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Systems biology approach to study permeability of paracetamol and its solid dispersion

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ABSTRACT

Physiological changes that take place at cellular level are usually reflective of their level of gene expression. Different formulation excipients have an impact on physiological behavior of the exposed cells and in turn affect transporter genes, enterocyte-mediated metabolism and toxicity biomarkers. The aim of this study was to prepare solid dispersion of paracetamol and evaluate genetic changes that occur in Caco-2 cell lines during the permeability of paracetamol alone and paracetamol solid dispersion formulations. Paracetamol–PEG 8000 solid dispersion was prepared by melt fusion method and the formulation was characterised using differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). Formulation of solid dispersion resulted in the conversion of crystalline drug into an amorphous form. Permeability studies showed that paracetamol absorption was higher from the solid dispersion formulation. DNA microarrays analysis was carried out in order to investigate the involvement of any efflux/uptake transporters in paracetamol or its solid dispersion permeability. Neither transporter carriers nor efflux proteins were found to be involved in the absorption of paracetamol or its PEG solid dispersion. Gene expression analysis established that paracetamol toxicity was potentially reduced upon formulation into solid dispersion when ATP binding cassette (ABC) and solute carrier transporter (SLC) genes were analyzed.

1. Introduction

Oral drug delivery is by far still the most popular route for the administration of drugs. The absorption of drugs from the gastrointestinal tract relies on two crucial stages: drug solubilisation and permeability. Intestinal permeability of therapeutic agents is considered as a prerequisite for oral bioavailability. Hence, assessing and improving drug transportation across intestinal membrane is the key process in drug discovery and development. Various *in vitro* techniques employed for this purpose include excised animal and human intestinal tissues and cell culture models such as Caco-2 cell lines. The extensive application of Caco-2 cell lines, is primarily due to its ability to exhibit many common features of human intestinal cells such as microvillous structure, carrier mediated transporter system and high enzyme activity (Artursson and Borchardt, 1997).

Interestingly, the majority of the physiological changes that take place at organism or cellular level are reflective on their level of gene expression. Gene regulation (expression or suppression) at cellular level varies under different conditions such as disease state, drug metabolism, exposure to toxins and response to treatment. The variations in gene regulation have led biologists to study and build data bases of gene expression matrixes that would allow the identification of the role of different genes during various cellular processes, diseases and differentiation. Analysis of gene expression at m-RNA levels was first discussed by Alwine et al. (1977) using Northern blotting. Unfortunately, Northern blot analysis allows the study of limited numbers of genes each time. In 1992 Liang and Pardee developed differential displaying technology which succeeded in measuring multiple gene expression changes in parallel. However, one of the major drawbacks for the technique was the qualitative assessment of gene changes and screening was dependent on the length of mRNA rather than its identity. All these techniques were limited when large numbers of simultaneous gene expression changes were required. The introduction of microarrays in 1995 has overcome these limitations and the sensitivity and throughput of gene expression have improved significantly. Microarrays allow the use of small amount of starting material and the small size of the arrays improves the sensitivity and enables the screening of vast number of genes simultaneously (Hal et al., 2000). Information gained from microarray analysis aids in understanding the role of various transporter proteins, enterocyte based metabolism and identification of various biomarkers.

The role of formulation excipients on drug dissolution and permeability has drawn significant attention in the recent past. Johnson et al. (2002) have demonstrated that polyethylene glycol (PEG 400) and Pluronic P85 inhibited drug efflux and metabolism of

