Profiling gene expression dynamics which underpin conventional testing to better inform pre-clinical evaluation of an age appropriate spironolactone formulation

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Profiling gene expression dynamics underpinning conventional testing approaches to better inform pre-clinical evaluation of an age appropriate spironolactone formulation.

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Profiling gene expression dynamics underpinning conventional testing approaches to better inform pre-clinical evaluation of an age appropriate spironolactone formulation.

Abstract

There is a need to accelerate paediatric formulation evaluation and enhance quality of early stage data in drug development to alleviate the information pinch point present between formulation development and clinical evaluation. This present work reports application of DNA microarrays as a high throughput screening tool identifying markers for prediction of bioavailability and formulation driven physiological responses. With a focus on enhancing paediatric medicine provision an oral liquid spironolactone suspension was formulated addressing a paediatric target product profile. Caco-2 cells cultured on transwell inserts were implemented in transport assays in vitro and DNA microarrays were used to examine gene expression modulation. Wistar rats were used to derive in vivo bioavailability data. In vitro, genomic and in vivo data sets were concurrently evaluated linking drug transport and the genomic fingerprint generated by spironolactone formulation exposure. Significant changes in gene expression are reported ~~for ABC transporters, SLC transporters, CYP enzymes and carboxylesterase enzymes resulting from~~as a result of formulation exposure. These include genes coding for ATP-binding cassette (ABC) transporters, Solute carrier (SLC) transporters, Cytochrome P450 (CYP) enzymes and carboxylesterase enzymes. Genomic findings better inform pre-clinical understanding of pharmacokinetic and pharmacodynamic responses to spironolactone and its active metabolites than current in vitro drug transport assays alone.

Key Words: - Microarray, Paediatrics, ABC transporters, SLC transporters, CYP enzymes, carboxylesterase enzymes.

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1. Introduction

The lack of suitable formulations in the paediatric patient segment is attributed largely to the dynamic physiological state, which is both vastly different to that of adults and changes significantly with age. As a result, the needs of the paediatric patient segment vary greatly to the needs of adults and represent a far more sensitive challenge [1]. Dosage form, route of administration, overall dose of active pharmaceutical ingredient (API) and excipients need careful consideration when producing an age appropriate dosage form. To aid researchers in this area there are a number of regulations and items of legislation in both Europe and the United States of America designed to promote development in this area and deliver appropriate medicines for children. These include the Best Pharmaceuticals for Children Act (BPCA) and the Paediatric Equity Act (PREA) in the USA and EU paediatric Regulation; EC No. 1901/2006; EC No.1902/2006 in Europe [2,3]. The latter has been in place since 2007 and applies to both new and off-patent medicinal products. Currently, pharmaceutical companies seeking marketing authorisation in Europe for products which are covered by intellectual property (IP) rights are required to submit a Paediatric Investigation Plan (PIP) to the Paediatric committee (PDCO) of the European Medicines Evaluation Agency (EMA). The PIP outlines a research plan for investigations in children targeting generation of sufficient data to permit marketing authorisation. This includes pre-clinical studies, clinical studies and quality studies describing formulation development. This is optional for off-patent products, however engaging with the PIP process when reformulating off-patent medicines to provide age appropriate formulations permits applications for paediatric use marketing authorisation (PUMA) [3-5]. This carries with it the benefit of 10 years of data protection (eight years of data exclusivity and two further years of market protection).

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Despite this incentive there has been poor success rates with only 4 PUMA's having been awarded since the inception of the scheme. This has been attributed to a number of factors, which hinder recruitment into clinical trials including current approaches to pre-clinical formulation evaluation [6].

Specifically, PIP research plans for investigations in children require data from pre-clinical studies including the prediction of pharmacokinetic responses in vivo. Current practice for the assessment of drug permeability includes human colon adenocarcinoma (Caco-2) cells cultured on transwell inserts or equivalent as it delivers many elements of the in vivo physiological equivalent such as microvilli, enzymatic activity and membrane transporter networks [7-9]. The carrier transporters in the intestine have relatively promiscuous specificity and are involved in the uptake and efflux of range of drug molecules. The genetic basis of these biological components and the dynamic nature of such networks means that microarray can be deployed for high throughput, cost effective screening to generate enriched large-scale data sets describing the physiological system.

This genome wide expression evaluation allows for a broad, network view of how a given molecule can impact cellular pathways and their interaction [10-13]. This provides a far more complete picture of what is happening in any given system and when combined with open access databases such as the Koyoto Encyclopaedia of Genes and Genomes (KEGG or other equivalents), delivers a true and informative systems biology based approach [14].

~~The aim of the present work was to investigate the application of DNA microarrays to identify markers suitable for the prediction of intestinal absorption and formulation effect to better inform drug development. This was investigated through the evaluation of an oral liquid spironolactone suspension which had been formulated in house to address the needs of the paediatric patient segment. Caco-2 cells cultured on transwell inserts were used for drug~~

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~~transport assay to model intestinal permeability in vitro and DNA microarrays were employed to examine gene expression changes occurring during drug transport. Wistar rats were used as a rodent model to derive in vivo bioavailability data for comparison. In vitro, genomic and in vivo data sets were evaluated to link drug transport and the gene expression changes generated by spironolactone as an approach to accelerate formulation evaluation and enhance the quality of early stage data generated in the drug development pipeline.~~

Spironolactone is a poorly soluble potassium sparing diuretic which acts as a receptor antagonist for the mineralocorticoid aldosterone. It is used clinically in adults for the treatment of a number of conditions including heart failure and hypertension [15]. However in paediatrics its licensed use is limited to the treatment of oedema in heart failure and in ascites, nephrotic syndrome and reduction of hypokalemia induced by diuretics or amphotericin [16]. Administration is via the oral route with dose regimes ranging from 1-2 mg/kg daily in 1 to 2 divided doses, up to a maximum of 7 mg/kg daily for neonates. 1-3 mg/kg daily in 1 to 2 divided doses up to 9 mg/kg for children aged from 1 month to 11 years and 50-100 mg daily in 1 to 2 divided doses, up to a maximum of 9 mg/kg daily in children aged between 12 – 17 years [16]. Currently, 25, 50 and 100 mg branded (Aldactone®, Pfizer) tablets and generic equivalents are the only licensed dosage forms. This limits use in paediatrics, particularly neonates as solid dosage forms are unsuitable for administration in neonates and young children and do not afford the dose flexibility required to effectively cover dose requirements in paediatric patients ranging from neonates through to teenagers.

Spironolactone has a solubility of 22 µg/ml in water whereas the desired dosages for a liquid spironolactone formulation are 10 mg/ml and 5 mg/ml. Therefore, to produce a clinically relevant formulation, the solubility of spironolactone would need to be increased 227-fold. Additionally, Spironolactone has been shown to be degraded by cyclodextrins meaning that this common method of solubilising poorly soluble drugs cannot be used. As such and with

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an onus on simplicity of formulation and limiting excipient use with paediatric application in mind, complex methods of solubilising the insoluble drug were rejected in favor of producing a suspension.

The aim of the present work was to investigate the application of DNA microarrays to identify markers suitable for the prediction of intestinal absorption and formulation effect to better inform drug development. This was investigated through the evaluation of an oral liquid spironolactone suspension which had been formulated in house to address the needs of the paediatric patient segment. Caco-2 cells cultured on transwell inserts were used for drug transport assay to ~~model~~evaluate intestinal permeability in vitro and DNA microarrays were employed to examine gene expression changes occurring during drug transport. Wistar rats were used as a rodent model to derive in vivo bioavailability data for comparison. In vitro, genomic and in vivo data sets were evaluated to link drug transport and the gene expression changes generated by spironolactone as an approach to accelerate formulation evaluation and enhance the quality of early stage data generated in the drug development pipeline.

2. Materials and Methods

2.1 Materials

Spironolactone was supplied by Discovery fine chemicals, UK. Xanatural, Pluronic F127, Sodium Metabisulphate, Sodium Benzoate and Xylitol were supplied by Sigma Aldrich U.K. with the exception of flavour concentrates which were samples provided by Azelis. Acetonitrile was supplied by Fisher Scientific U.K. Caco-2 cells (passage 45) were kind gift from Dr Andrew Collett and Dr Daniel Patten at the University of Huddersfield.

Polycarbonate transwell permeability supports in 6 well format ~~Permeability supports~~ were purchased from Appleton Woods Ltd, U.K. and all tissue culture media components

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including DMEM, Glutamate, Penstrep, Non-essential amino acids, FBS and HBSS were supplied by Sigma Aldrich UK. RNeasy kits for RNA extraction were supplied by Qiagen, U.K. One-colour microarray-based gene expression analysis low input quick amp labelling kit were purchased from Agilent Technologies. Agilent 4 x 44K whole genome arrays were used for microarray experimentation (Agilent Technologies, Santa Clara, CA)

2.2 Formulation development

An oral spironolactone suspension suitable for the paediatric patient segment was developed in house at dosages of 5 mg/ml and 10 mg/ml meeting the following target product profile (Table 1).

