of intestinal epithelial cells and transports neutral amino acids into and out of the blood.(24,25) SLC16A10 was seen to be significantly up regulated for all of the ramipril formulations. There were significant expressional changes for numerous SLC transporters in the protein absorption pathway across the intestinal epithelial but not SLC15A1, the transporter for which ramipril is a substrate. 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.(26,27) The pathways and proteins, for which the SLC genes displayed significant modification in gene expression could be linked to ramipril absorption as TAT1 similar to PETP1 is involved in protein absorption pathways. 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. This lends strength to the recently developing ideology that excipients potentially possess biological activity and are not inactive ingredients. (28,29)

Assessment of Absorption In Vivo

The plasma concentration-time profile for the formulations for in vivo absorption is shown in Figure 5. From the plasma concentration-time profile, values for area under the curve (AUC), CMAX and TMAX were determined and are shown in Table 2. Ramipril AUC values were within the anticipated range as its bioavailability is known to be highly variable between individuals with reports showing variation ranging between 6-60%. (4) Ramipril showed a CMAX of 10.48 µg/mL for the solution produced using co-solvent, 13.04µg/ml for the ramipril
suspension and 29.58µg/mL for the ramipril solution produced using cyclodextrin as a solubiliser.

The findings for $C_{\text{MAX}}$ and $T_{\text{MAX}}$ reveal interesting findings; ramipril solution produced using co-solvent and the ramipril suspension showed $T_{\text{MAX}}$ at 2.5h after administration. However, ramipril solution produced using cyclodextrin showed a much earlier $T_{\text{MAX}}$ 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 $T_{\text{MAX}}$ and higher $C_{\text{MAX}}$. There is evidence that HP-β-CD decreases GI transit time in rats thus shortening the time taken for the drug to reach the site of absorption.(30) 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. 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.

**Conclusions**

Oral liquid ramipril formulations targeting the paediatric population were produced addressing the needs outlined in EMA/480197/2010. The current study has outlined for the first time the different oral liquid formulations of ramipril that can be produced without the need for inclusion of high concentration of excipients that are not recommended for use in paediatric formulations. The results provide the formulator with the option of developing either a solution or suspension based formulation which has a shelf life of twelve months. Biopharmaceutical
evaluation of the formulated products has shown that they exhibit similar permeability as well as pharmacokinetic profile as reported in clinical literature. Results from these investigations provide the platform to formulate better medicines for children using simple, cost effective and scalable processing parameters.

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Figure 1 - Phase solubility profile for Ramipril complexed with HP-β-CD. Six concentrations of cyclodextrin were produced in distilled water from 1.2mM to 3.75µM using serial dilution. To these excess Ramipril was added. Solutions were left stirring for 1 hour before samples were filtered and prepared for HPLC analysis (n=3).
Six concentrations of cyclodextrin were produced in distilled water from 1.2mM to 3.75µM using serial dilution. These were produced in solutions of pH 3, 5 and 7 and to these excess Ramipril was added. Solutions were left stirring for 1 hour before samples were filtered and prepared for HPLC analysis (n=3).

Figure 2 - Effect of pH on Ramipril solubility in the presence of HP-β-CD. Six concentrations of cyclodextrin were produced in distilled water from 1.2mM to 3.75µM using serial dilution. These were produced in solutions of pH 3, 5 and 7 and to these excess Ramipril was added. Solutions were left stirring for 1 hour before samples were filtered and prepared for HPLC analysis (n=3).
Figure 3 - Suspension viscosity under variable shear stress in Ramipril suspensions. Ramipril suspensions \((n=3)\) in concentrations of Xanatural from 0.1\%-0.5\%w/v were prepared and 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. 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.
Figure 4 - Apparent permeability values for Ramipril formulations. It is generally accepted that completely absorbed drugs have $P_{app} > 1 \times 10^{-6}\text{cm/s}$. ($\log P_{app} > -6$) and incompletely or poorly absorbed drugs have $P_{app} < 1 \times 10^{-6}\text{cm/s}$. ($\log P_{app} < -6$). Permeability coefficients for Ramipril formulations are in keeping with these values, Ramipril has limited absorption and this is supported with all of the Ramipril formulations displaying $\log P_{app} < -6\text{cm/s}$ ($n=3$).

Figure 5 - Plasma Concentrations Following Oral Administration ($n=3$). Ramipril showed a $C_{\text{max}}$ range of 10.48 $\mu\text{g/mL}$ for the Ramipril solution produced using a co-solvent, 13.04$\mu\text{g/mL}$ for the Ramipril Suspension and 29.58$\mu\text{g/mL}$ for the Ramipril solution produced using cyclodextrin as a solubiliser. Ramipril solution produced using
a co-solvent and the Ramipril suspension of $T_{\text{MAX}}$ 2.5h. The Ramipril solution produced using cyclodextrin however shows a much earlier $T_{\text{MAX}}$ occurring only 0.75h following administration.