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digitoxin and verpamil respectively. Such effects were attributed to the direct inhibitory effect on P-glycoprotein (P-gp) and the indirect effect of these compounds on buffer osmolarity, membrane fluidity and mitochondrial toxicity which results in depleting adenosine triphosphate (ATP). Similar studies were reported by other research groups investigating the role of surfactants (Cornaire et al., 2004). However, the linear approach of concentrating research efforts on the role of single/couple of genes to study excipient/formulation influence can lead to loss of vital information such as their role in metabolism of active ingredient, role of other potential gene targets to improve drug absorption and the interconnected influence of different genes. Microarrays provide a useful tool to study and develop a holistic view of the various events occurring at cellular levels. It enables to examine multiple aspects of drug delivery including genes regulating drug absorption, metabolism and toxicity. The current study investigates the genomic signature for paracetamol as a model drug as its bioavailability is limited by permeability. Paracetamol (acetaminophen) is a weakly acidic drug with pK_a of 9.5 (Kalantzi et al., 2006). It is usually prescribed as an analgesic and anti-inflammatory for the treatment of moderate pain and fever. Permeability studies and gene regulation were studied for paracetamol and its solid dispersion (using polyethylene glycol 8000).

2. Materials and methods

2.1. Materials

Paracetamol, phosphate buffered saline (PBS) tablets, potassium bromide, ethylenediaminetetraacetic acid (EDTA), agarose, trypan blue, albumin from bovine serum (BSA V), sodium citrate, sodium chloride, formamide, sodium carbonate and sodium bicarbonate were purchased from Sigma–Aldrich, UK. Sodium hydroxide was provided by Anala R, UK. Polyethylene glycol 8000 (PEG 8000) was obtained from Fluka (Biochemika), UK.

Caco-2 cell line was purchased from American Type Culture Collection (ATCC), UK. RNeasy Mini Kit (50) was provided by Qiagen, UK. Millex-GV syringe filter and MilliPore Pronto background reduction kit were provided by Corning, UK. Poly dA (1 μg/μL) and Human Cot1 DNA were purchased from Invitrogen, UK. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids (NEAA) and Hank's balanced salt solution (HBSS) were purchased from Bio Sera, UK. 1% Trypsin–EDTA, 1% penicillin–streptomycin supplemented with 2 mM glutamine and RNase free water were purchased by Gibco Lab., UK.

2.2. Analytical techniques

2.2.1. Spectrophotometric analysis

Drug concentration was measured and determined in solution via spectrophotometric technique. Paracetamol (measured amounts) was dissolved in phosphate buffered saline solution (pH 7.4) and Unicam UV–visible spectrophotometer (200–400 nm) was used to determine the wavelength for maximum absorption. A stock solution of paracetamol was prepared at 30 μg/mL in PBS (pH 7.4) to carryout calibration. The linearity of calibration curve was obtained in a concentration range from 2 μg/mL to 14 μg/mL and analyzed by UV spectroscopy at λ_{max} of 240 nm.

2.2.2. Hyper differential scanning calorimetry (DSC)

Perkin-Elmer Diamond DSC with a thermal analyzer, equipped with Pyris software was employed to obtain Hyper DSC data. 2–5 mg samples were crimped and placed on the sample furnace after weighing into a non-hermetically sealed DSC sample pan. Heat flow rate of 500 °C/min was used to heat the samples from 0 °C to 300 °C. Helium was used as a purge gas. In order to derive the melting points of each peak onset temperature was measured. For reference an empty pan was crimped. All the measurements were performed in triplicate.

2.2.3. Infrared spectroscopy

FTIR spectrometer Pye Unicam Ltd. (Cambridge, England) was used to obtain FTIR spectra. The samples were mixed thoroughly with potassium bromide at 1:100 (sample: potassium bromide) weight ratio after being ground. Pressure of 5 tons was applied for 5 min to compress the powder in order to prepare potassium bromide discs in a hydraulic press. Scans were obtained at a resolution of 4 cm^{-1}, from 4000 to 400 cm^{-1} at a scan rate of 16. All the studies were performed in triplicate.

2.2.4. Scanning electron microscopy (SEM)

SEM data was achieved by using a scanning electron microscope, Stereoscan 90 (Cambridge, UK). Double-sided adhesive carbon tape was used to fix the sample powders to an aluminium stub and was made conductive for use in Emscope Sputter (SC 500) for 360 s at 10 mA by coating in vacuum (4 psi) with a double gold layer. The samples were then loaded into the SEM to obtain scanning electron micrographs of the sample.