~~Table 1 – Target product profile for spironolactone suspension detailing target components for each quality attribute.~~

~~Quality Attribute~~	~~Target~~
~~Route of Administration~~	~~Oral~~
~~Dosage form~~	~~Acceptable for patients aged from birth to <18 years~~
~~Dose Range~~	~~1-2mg/kg daily in 1 to 2 divided doses, up to a maximum of 7mg/kg daily for neonates. 1-3mg/kg daily in 1 to 2 divided doses up to 9mg/kg for children aged from 1 month to 11 years and 50-100mg daily in 1 to 2 divided doses, up to a maximum of 9mg/kg daily in children aged from 12 – 17 years. Includes dose titration.~~

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~~Pharmacokinetics~~	~~Immediate Release~~
~~Palatability~~	~~Neutral/Flavored/Sweetened preferred~~
~~Shelf life~~	~~Minimum of 12 Months~~
~~Container closure system~~	~~Multi-dose~~
~~Additional Information~~	~~All excipients must be acceptable for the paediatric patient population~~

2.3 Spironolactone HPLC Method

A Dionex GP50 gradient pump coupled to a Dionex UVD170U detector and a Dionex A550 auto sampler were combined with a Phenomenex Gemini 5 µm C18 reverse phase HPLC column (150 x 4.5 mm with 5 µm Particle Size). Mobile phase was acetonitrile and water (50:50) and the detection wavelength was set at 254 nm. The injection volume was 50 µl with a run time of 12 minutes and retention time of 8 minutes. Preparation of calibration standards involved production of six standards via serial dilution in mobile phase. Standards ranged from 0.0625 to 0.5 µg/ml and included a blank. Method validation was carried out following ICH Guidelines (Q2(R1)) [17].

2.4 Assessment of Absorption in Vitro

2.4.1 Culture of Caco-2 Cells for permeability assay

Caco-2 cells (passage 48) were seeded at a density of 1.3 x 105 cells/cm^2 onto polycarbonate transwell permeability supports in 6 well format. Cells were cultured in an incubator (Sanyo) at 37 °C in a humidified 5% CO_2/95% 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

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EVOM – Epithelial Voltohmmeter (World Precision Instruments Ltd). Monolayer integrity was confirmed with TEER values greater than 350 Ωcm^2.

2.4.2 Trans-epithelial flux of spironolactone across Caco-2 monolayers

Culture media was removed, and the monolayers washed with HBSS (pH range 6.7 - 7.8) before the monolayers were incubated at 37 °C for 30 minutes with 2.5 ml of HBSS basolaterally and 1.5 ml apically. In each case, HBSS was then replaced in the apical compartment with 1.5 ml of the optimised formulation to be tested. Experiments using cells which were not exposed to the drug were used as controls. 200 µl samples were then taken from the basolateral compartment at time points of 5, 10, 15, 20, 30, 60, 90, and 120 minutes. 200 µl of HBSS was added ~~in each case~~back into each well to maintain volume of solution in the basolateral compartment. This was accounted for in calculations. Samples were analysed via HPLC. Method validity has been confirmed ~~in line~~ with ICH M9 guidance and all experiments were performed in triplicate. The level of transport is described as percentage of drug arriving in the basolateral compartment, and from this, P_{app} was calculated.

Following the production of a transport/time graph Papp was calculated using the following equation [18];

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

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).

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

2.5 Genomic Evaluation

2.5.1 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).

2.5.2 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, 1.5 µl of 50 ng total RNA was mixed with 2 µl 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.

All samples were then hybridised using Agilent 4 x 44K 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.

Slide scanning was carried out using an Agilent Scanner (Agilent Technologies, Santa Clara, CA) ran 20-bit scans at a resolution of 50 n.

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2.5.3 Microarray Slide Scanning Validation TIFF Image analysis

Spot centroids located in the corner of each array were used to confirm quality and position. Feature extraction software assessed the signal quality of the array determined by the normal distribution of the data. The Log values of the processed signal were confirmed to show good linearity with the Log values of the corresponding concentrations of SpikeIns for all arrays used. 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 minimal variation. Multiple reference probes across the array confirm reproducibility across the length and breadth of the array where a low median coefficient of variation in the signal level from these probes confirmed. Following TIFF image verification following microarray scanning, data normalisation was performed prior to analysis.

2.5.4 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. These images were assessed on grid alignment, signal quantification and overall slide quality.

2.5.5 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

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down regulation in gene expression. Delta values were selected so as that the median false discovery rate (FDR) was lower than 2%. 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/. Pathway analysis was undertaken using KEGG Pathway and Orthology features.

2.6 Assessment of Absorption in Vivo.

All animal experiments complied with the ARRIVE guidelines and experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated facility under the project license number PPL 30/2743. Male Wistar rats with a body weight of 250g – 300 g 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 and formulations were diluted in H_2O, Spironolactone was dosed at 40 mg/kg and 20 mg/kg depending on formulation. Following dosing, an additional 1 ml of H_2O was administered to rinse in the formulations. Blood samples (45 µl) were taken via tail bleeds at 15 minutes, 30 minutes, 45 minutes and, 1 hour, 1.5 hour, 2 hour, 2.5 hour, 3 hour and 4 hour time points. Plasma was extracted from blood samples by centrifugation at 2800 x g for 10 minutes. The plasma samples were then diluted 1:5 in mobile phase and analysed using HPLC. Test/reverence ratios were calculated using data published for spironolactone bioequivalence studies to allow for comparison of mean plasma pharmacokinetic parameters without the need to use an intravenous reference standard.

3. Results and Discussion

3.1 Formulation development and characterisation

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The first stage of developing an age appropriate formulation of Spironolactone commenced with scoping a target product profile comprising of small dose volume, long term stability, flexibility for dose manipulation and an acceptable formulation presentation. Spironolactone is most stable at pH 4.5 [19] and as such the formulation required pH control so as to maintain optimum conditions for drug stability and maximize shelf life. Experimental investigation into vehicle buffer production revealed that the ratio of 0.1 M citric acid to 0.1 M trisodium citrate resulted in solvent vehicle with pH 4.5. Following this, Xantural 180 and Pluronic F127 were selected as viscosity modifier and surfactant respectively and investigated across concentration ranges of 0-5% w/v and 0-0.5% w/v respectively in order to develop a vehicle offering optimized physical stability to the formulation. Pluronic F127 is known to undergo transition from a solution to a gel at the sol-gel transition temperature which would be undesirable in this formulation. Although this normally occurs at concentrations greater than 5% w/v, the effect of Xanatural 180 on this is unknown. All concentration of Pluronic and Xanatural were investigated at temperatures up to 55 °C. No evidence of Sol-Gel was observed for any of the vehicle compositions tested. Evaluation of formulation sedimentation in different concentrations of Pluronic and Xanatural showed that inclusion of Pluronic was necessary however increasing the concentration above 1% to 2, 3, 4 or 5% had no impact on rate and volume of sedimentation. Conversely, increasing Xanatural 180 concentration up to 0.4% gradually reduced the amount of sedimentation until at 0.4% w/v, whereby no sedimentation was noticed. Table 2 summarises the final spironolactone suspension formulation components.

~~Table 2 – Oral spironolactone suspension composition – Spironolactone formulations were produced at doses of 5mg/ml and 10mg/ml.~~

~~Spironolactone formulation components~~	
~~Spironolactone~~	~~API~~

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~~Xanatural 180~~	~~Viscosity Modifier~~
~~Pluronic F127~~	~~Surfactant~~
~~Sodium Metabisulphate~~	~~Antioxidant~~
~~Sodium Benzoate~~	~~Preservative~~
~~Xylitol~~	~~Sweetener~~
~~Strawberry Flavour~~	~~Flavouring agent~~

Formulations were assessed for stability at accelerated and long-term conditions for storage as set out in the ICH Harmonisation Guidelines (Q1A(R2)) [17]. Over the six month course of the stability testing in accelerated conditions ~~with~~at 40 °C and 75% relative humidity, the drug content of the formulations remained above 95% of the starting dose indicating that the formulations displayed adequate stability in accordance with ICH guidelines and the pH remained constant for the duration of the stability testing (Figure 1). Similar results were seen for 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 2). The results show formulations were physically and chemically stable over 12 months with drug content and pH within acceptable limits.