2.3. Preparation of solid dispersion and physical mixture

Solid dispersions were prepared by melt fusion containing 15% (w/w) paracetamol loading in PEG 8000 (Dubois and Ford, 1985). Homogeneous solution was formed by heating the drug and the polymer while stirring until the drug dissolved completely in the melt. The solution was solidified under ambient conditions after pouring it into tablet moulds. Paracetamol 15% (w/w) and PEG 8000 were ground for around 15 min in a mortar to make physical mixtures.

2.4. HPLC analysis (transport studies)

HPLC studies were performed using a Dionex 1100 HPLC system with autosampler (AS50), gradient pump (GP50), detector (UVD170U) and a C18 analytical column (Phenomenex ODS 3 Column, 4.6 μm × 150 mm) with a particle size of 5 μm. The mobile phase consisted of acetonitrile (10%):double distilled water (90%) for paracetamol and was detected at a wavelength of 240 nm, at a flow rate of 1 mL/min. 20 μL of paracetamol was injected and retention time of paracetamol at this flow rate was between 5 and 6 min.

2.5. Procedure for Caco-2 cell culture

Caco-2 cells with passage 70 were obtained from American Type Culture Collection (ATCC) and used at passages 90–100. Cells were grown to 90% confluences in 75 cm^2 T-flasks with DMEM supplemented with 10% FBS, 1% penicillin–streptomycin supplemented with 2 mM glutamine and 1% NEAA. Culture medium was changed every second day and cells were grown at a temperature of 37 °C and 5% CO_2. For the transport assay, cells were seeded on top of 6 well transwell culture plate inserts (24 mm, 4.7 cm^2) at a density of 2×10^5 cells/cm^2. Transwell inserts were used by first adding medium to the 6 well plates, then adding the transwell insert, followed by the addition of the medium and cells to the inside compartment of the transwell insert. Recommended 6 transwell permeable medium volume is 2.6 mL for plate well and 1.5 mL for the inside of transwell. An initial equilibrium period was used to improve cell attachment by adding medium to the 6 well plate and then to the transwell insert. The plate was then incubated for at least 1 h. The cells were then added to fresh medium in

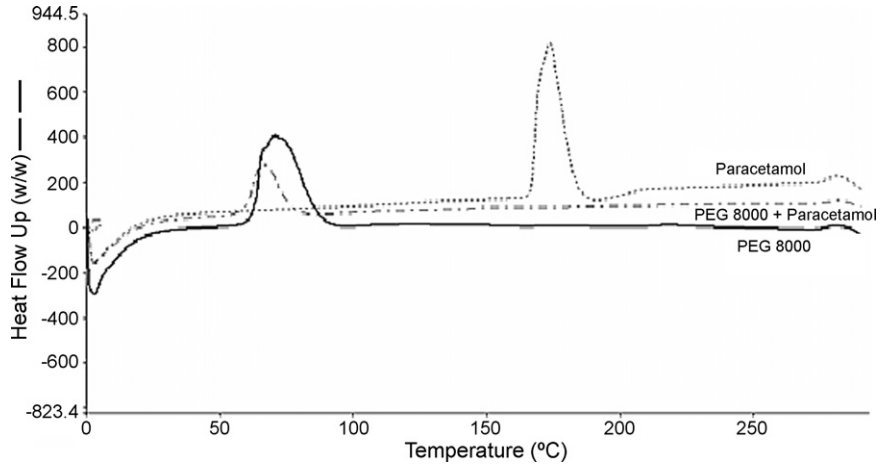

Fig. 1. Hyper-DSC thermograms of solid dispersion consisting of 15% (w/w) paracetamol (formulation—PEG 8000 + paracetamol) paracetamol alone and PEG 8000 alone.

the transwell insert and returned to the incubator. The level was checked periodically and fresh medium added as required. The culture medium was replaced every 24–48 h. Transepithelial electrical resistance (TEER) was measured at the start and end of every study.