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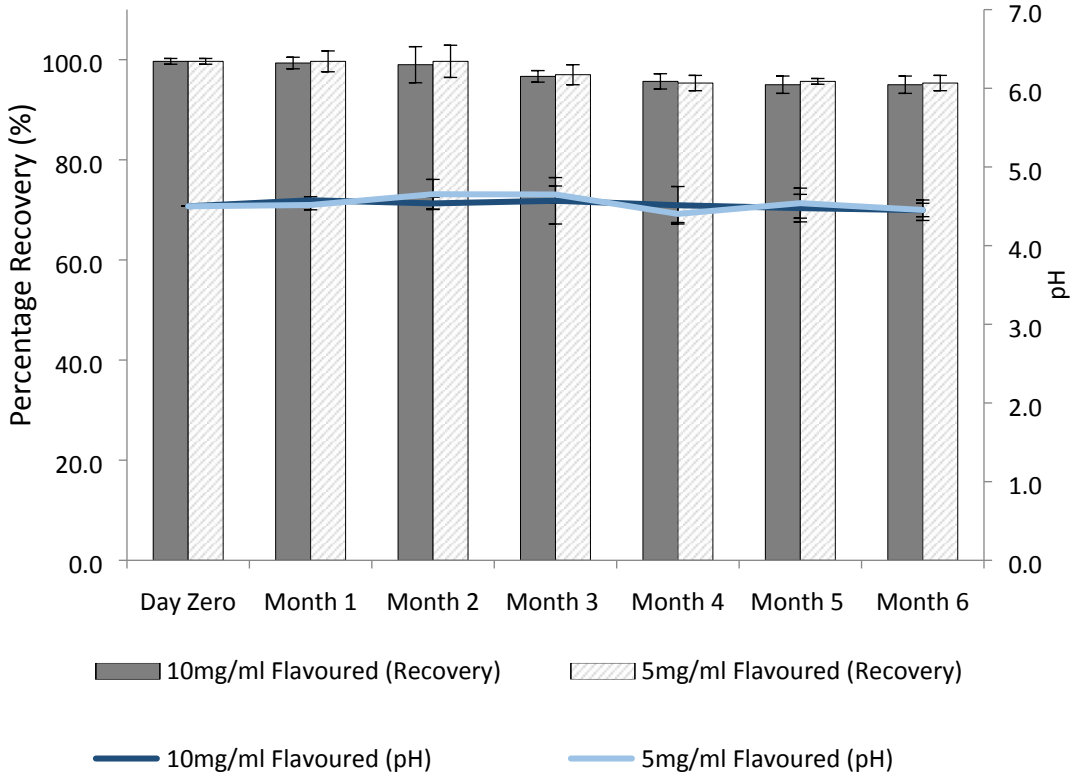

~~Figure 1 - Spironolactone Stability in Accelerated Conditions - Spironolactone recovery following HPLC for formulations in accelerated storage conditions is illustrated by the vertical bars. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD). Spironolactone pH stability for formulations in accelerated storage conditions is represented by the horizontal lines. 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).~~

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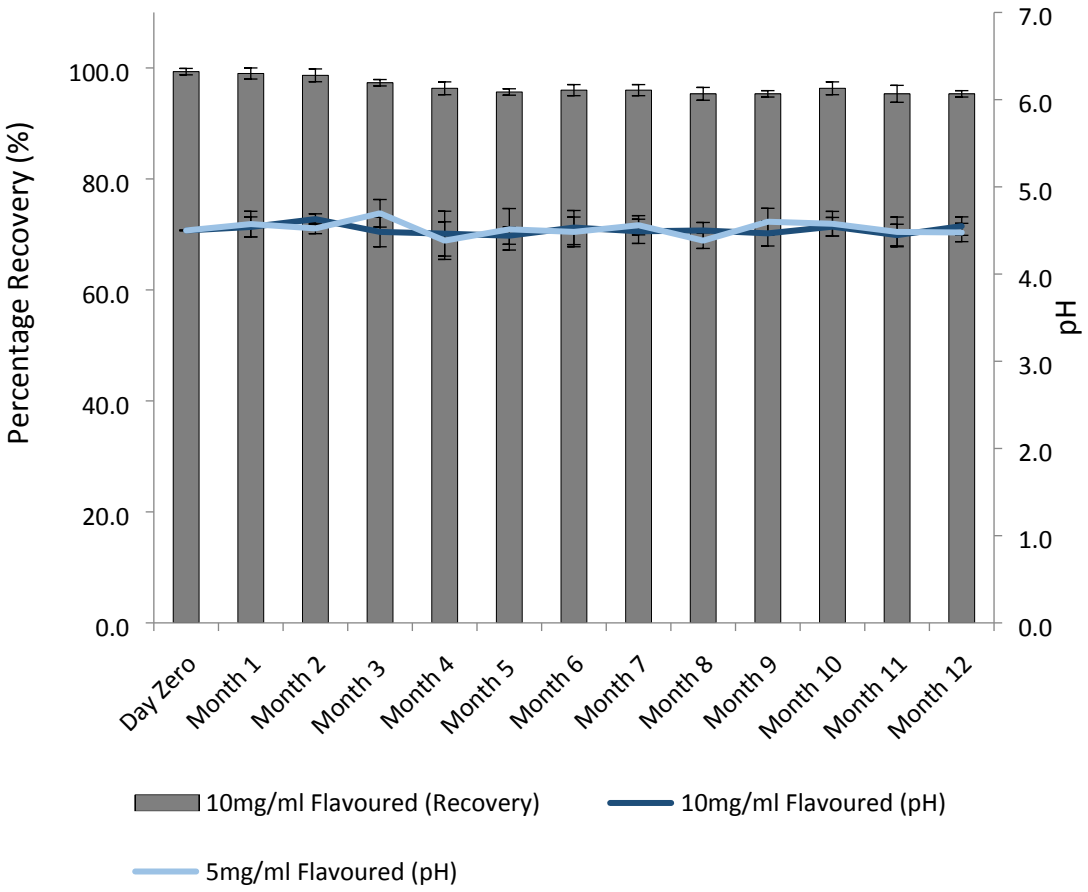


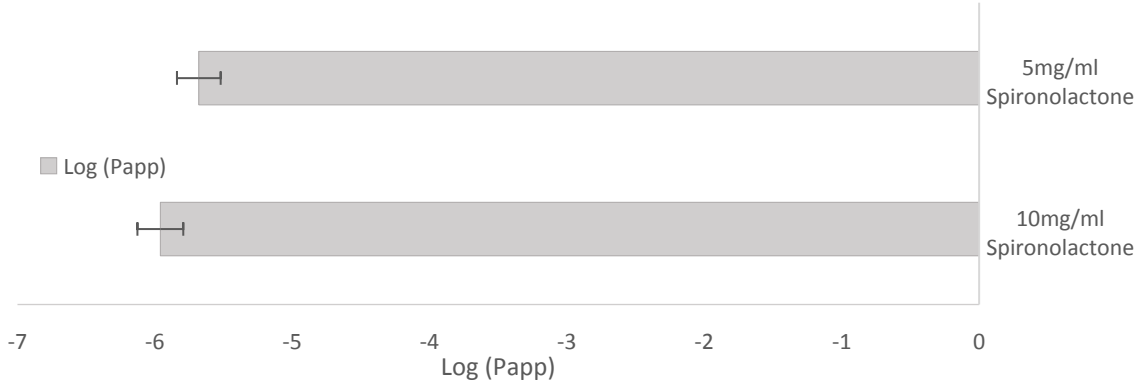
~~Figure 2 – Spironolactone Stability in Long Term Conditions - Spironolactone recovery following HPLC for formulations in long term storage conditions is illustrated by the vertical bars. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD). Spironolactone pH stability for formulations in long term storage conditions is represented by the horizontal lines. 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).~~

3.2 Assessment of Absorption in Vitro

The next phase of investigation involved, in vitro formulation evaluation (10 mg/ml and 5 mg/ml) using Caco-2 cells for drug transport studies, followed by genome fingerprinting to evaluate dynamic gene expression responses to formulation exposure.

~~Caco-2 cells were grown on transwell inserts for a minimum of 21 days to allow for full differentiation and monolayer integrity was confirmed through trans-epithelial electrical resistance (TEER). This was measured before, during and after permeability experiments. At appropriate time points during permeability experiments, samples were taken from the basolateral chamber and analysed via HPLC.~~

The results from transport vs time analysis wereas used to calculate apparent permeability, $P_{app,}$ for both the formulations. The average log value in the current study was -5.81 (Figure 3). P_{app}, from previous investigations ranged from $5x10^{-8}$ to $5x10^{-5}$ [7,12]. The permeability coefficients for the 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. (LogPapp >-6) whereas incompletely or poorly absorbed drugs have Papp $<1x10^{-6}$ cm/s. (LogPapp <-6).



~~Figure 3 - Apparent permeability of spironolactone in oral liquid suspension formulations – Average Log P_{app} values calculated for spironolactone formulations range from -5.96 to -5.70 with an average of -5.81. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).~~

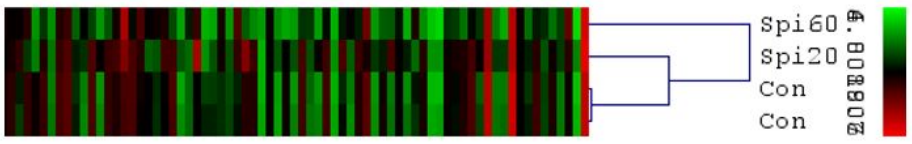
3.3 Genomic Evaluation of Caco-2 cells following drug transport experiments

Investigation into gene expression changes of Caco-2 cells during drug transport studies were intended to identify links between the predicted drug permeability and the expression of the genes which code for the intestinal transporters of spironolactone [20-22]. The aim was to study the response of transporter super families such as ABC and SLC, cytochrome P450 (CYP) and carboxylesterase (CES) enzymes. ~~Drug transport studies were carried out using transwell arrangements and the genetic profiles of the subsequently harvested Caco-2 cells were examined. Cells were harvested at 20 minutes and 60 minutes following initiation of the drug transport experiments. The expression patterns for Caco-2 cells in their basal state were used as a control and compared to cells exposed to the spironolactone formulation.~~

~~Following successful RNA extraction, labelling and amplification, samples were hybridised onto microarrays and processed to identify gene expression changes. Data normalisation was undertaken before statistical analysis of findings was performed.~~

3.3.2 Hierarchical clustering algorithm (HCA)

HCA was used to cluster samples into groups based on the similarity in gene expression profiles. Control samples were compared to formulation samples at each time point (20, 60 min) (Figure 4). The HCA clusters showed that gene expression levels in Caco-2 cells which had undergone treatment with the drug formulations had been altered from that of the control samples where spironolactone formulation was absent. In addition to this, time was indicated as a factor in the magnitude of this change as greater difference was seen for cell samples taken at 60 minutes compared to 20 minutes.