2.6. Paracetamol permeability study

Caco-2 monolayers were used 21–25 days after seeding. Apical to basolateral permeability of drug and the solid dispersion was assessed. After 1 h of pre-incubation with drug-free transport medium (Hanks balanced salt solution), the medium containing the drug and the solid dispersion was introduced to the apical side (1.80 mL). To determine the initial concentration (C_0), a sample of 300 μL was taken from the apical side (1.50 mL remaining at the apical side). Sample aliquots (300 μL) were taken from the basolateral side at given time intervals (0, 5, 10, 15, 20, 25, 30, 60 min). After each sampling, an equal volume of fresh transport buffer (prewarmed at 37 °C) was added to the receiver compartment (basal side) and kept the cells at a temperature of 37 °C and 5% CO_2 during experiment. Samples were subsequently analyzed by HPLC. All experiments were performed at 37 °C ($n = 3$).

Apparent permeability P_{app} (cm/s) was calculated according to equation

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

where dQ/dt is the rate of appearance of the drugs on the basolateral side (nmol s^{-1}), C_0 is the initial concentration on the apical side (mM) and A is the surface area of the monolayer (cm^2).

The data presented was validated using HPLC measurement of drug transfer during the process of permeability. This included repetition of drug permeability studies in the preparations used for microarray analysis.

2.7. Microarrays

The samples were collected at various time points for transcriptomics directly into a mixture of phenol (5%) and ethanol (95%) in the ratio of 1:5 of phenol–ethanol and sample. The samples were incubated on ice and then pelleted by centrifugation at 5000 rpm at 4 °C for 10 min and the pellets were stored at −80 °C until RNA extraction. Total RNA was extracted from the cell pellets by using an RNeasy kit (Qiagen) and reverse transcribed and fluorescently labelled with cy3 and cy5 dye by using a CyScribe indirect postlabelling kit (GE-healthcare). All the controls and test samples were labelled with cy3 and cy5 dyes and single channel hybridization was carried out. Prior to hybridization slides were treated with sodium borohydride from Pronto background reduction kit (Corning) followed by prehybridization of slides for a minimum of 2 h in (20× SSC, 0.1% SDS, BSA 0.1%). For each experiment 80 pmol of cy3 and cy5 labelled cDNA was added to a final volume of 80 μL of hybridization solution containing 30% formamide, 20× SSC, 0.1% SDS, Human Cot1 DNA and Poly dA (1 μg/μL). The cDNA probes were denatured at 95 °C for 5 min, centrifuged for 2 min at full speed and hybridized for 16 h at 42 °C. Slides were then washed at 42 °C with 2× SSC–0.1% SDS for 15 min and at room temperature with 0.2× SSC and twice with 0.05× SSC for 15 min using Advawash slide washing machine. Slides were dried by low speed centrifugation at 1500 rpm at 25 °C for 10 min and scanned by using Perkin Elmer Scanarray scanner. The signal intensity of each spot in the microarray was quantified by using scanarray software.

Quantile normalization, hierarchical clustering, PCA and significant analysis of microarrays (SAM) were performed using TMEV (Saeed et al., 2006) TM4: a free, open-source system for microarray data management and analysis.

2.8. Statistical analysis of data

Student's unpaired t-test with $P < 0.05$ was considered as significant. Statistical significance was calculated by GraphPad prism software.

3. Results and discussion

3.1. Characterisation studies for paracetamol

Paracetamol solid dispersion with PEG 8000 was prepared by melt fusion method and both the prepared solid dispersion and paracetamol free drug were characterised using hyper differential scanning calorimetry (hyper DSC), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM).

Fig. 1 shows the DSC thermograms of paracetamol, PEG 8000 and solid dispersion 15% (w/w) (paracetamol–PEG 8000). Paracetamol exhibited an endothermic peak at 166.71 °C corresponding to its melting point while, PEG polymer shows a melt at around 59.13 °C. Upon dispersing paracetamol into PEG, the melting endotherm of paracetamol free drug disappeared and only the polymer peak was observed at 59 °C. The absence of paracetamol melting endotherm in the prepared solid dispersion could be attributed to the solubilisation and distribution of the drug within the hydrophilic polymer matrix which in turn possibly resulted in the conversion of the crystalline paracetamol form into the amorphous form. Similar

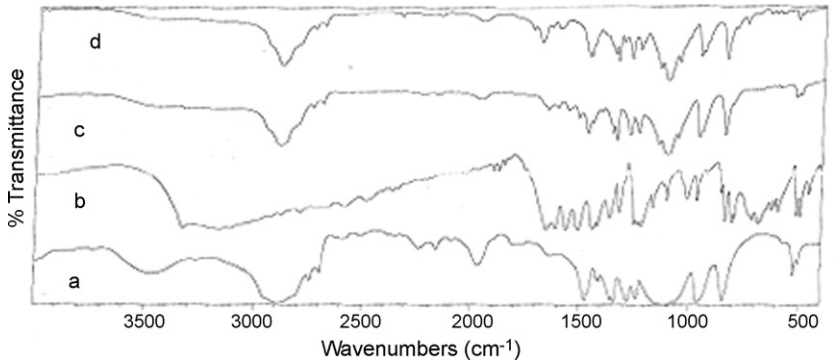

Fig. 2. Fourier transform infrared spectra of (bottom to top): (a) PEG 8000, (b) paracetamol, (c) physical mixture of PEG 8000 and paracetamol and (d) solid dispersion of PEG 8000 and paracetamol.

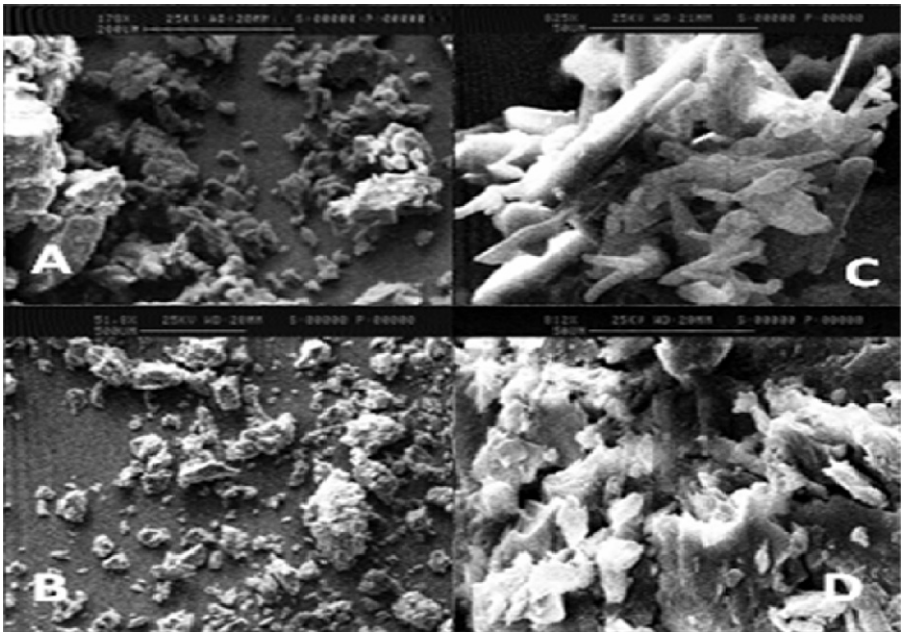

Fig. 3. Scanning electron microphotographs of (A) PEG 8000; (B) paracetamol alone; (C) physical mixture of PEG 8000-paracetamol binary systems with 15% (w/w) drug content; (D) solid dispersion of paracetamol binary system with 15% (w/w) drug content.

results were suggested by Guyot et al. (1995) and Damian et al. (2000).

On the other hand, paracetamol physical mixture with PEG 8000 showed two separate peaks at 59 and 166 °C corresponding to the melting point of the polymer and paracetamol respectively (data not shown).