~~Figure 4 - Hierarchical clustering algorithm (HCA) – Gene expression data for control samples was compared to gene expression data for samples exposed to~~

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formulations at each time point using HCA analysis. Clustering analysis groups similar data sets and as shown above, there is significant difference indicated between the control gene expression data and the expression data seen at each time point.

3.3.3 Principal Component Analysis (PCA)

Eigenvector decomposition (EVD) generated 4 principal components for PCA of samples relating to the spironolactone suspension of which 88.989<u>9.99</u>% of the variance was described in components 1 and 2 (Table 3). Components 3 and 4 were therefore discounted to focus data analysis.

Table 3 – Eigenvector decomposition (EVD) - EVD 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 %		

Following HCA, principal component analysis (PCA) plots were generated which showed that there was a clear difference in the gene expression levels between the control and treated samples; control samples clustered centrally compared to spironolactone formulation at time

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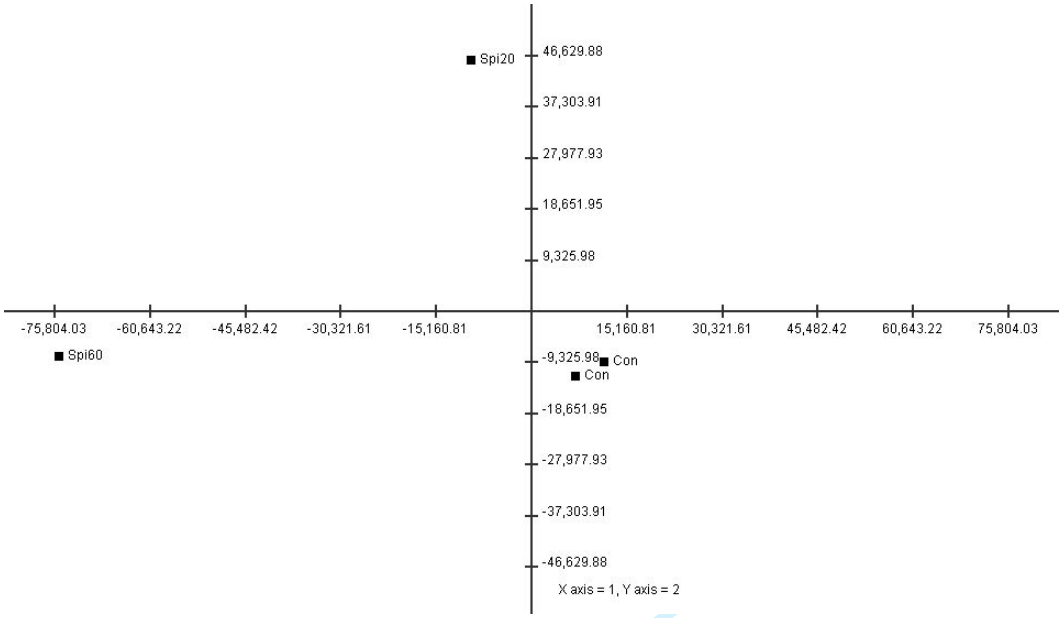
points 20 ~~minutes~~ and 60 minutes (Figure 5). This difference was seen along component one and two of the first and second PCA for the suspension. The differences between the time points indicated greater change in the expression levels after 60 minutes across the first component (X-axis) when compared to the change after 20 minutes. This further supports the trends identified by HCA analysis.



~~Figure 5 – 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.~~

3.3.4 Significance Analysis of Microarrays (SAM)

With definitive differences confirmed for the sample profiles, SAM was carried out on the microarray data to identify specific genes which showed a significant change in their level of expression following transport experiments using spironolactone formulations. Genes with a fold difference >2 in the SLC, ABC, CYP and CES families were tabulated and positive or negative change in expression indicated. For the spironolactone suspensions, the significant gene expression information generated from the SAM analysis is presented in Figure 6. A total of 2576 significantly upregulated genes (positive change) and 1235 significantly

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downregulated genes (negative change) were identified at a FDR rate of 1.5 % and delta value of 0.35.

SAM Cluster Information - Gene Expression

~~Figure 6 - SAM Cluster Information - Gene Expression - A total of 2576 upregulated (positively significant) genes and 1235 downregulated (negatively significant) genes were identified, 37282 genes were not significant.~~

For the spironolactone suspensions there was significant effect on 9 genes in the ABC family, 71 genes in the SLC family, 51 genes in the CYP family and 5 genes in the CES with a fold change >2. In each instance it was interesting to note that all of these genes were up regulated. Following the production of the SLC, ABC and CYP gene tables the gene names were entered into KEGG to identify the pathways affected by the changes in gene expression.

3.3.5 KEGG Pathway analysis

The genes from the SLC, ABC, CYP and CES gene families for which a significant change in gene expression was seen were listed and KEGG pathway identification performed to identify the role of each (comprehensive gene lists exist as supplementary material). Where no pathway information was available genes are omitted from the tables. Pathway information was available for 91 of the 136 genes identified.

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When considering permeability through the intestinal epithelial it is important to consider the inherent properties which limit absorption. This includes the physiochemical makeup of the cell membranes as well as the tight junctions between the cells which are tightly regulated and highly selective. For instance, SLC9A3R1 is of interest when examining intestinal permeability as it codes for SLC9A3 Regulator 1 (NHERF1). This protein interacts with villin and actin which function as linkers between integral membrane and cytoskeletal proteins involved with the formation and maintenance of tight junctions [23]. Tight junctions exist between intestinal enterocytes and are one of the key limiters in modulating paracellular intestinal permeability and maintaining membrane barrier function. Although spironolactone is not reported to be absorbed paracellularly this may indicate that tight junctions were closed in response to exposure to the spironolactone formulation. Evidence of cellular response to formulation exposure can also be seen through expression changes in genes involved in signalling pathways including SLC25A6, SLC3A2, SLC7A5, SLC27A1 and SLC2A2. cGMP-PKG signalling has been shown previously to control dynamic responses of tight junctions in the blood brain barrier (BBB) through voltage-dependent anion channel protein 1 which is coded for by SLC25A6 [24]. The tight junctions that form the paracellular barrier at the BBB and intestinal enterocytes display remarkable molecular similarities [25].

Beyond the barrier function of the intestine, efflux transporters and enzymatic activity present an additional barrier to drug absorption and their combined action can limit bioavailability [26]. Spironolactone has been shown to interact with ABCB1, multidrug resistance protein 1 (MDR) or P-glycoprotein (P-gp) which is supported by findings of this study with a 8.75 fold increase in expression levels [21]. However, this observation suggests spironolactone co-administration requires clinical monitoring for drugs prone to efflux which may have deleterious clinical impact on their bioavailability [21].

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Spironolactone is rapidly metabolised following administration via carboxylesterases which are known to be expressed in Caco-2 cells and their expression has been shown to be upregulated in this investigation with carboxylesterase 1 (hCE-1) showing a 9.26 fold increase in expression and carboxylesterase 2 (hCE-2) showing a 7.10 fold increase in expression [27]. Carboxylesterase metabolism of spironolactone produces a number of active metabolites including 7α-thiomethylspironolactone (7α-TMS), 6β-hydroxy-7α-thiomethylspironolactone (6β-OH-7α-TMS), and canrenone. Of these, 7α-TMS and 6β-OH-7α-TMS are known to be substrates for CYP3A4 and likely responsible for gene regulation changes (CYP3A4 is upregulated 7.07 fold). CYP3A4 is the most prominent oxidative CYP enzyme present in the human intestine and has been shown to significantly metabolise orally administrated drugs and limit bioavailability [28].