The DSC results were further confirmed by SEM images (Fig. 3) showing that PEG 8000 particles were irregular in shape and size as previously reported by Badens et al. (2009). Paracetamol–PEG physical mixture showed elongated crystals of the drug on the surface of the polymer (Fig. 3C). However, analysis of solid dispersion revealed the absence of these characteristic crystals possibly due to the formation of amorphous drug upon melting and solidification with PEG (Fig. 3D) which was confirmed from DSC scans.

Further characterisation of the drug and formulation was carried out using FTIR. The infrared spectra for PEG 8000 was characterised by sharp peaks at 3450, 2891 and 1490 cm^{-1} corresponding to the stretching associated with O–H, C–H and C–O bonds respectively. Analysis of spectra for paracetamol showed specific absorption bands at wave numbers 3324, 3289 and 1690 cm^{-1} corresponding to the stretching associated with O–H, N–H and C–O respectively. Interestingly, the hydroxyl (–OH) peak characteristic for PEG disappeared when the solid dispersion was prepared, moreover the carboxyl group characteristic for paracetamol had shifted to 1640 cm^{-1} which in turn suggest the formation of H-bonds between the two moieties (Fig. 2).

In summary the characterisation data suggests the formation of amorphous paracetamol which formed hydrogen bonds with the polymer upon formulation as solid dispersion.

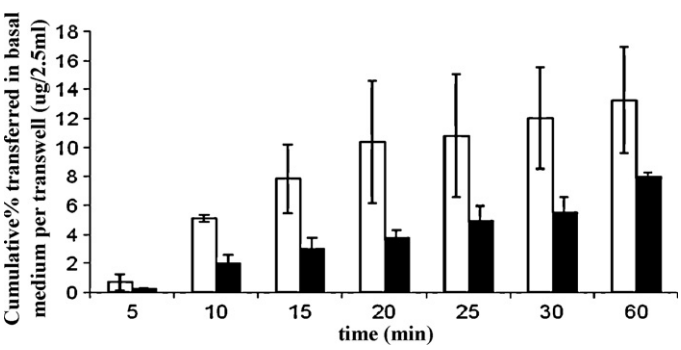

Fig. 4. Apical-to-basal permeability of solid dispersion of paracetamol (□) and paracetamol alone (■) across Caco-2 monolayers. Each column indicates mean ± S.D. ($n = 3$).

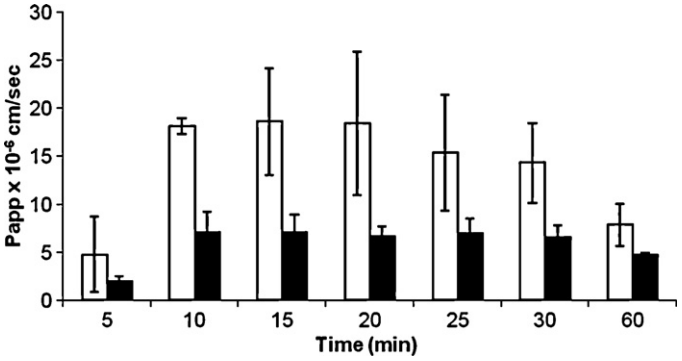

Fig. 5. The apparent permeability coefficients (P_{app}) of solid dispersion containing paracetamol (□) compared to the paracetamol alone (■). Each column indicates mean ± S.D. ($n = 3$).

3.2. Permeability studies

Caco-2 cell monolayers have been recognised as vital *in vitro* model for the rapid screening of intestinal drug absorption. Permeability studies for drug absorption for paracetamol alone and its solid dispersion were carried out to study the influence of molecular state of drug (amorphous form against crystalline form) and PEG 8000 on drug permeation. The cumulative % transfer of paracetamol from apical to basal (μg/2.5 mL) across Caco-2 monolayer at different time points for paracetamol alone and its solid dispersion is presented in Fig. 4.

Paracetamol permeability across Caco-2 cells was higher from the solid dispersion formulation at all the time points when compared against paracetamol alone. Paracetamol absorption from the solid dispersion increased from less than 1 μg/2.5 mL during the first 5 min of incubation to 5 μg/2.5 mL after 10 min of incubation and the permeability increased to a maximum of 12.7 ± 4.1 μg/2.5 mL after 60 min. However, permeability measurements of paracetamol alone resulted in relatively poor drug absorption and only 7.6 ± 0.3 μg/2.5 mL was absorbed after 60 min of incubation.

The apparent permeability coefficients (P_{app}) for paracetamol and its solid dispersion at different time points were calculated and found to be $5.12 \pm 0.76 \times 10^{-6}$ cm/s and $7.09 \pm 3.76 \times 10^{-6}$ cm/s respectively (Fig. 5) suggesting that paracetamol and its solid dispersion can be classified as moderately permeable (Yee, 1997). Overall, solid dispersion of paracetamol exhibited twofold increase in permeability when compared to drug alone.

The improvement in paracetamol permeability from the solid dispersion could be assigned to two critical factors. Firstly, the conversion of crystalline paracetamol into amorphous form upon inclusion into PEG 8000 hydrophilic matrix could have possibly resulted in an increase in solubility which allows the drug to readily associate with aqueous environment with relatively low enthalpy and ultimately be presented to the site for drug absorption (Prabhu et al., 2005). Secondly, the presence of PEG 8000 within the matrix could have resulted in microenvironmental changes of cell surface (membrane fluidity) as suggested by Johnson et al. (2002) leading to enhancement in drug absorption.

As majority of the research investigating drug permeability is restricted to study the influence of single transporter gene with P glycoprotein occupying the forefront, our research was directed to study a holistic view of various transporter genes using microarrays. The motivation to further explore gene expression changes during paracetamol permeability was driven by the desire to study regulation of transporter genes such as ATP binding cassette (ABC) transporters and solute carrier transporters (SLC) and investigate their role in drug absorption, metabolism and toxicity. Microarray studies were conducted and gene expression was assessed during permeability of paracetamol and its solid dispersion at three different time points (15, 30 and 60 min).

3.3. Microarray

3.3.1. Data clustering and normalization

Analysis of data generated from microarrays can be performed using different computational and statistical approaches. Among them data clustering helps understand expression patterns when comparing different data sets from similar experiments (Eisen et al., 1998; Tibshirani et al., 1999; Getz et al., 2000). Data clustering was carried out for the different data sets at various time points. Initial analysis was done on the control data sets (Caco-2 cells at 15, 30 and 60 min). The results showed that the different data sets for the controls were grouped together when analysis was carried out on triplicate data sets at each time point and also at different time points throughout the course of the experiments indicating that there were minimal variations in gene expression profiling between control samples (data not shown). As analysis for control data sets revealed that there was minimal variation during the entire 60 min of experimental study, a mean value was employed for comparing data sets of control against data sets for drug alone treated cells and solid dispersion treated cells.

To further study and compare gene expression patterns between control and drug treated (both drug alone and solid dispersion) hierarchical agglomerative data clustering analysis was carried out. Additionally, when multiclass significant analysis of microarrays (SAM) was performed for the control samples, it was found that at a false discovery rate (FDR) of 12%, 1000 out of the total 38000 genes significantly changed between control samples at the three different times. However, SAM analysis on data sets comparing control against drug alone and solid dispersion treated cells showed that 25% of the total genes exhibit variations at an FDR of 1%.

The next phase of data analysis involved the study of principal component analysis (PCA) to determine dynamic trends during drug permeability using averaged normalized data for the different time point data sets treated with paracetamol alone and its solid dispersion when compared to the control (Fig. 6). PCA analysis results revealed that all the data sets were clustered at the origin for control samples as well as samples treated with paracetamol and its solid dispersion (15 min only) suggesting that there was no significant variation in gene expression pattern in the first 15 min of the study. However, maximum variability in gene expression profile was obtained after 30 min of drug alone treatment as well as solid dispersion treatment suggesting that maximum variation in gene expression was obtained with data sets reverting back to the original state after 60 min (Fig. 6). PCA analysis showed that molecular expression pattern for genes followed a clockwise trajectory for paracetamol alone treated cells as well as cells treated with solid dispersion and that maximum variation occurred at 30 min.