With a focus on transport mediated absorption and intestinal permeability, there were a large proportion of upregulated genes from the SLC superfamily linked to absorption pathways. SLC1A5, SLC3A2, SLC1A1, SLC3A1, SLC6A19 and SLC7A7 are all active in protein absorption while SLC31A1, SLC11A2, SLC26A3, SLC5A6 and SLC6A19 are involved with mineral absorption. SLC2A2 and SLC5A6 were similarly identified as being significantly upregulated and these are functional in carbohydrate absorption and vitamin absorption pathways respectively [14]. Upregulated SLC3A2 codes for the chaperone protein CD98 which can heterodimerize with a number of amino acid transporters including LAT1, LAT2, y+LAT1, y+LAT2, and xCT and thereby influence a number of cellular functions including transport mechanisms. Of the transporters listed, increases in expression levels were seen for LAT1 (SLC7A5) and y+LAT1 (SLC7A7). CD98 dimerises with both, y+LAT1 (SLC7A7) can be found in the basolateral border of enterocytes to transfer cationic and large neutral amino acids from the cell to the extracellular space [29]. LAT1 (SLC7A5) dimerises with CD98 to form a functional unit for both the uptake of large neutral amino acids and a number

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of pharmaceutical drugs. Evidence generated by Amaral and Pinho suggests that tight binding of spironolactone to the cytosolic mineralocorticoid receptor in collecting duct principle cells drives transcriptional changes, these have been shown to modulate subsequent downstream changes in LAT1, LAT2 and ASCT2 expression [30]. Our findings indicate that this same pathway is also active in intestinal enterocytes and this argument is strengthened as the cytosolic mineralocorticoid receptor (NR3C2) for which the expression profile was extracted individually was found to be upregulated indicating both that it is present and that capacity for activity is increased following exposure of Caco-2 cells to spironolactone formulations [31].

Amino acid transporters have roles beyond drug transport and LAT1 is active in the mammalian target of rapamycin (mTOR) signalling pathway. This pathway is activated during various cellular processes including tumor formation and angiogenesis, insulin resistance, adipogenesis and T-lymphocyte activation and is deregulated in human diseases such as cancer and type 2 diabetes [14,32]. It is not possible to be definitive given the scope of this current study, however modulation of LAT1 gene expression as a result of spironolactone formulation exposure may provide early indication of long-term adverse effects resulting from mTOR pathway activation. Evidence in support of this is strengthened as SLC27A1 and SLC2A2 are seen to be upregulated and code for proteins involved in the Insulin resistance pathway. Likewise, SLC2A2 and ABCC8 are both upregulated and linked to Type II diabetes mellitus.

3.4 Assessment of Absorption in Vivo.

In vivo drug absorption using rodent models is used routinely in a pre-clinical setting to provide the most representative model of pharmacokinetics and pharmacodynamics. Wistar rats were used in this investigation and spironolactone formulations were loaded into a 1ml

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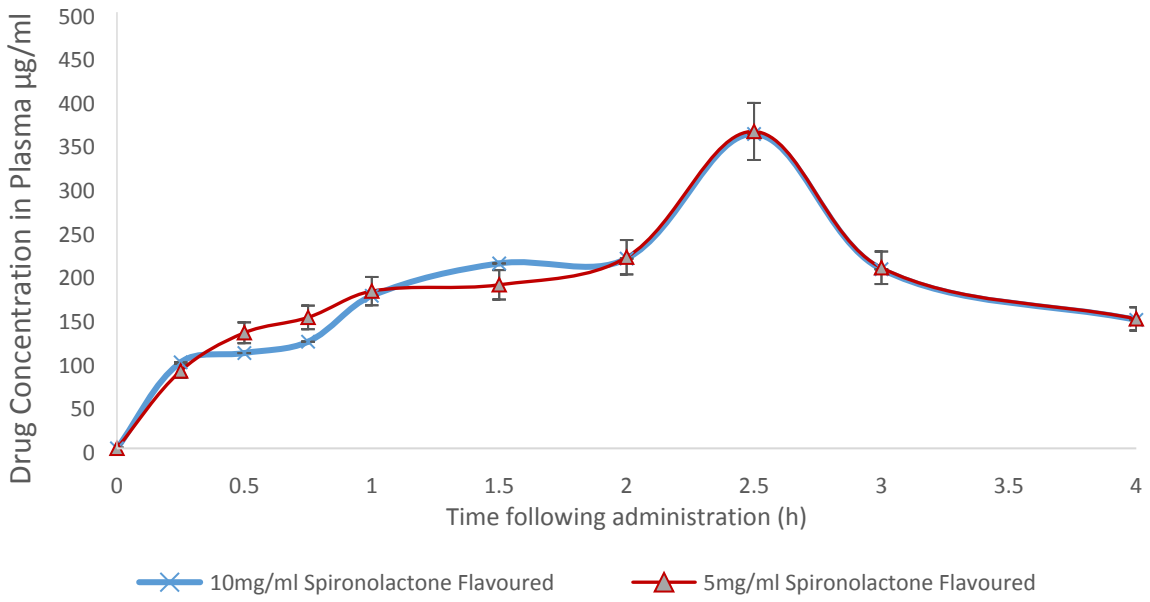
syringe prior to administration via oral gavage. Dosing was calculated depending upon the weight of each individual animal and blood samples were taken via tail bleeds prior to HPLC analysis. The plasma concentration-time profile for the formulations for *in vivo* absorption is shown in figure 7.



~~Figure 7 – *In vivo* plasma responses in rats following spironolactone administration – Plasma concentrations following oral administration. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).~~

The plasma concentration-time profile for all formulations was comparable and representative of expected results with T_{MAX} occurring 2.5hours after administration. From the plasma concentration-time profile, values for AUC, C_{MAX} and T_{MAX} were determined and are shown below in table 4. Values are in keeping with published bioequivalence studies examining spironolactone formulations [33]. AUC and C_{MAX} are within 5% of published values with test/reference (T/R) ratios ranging from 0.91-1.11 with a mean value of 1.02

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while T_{MAX} was located centrally within 1.5 hours to 4.5 hours. It can therefore be deduced that the spironolactone formulations resulted in 80-90% *in vivo* absorption.

Table 4 – *In vivo* results for AUC, C_{MAX} and T_{MAX} – AUC was identified to be 992.80µg*h/mL and 988.97µg*h/mL for the 10mg/ml and 5mg/ml formulations respectively. C_{max} was shown to range from 363.24µg/ml to 375.37µg/ml and T_{MAX} was at 2.5hours for all formulations.

Formulation	AUC (µg*h/mL)	C_{MAX} (µg/ml)	T_{MAX} (h)
Spironolactone Suspension (10mg/ml)	992.80	375.37	2.5
Spironolactone Suspension (5mg/ml)	988.97	363.24	2.5

Previously reported findings for spironolactone in animal models showed gastrointestinal absorption estimated to be 82% in rats, 62% in dogs and 103% in monkeys [34]. Our findings are in keeping with the values for reported rat models. In comparison, human absorption is estimated to be in the range of 70%-90% and a fed state is reported to enhance absorption [35]. Following a 100 mg dose administered to human subjects, plasma half-life of spironolactone is reported to be 1–2 h with time to C_{MAX} being 2–3.2 h. Maximum blood concentration is reported as 92–148 ng/mL with area under the concentration–time (0–24 h) curve 1430–1541 ng/mL per h and elimination half-life of 18–20 h [36,37]. These values represent data as would be expected based on predictions using *in vitro* and *in vivo* findings of this current study.

4. Conclusions

The purpose of this study was to evaluate an oral liquid spironolactone formulation to address the current need for an age appropriate oral liquid formulation. Microarray technology has been implemented to profile genome dynamics as a response to formulation exposure and examine the findings in comparison with conventional *in vitro* and *in vivo* screening.

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Shelf life of 12 months has been confirmed with stability testing following ICH guidelines. *In vitro* drug transport studies returned a Log Papp value of -5.81 indicating good *in vivo* absorption. Microarray based gene expression changes provided comprehensive assessment of drug-transporter/absorption interaction which in turn could be used as a high throughput method to develop generic formulations and with refinement, predict indicative *in vivo* performance of alternative dosage forms. This strategy will allow screening of multiple variations of formulations and provide sufficient confidence for subsequent pre-clinical and clinical testing.

5. Conflict of interest

The authors declare no conflict of interest

6. Funding

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7. Data Sharing

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

8. Acknowledgements

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Supplement - Fingerprinting genome dynamics for comparison with in vitro and in vivo formulation evaluation in the development of age appropriate dosage forms – Gene information tables

SLC transporter gene expression response following spironolactone formulation exposure - Gene identification, expression fold change and pathway identification is listed for all genes in the SLC transporter family for which a >2 fold change was seen and pathway information was available.

SLC Transporters

Transporter	Fold Change	+ve or -ve	KEGG Pathway Identification
SLC17A9	6.4354215	+ve	Linked to Porokeratosis
SLC1A5	13.079359	+ve	Protein digestion and absorption pathway, Central carbon metabolism in cancer pathway
SLC25A1	10.693407	+ve	Linked to Congenital myasthenic syndrome, Linked to Combined D-2- and L-2-hydroxyglutaric aciduria
SLC25A29	9.254947	+ve	Thermogenesis pathway
SLC25A6	10.979601	+ve	Calcium signalling pathway, cGMP-PKG signalling pathway, Necroptosis pathway, Cellular senescence pathway, Parkinson disease pathway, Huntington disease pathway, Influenza A pathway, Human T-cell leukaemia virus 1 infection pathway

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SLC29A2	15.156823	+ve	Alcoholism pathway
SLC31A1	16.562778	+ve	Platinum drug resistance pathway, Mineral absorption pathway
SLC38A5	7.1403027	+ve	GABAergic synapse pathway
SLC39A5	7.296592	+ve	Linked to Myopia
SLC3A2	14.07006	+ve	mTOR signalling pathway, Ferroptosis pathway, Protein digestion and absorption pathway
SLC6A8	16.636656	+ve	Linked to Cerebral creatine deficiency syndrome, Linked to X-linked creatine deficiency syndrome
SLC7A5	13.480293	+ve	mTOR signalling pathway, Central carbon metabolism in cancer pathway
SLC7A8	8.355325	+ve	Protein digestion and absorption pathway
SLC11A2	31.62154	+ve	Lysosome pathway, Ferroptosis pathway, Mineral absorption pathway
SLC1A1	19.64092	+ve	Synaptic vesicle cycle pathway, Glutamatergic synapse pathway, Protein digestion and absorption pathway
SLC22A5	8.93393	+ve	Choline metabolism in cancer pathway
SLC25A10	17.145796	+ve	Proximal tubule bicarbonate reclamation pathway
SLC25A13	9.850117	+ve	Linked to Citrullinemia, Linked to Primary hyperammonemic disorders (Urea cycle disorders)

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SLC25A46	10.360896	+ve	Linked to Charcot-Marie-Tooth disease
SLC26A3	9.05577	+ve	Pancreatic secretion pathway, Mineral absorption pathway
SLC27A1	13.418841	+ve	PPAR signalling pathway, Insulin resistance pathway
SLC29A3	13.328121	+ve	Alcoholism pathway
SLC2A2	7.823031	+ve	Insulin secretion pathway, Prolactin signalling pathway, Glucagon signalling pathway, Type II diabetes mellitus pathway, Insulin resistance pathway, Maturity onset diabetes of the young pathway, Carbohydrate digestion and absorption pathway, Central carbon metabolism in cancer pathway. Linked to Glycogen storage disease, Linked to Fanconi-Bickel syndrome
SLC35A2	8.219815	+ve	Linked to Congenital disorders of glycosylation type II, Linked to Early infantile epileptic encephalopathy
SLC35C1	20.138887	+ve	Linked to Leukocyte adhesion deficiency, Linked to Congenital disorders of glycosylation type II
SLC38A1	18.407154	+ve	Glutamatergic synapse pathway, GABAergic synapse pathway
SLC39A13	9.199426	+ve	Linked to Ehlers-Danlos syndrome, spondylodysplastic type

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SLC39A14	24.078133	+ve	Ferroptosis pathway
SLC3A1	18.950901	+ve	Protein digestion and absorption pathway
SLC44A1	10.62931	+ve	Choline metabolism in cancer pathway
SLC44A2	13.139595	+ve	Choline metabolism in cancer pathway
SLC45A3	6.9271073	+ve	Transcriptional mis-regulation in cancer pathway, MicroRNAs in cancer pathway
SLC5A6	20.858412	+ve	Vitamin digestion and absorption pathway
SLC6A19	6.3885436	+ve	Protein digestion and absorption pathway, Mineral absorption pathway. Linked to Hartnup disorder, Linked to Iminoglycinuria, Linked to Hyperglycinuria
SLC6A4	6.203496	+ve	Synaptic vesicle cycle pathway, Serotonergic synapse pathway. Linked to Obsessive-compulsive disorder
SLC7A7	11.712494	+ve	Protein digestion and absorption pathway. Linked to lysinuric protein intolerance, Linked to secondary hyperammonemia
SLC9A3R1	6.900848	+ve	Tight junction pathway, Parathyroid hormone synthesis secretion and action pathway, Pathogenic Escherichia coli infection pathway, Human papillomavirus infection pathway. Linked to Nephrolithiasis/osteoporosis, hypophosphatemic

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ABC transporter gene expression response following spironolactone formulation exposure - Gene identification, expression fold change and pathway identification is listed for all genes in the ABC transporter family for which a >2 fold change was seen and pathway information was available.

ABC Transporters

Transporter	Fold Change	+ve or -ve	KEGG Pathway Identification
ABCC8	7.0988774	+ve	Insulin secretion pathway, Type II diabetes mellitus pathway. Linked to permanent neonatal diabetes mellitus, linked to transient neonatal diabetes mellitus, Linked to familial hyperinsulinemic hypoglycemia
ABCF2	8.409261	+ve	Pathogenic Escherichia coli infection pathway
ABCB1	8.749464	+ve	ATP-binding cassette, subfamily B (MDR/TAP), member 1
ABCB6	11.907367	+ve	Linked to hereditary stomatocytosis, linked to microphthalmia, Linked to familial pseudohyperkalemia
ABCB7	7.421333	+ve	Linked to sideroblastic anaemia
ABCC3	6.1075015	+ve	Antifolate resistance pathway, Bile secretion pathway
ABCC5	9.429524	+ve	Antifolate resistance pathway
ABCD4	16.697014	+ve	Peroxisome pathway

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15 **Cytochrome P450 Enzyme gene expression response following spironolactone formulation exposure** - Gene identification, expression fold change and pathway identification is listed for all genes in the Cytochrome P450 Enzyme family for which a >2 fold change was seen and pathway information was available.

Cytochrome P450 Enzymes			
Enzyme	**Fold Change**	**+ve or -ve**	**KEGG Pathway Identification**
CYP2E1	5.901765057	+ve	Steroid hormone biosynthesis, Arachidonic acid metabolism, Linoleic acid metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Drug metabolism - other enzymes, Metabolic pathways, Non-alcoholic fatty liver disease (NAFLD), Chemical carcinogenesis
CYP2J2	5.749191333	+ve	Arachidonic acid metabolism, Linoleic acid metabolism, Metabolic pathways, Serotonergic synapse, Inflammatory mediator regulation of TRP channels, Ovarian steroidogenesis
CYP26B1	4.599662301	+ve	Retinol metabolism, Metabolic pathways
CYP3A7	9.287605916	+ve	Steroid hormone biosynthesis, Retinol metabolism, Metabolic pathways, Chemical carcinogenesis
CYP11B2	6.987923906	+ve	Steroid hormone biosynthesis, Metabolic pathways, Aldosterone synthesis and secretion

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CYP4F3	4.781126103	+ve	Arachidonic acid metabolism, Metabolic pathways
CYP4V2	6.479034553	+ve	Familial flecked retina syndrome, Bietti crystalline corneoretinal dystrophy
CYP2C8	5.342657131	+ve	Arachidonic acid metabolism, Linoleic acid metabolism, Retinol metabolism, Drug metabolism - cytochrome P450, Metabolic pathways, Serotonergic synapse, Chemical carcinogenesis
CYP1A2	6.028728553	+ve	Steroid hormone biosynthesis, Caffeine metabolism, Tryptophan metabolism, Linoleic acid metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Metabolic pathways, Biosynthesis of secondary metabolites, Chemical carcinogenesis
CYP51A1	6.701533528	+ve	Steroid biosynthesis, Metabolic pathways
CYP1B1	5.581066368	+ve	Steroid hormone biosynthesis, Tryptophan metabolism, Metabolism of xenobiotics by cytochrome P450, Ovarian steroidogenesis, Chemical carcinogenesis, MicroRNAs in cancer
CYP27A1	5.154637741	+ve	Primary bile acid biosynthesis, Metabolic pathways, PPAR signalling pathway, Cholesterol metabolism
CYP3A43	6.55835254	+ve	Chemical carcinogenesis
CYP2R1	7.519928796	+ve	Steroid biosynthesis, Metabolic pathways

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CYP24A1	6.882079062	+ve	Steroid biosynthesis, Metabolic pathways, Parathyroid hormone synthesis, secretion and action, MicroRNAs in cancer
CYP2D6	8.501487764	+ve	Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Endocrine resistance, Serotonergic synapse
CYP19A1	6.973407083	+ve	Steroid hormone biosynthesis, Metabolic pathways, Ovarian steroidogenesis
CYP4X1	5.080183496	+ve	Serotonergic synapse
CYP2U1	9.482126701	+ve	Arachidonic acid metabolism, Metabolic pathways
CYP4A11	5.901765057	+ve	Fatty acid degradation, Arachidonic acid metabolism, Retinol metabolism, Metabolic pathways, PPAR signalling pathway, Vascular smooth muscle contraction, Inflammatory mediator regulation of TRP channels
CYP4F8	6.61709587	+ve	Arachidonic acid metabolism, Metabolic pathways
CYP3A5	5.635286563	+ve	Steroid hormone biosynthesis, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Metabolic pathways, Chemical carcinogenesis
CYP2C19	6.42128861	+ve	Arachidonic acid metabolism, Linoleic acid metabolism, Drug metabolism - cytochrome P450, Metabolic pathways, Serotonergic synapse, Chemical carcinogenesis

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CYP11A1	10.81332671	+ve	Arachidonic acid metabolism, Linoleic acid metabolism, Drug metabolism - cytochrome P450, Metabolic pathways, Serotonergic synapse, Chemical carcinogenesis
CYP2F1	14.46130063	+ve	Steroid hormone biosynthesis, Metabolic pathways, Ovarian steroidogenesis, Aldosterone synthesis and secretion, Cortisol synthesis and secretion, Cushing syndrome
CYP11B1	12.92451789	+ve	Steroid hormone biosynthesis, Metabolic pathways, Cortisol synthesis and secretion, Cushing syndrome
CYP27B1	5.85475736	+ve	Steroid biosynthesis, Metabolic pathways, Parathyroid hormone synthesis, secretion and action, Tuberculosis
CYP17A1	7.235173816	+ve	Steroid hormone biosynthesis, Metabolic pathways, Ovarian steroidogenesis, Prolactin signalling pathway, Cortisol synthesis and secretion, Cushing syndrome
CYP2C9	5.755881365	+ve	Arachidonic acid metabolism, Linoleic acid metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Metabolic pathways, Serotonergic synapse, Chemical carcinogenesis
CYP2S1	12.17852083	+ve	Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Metabolic pathways

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CYP1A1	11.13978829	+ve	Steroid hormone biosynthesis, Tryptophan metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Metabolic pathways, Ovarian steroidogenesis, Chemical carcinogenesis
CYP2B6	7.538801038	+ve	Arachidonic acid metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Metabolic pathways
CYP46A1	6.36113458	+ve	Primary bile acid biosynthesis
CYP39A1	10.68083174	+ve	Primary bile acid biosynthesis
CYP7A1	5.342657131	+ve	Primary bile acid biosynthesis, Steroid hormone biosynthesis, Metabolic pathways, PPAR signalling pathway, Bile secretion, Cholesterol metabolism
CYP7B1	9.51232016	+ve	Primary bile acid biosynthesis, Steroid hormone biosynthesis
CYP21A2	9.560005811	+ve	Steroid hormone biosynthesis, Metabolic pathways, Aldosterone synthesis and secretion, Cortisol synthesis and secretion, Cushing syndrome
CYP4F2	9.09313066	+ve	Arachidonic acid metabolism, Metabolic pathways
CYP26A1	5.933585535	+ve	Retinol metabolism, Metabolic pathways
CYP2A6	6.028728553	+ve	Caffeine metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450,

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CYP3A4	7.074985738	+ve	Drug metabolism - cytochrome P450, Drug metabolism - other enzymes, Metabolic pathways, Chemical carcinogenesis Steroid hormone biosynthesis, Linoleic acid metabolism, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Drug metabolism - other enzymes, Metabolic pathways, Bile secretion, Chemical carcinogenesis
CYP2C18	5.743592511	+ve	Retinol metabolism, Metabolic pathways, Serotonergic synapse, Chemical carcinogenesis
CYP8B1	5.196221969	+ve	Primary bile acid biosynthesis, Metabolic pathways, PPAR signaling pathway
CYP2A13	8.771337202	+ve	Metabolism of xenobiotics by cytochrome P450, Chemical carcinogenesis

Carboxylesterase enzyme gene expression response following spironolactone formulation exposure – Gene identification, expression fold change and pathway identification is listed for all genes in the Carboxylesterase enzyme family for which a >2 fold change was seen and pathway information was available.

Carboxylesterase Enzymes

Enzyme	Fold Change	+ve or -ve	KEGG Pathway Identification
CES1	9.266629724	+ve	Drug metabolism - other enzymes
CES2	7.103678207	+ve	Drug metabolism - other enzymes

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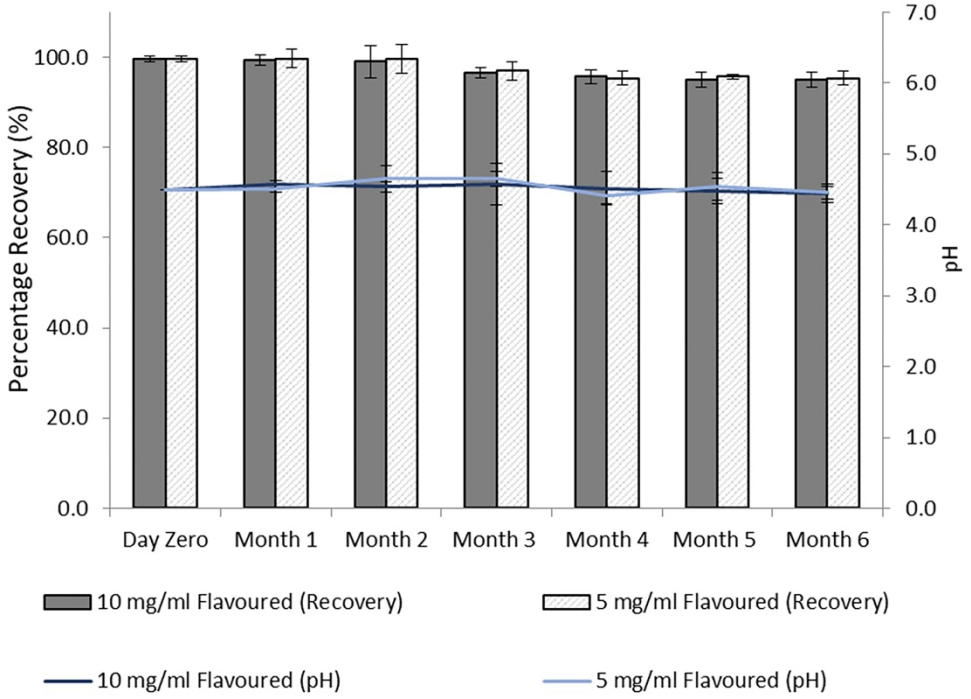

Figure 1 - Spironolactone Stability in Accelerated Conditions - Spironolactone recovery following HPLC for formulations in accelerated storage conditions is illustrated by the vertical bars. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD). Spironolactone pH stability for formulations in accelerated storage conditions is represented by the horizontal lines. 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).

127x95mm (600 x 600 DPI)

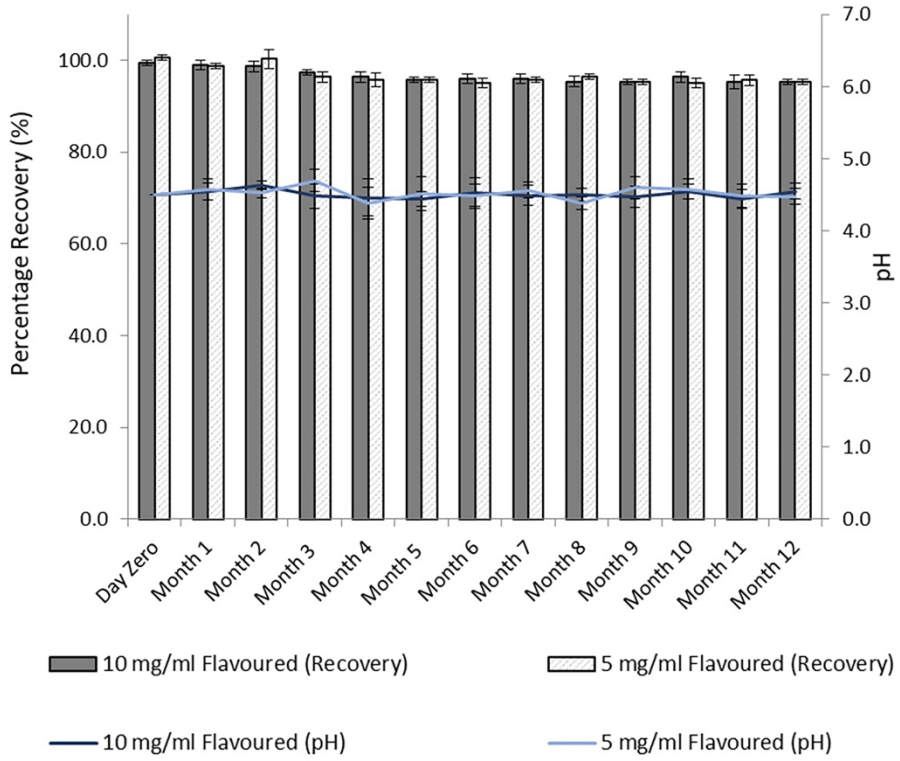

Figure 2 – Spironolactone Stability in Long Term Conditions - Spironolactone recovery following HPLC for formulations in long term storage conditions is illustrated by the vertical bars. The drug content of the formulations remained above 95% of the starting dose indicating that the formulations display adequate stability in accordance with ICH guidelines. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD). Spironolactone pH stability for formulations in long term storage conditions is represented by the horizontal lines. 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).

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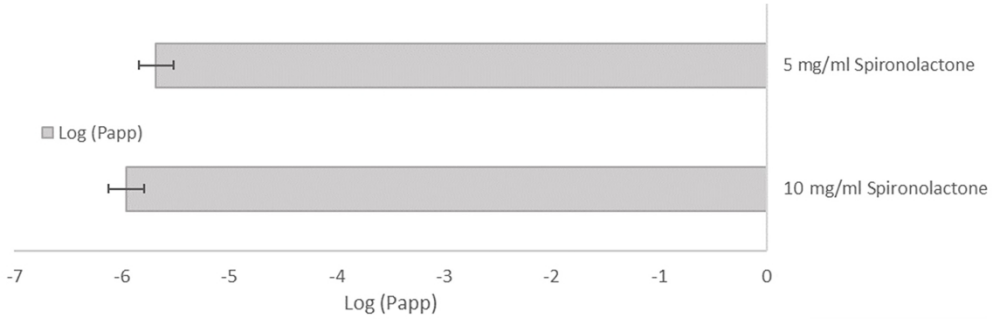

Figure 3 - Apparent permeability of spironolactone in oral liquid suspension formulations – Average Log Papp values calculated for spironolactone formulations range from -5.96 to -5.70 with an average of -5.81. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

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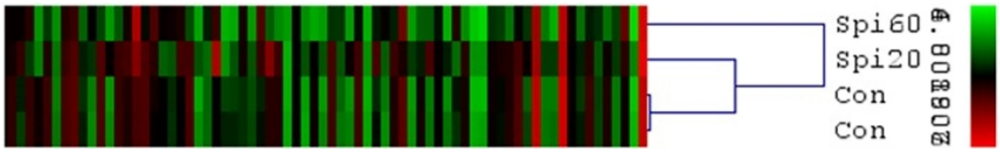

Figure 4 - Hierarchical clustering algorithm (HCA) – Gene expression data for control samples was compared to gene expression data for samples exposed to formulations at each time point using HCA analysis. Clustering analysis groups similar data sets and as shown above, there is significant difference indicated between the control gene expression data and the expression data seen at each time point.

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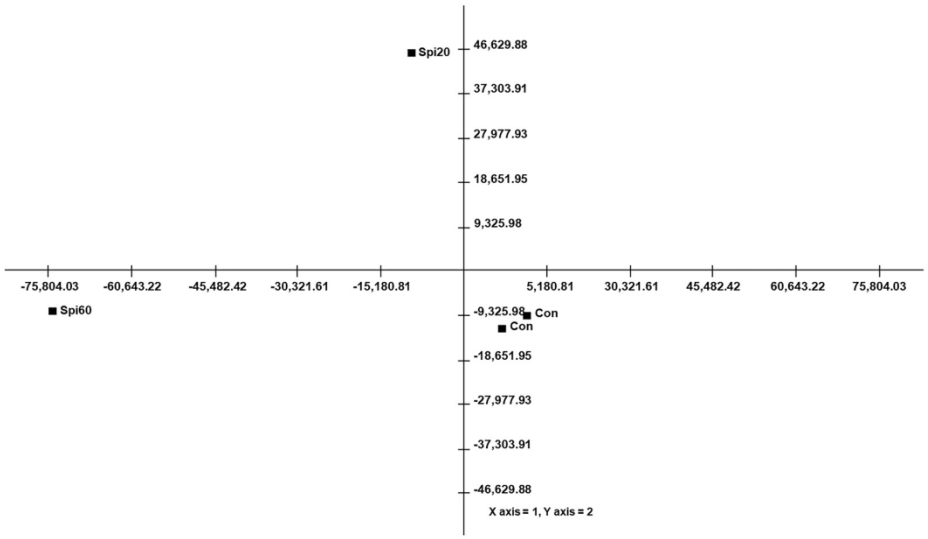

Figure 5 – First and Second PCA - There is a clear difference in the gene expression levels between the control samples and the formuation time points. This is seen along component one and two. The difference seen bewteen the time points shows a greater change in the gene expression after 60 minutes when compared to the change after 20 minutes.

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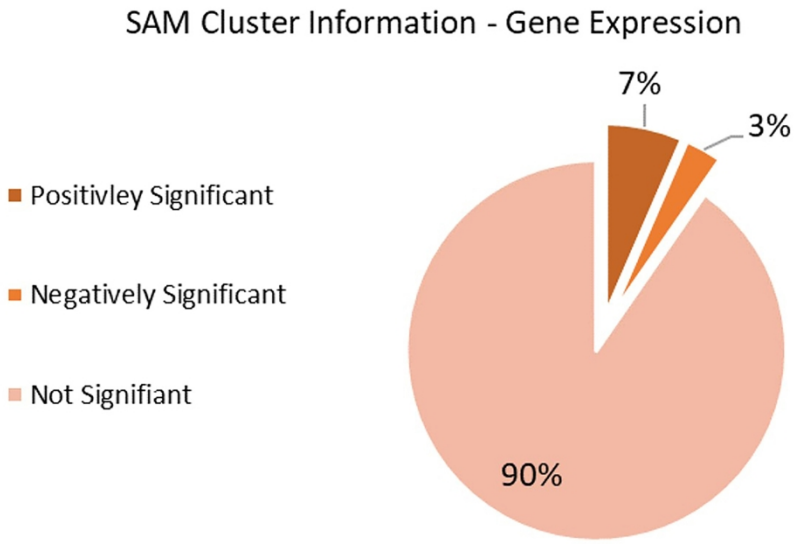

Figure 6 - SAM Cluster Information - Gene Expression - A total of 2576 upregulated (positively significant) genes and 1235 downregulated (negatively significant) genes were identified, 37282 genes were not significant.

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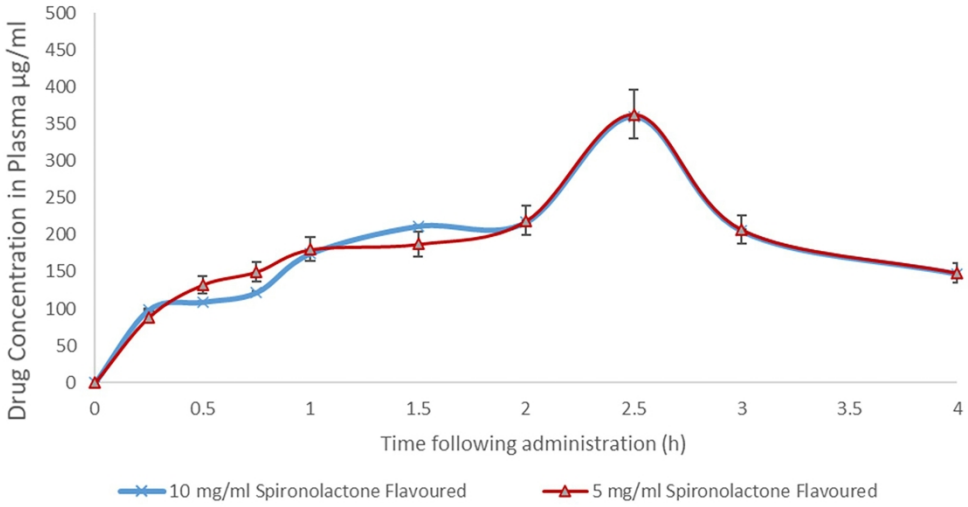

Figure 7 – In vivo plasma responses in rats following spironolactone administration – Plasma concentrations following oral administration. Results are generated from triplicate repeats (n=3) and error bars indicate standard deviation (RSD).

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Table 1 – Target product profile for spironolactone suspension detailing target components for each quality attribute.

Quality Attribute	Target
Route of Administration	Oral
Dosage form	Acceptable for patients aged from birth to <18 years
Dose Range	1-2 mg/kg daily in 1 to 2 divided doses, up to a maximum of 7 mg/kg daily for neonates. 1-3 mg/kg daily in 1 to 2 divided doses up to 9 mg/kg for children aged from 1 month to 11 years and 50-100 mg daily in 1 to 2 divided doses, up to a maximum of 9 mg/kg daily in children aged from 12 – 17 years. Includes dose titration.
Pharmacokinetics	Immediate Release
Palatability	Neutral/Flavored/Sweetened preferred
Shelf life	Minimum of 12 Months
Container closure system	Multi-dose
Additional Information	All excipients must be acceptable for the paediatric patient population

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Table 2 – Oral spironolactone suspension composition – Spironolactone formulations were produced at doses of 5 mg/ml and 10 mg/ml.

Spironolactone formulation components	
Spironolactone	API
Xanatural 180	Viscosity Modifier
Pluronic F127	Surfactant
Sodium Metabisulphate	Antioxidant
Sodium Benzoate	Preservative
Xylitol	Sweetener
Strawberry Flavour	Flavouring agent

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Table 3 – Eigenvector decomposition (EVD) - EVD 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 %		

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Table 4 – In vivo results for AUC, C_{MAX} and T_{MAX} – AUC was identified to be 992.80 µg*h/mL and 988.97 µg*h/mL for the 10 mg/ml and 5 mg/ml formulations respectively. C_{max} was shown to range from 363.24 µg/ml to 375.37 µg/ml and T_{MAX} was at 2.5 hours for all formulations.

Formulation	AUC (µg*h/mL)	C_{MAX} (µg/ml)	T_{MAX} (h)
Spironolactone Suspension (10 mg/ml)	992.80	375.37	2.5
Spironolactone Suspension (5 mg/ml)	988.97	363.24	2.5

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