A study conducted by Bagnall et al. (1979) suggested that paracetamol is mainly transported across the intestinal membrane by passive diffusion. However, paracetamol metabolism by conjugation with sulphates and glucuronides increased the polarity of the drug and the primary active transporters were more likely to get involved in paracetamol metabolite absorption (Evans, 2007). Hence, our next stage of work was to investigate the analysis of transporter genes with primary focus on ABC and SLC transporters. ABC family serve as primary active transporters and play important roles in modulating drug absorption, metabolism, toxicity and effectiveness (Glavinas et al., 2004). HPLC analysis was carried out throughout the study to validate the results for gene expression analysis similar to our recent studies on genomic analysis of indomethacin based formulations (Khan et al., in press).

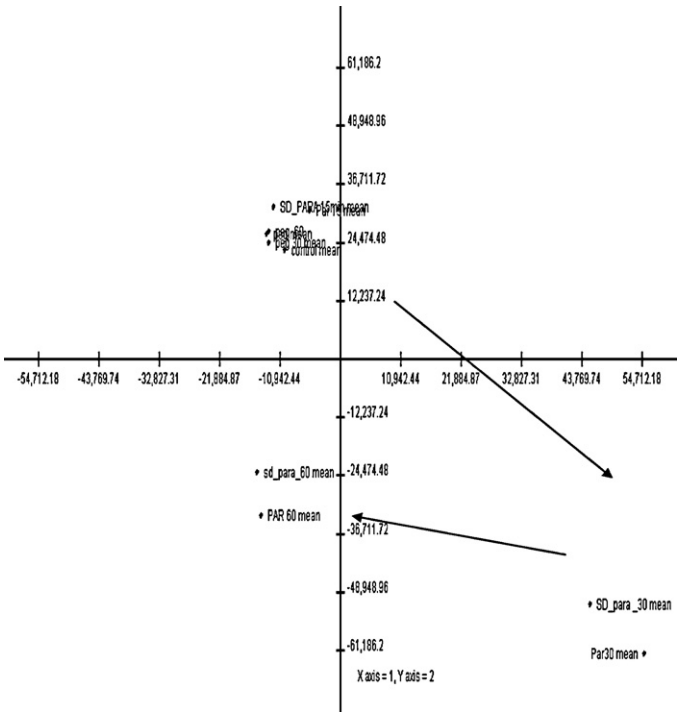

Fig. 6. Principal component analysis on the transcriptional time course for paracetamol (PARA) alone and solid dispersion of paracetamol (SD-PARA). The number represents the time points. The plot represents the data for mean values ($n = 3$) at each time point.

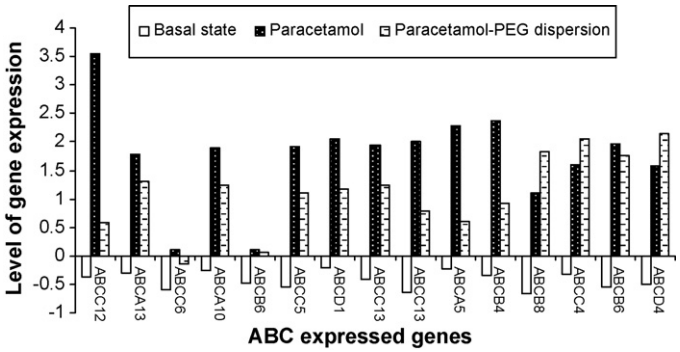

Fig. 8. Gene expression profiles of ABC transporters on Caco-2 cells at basal state (□), after 30 min of exposure to paracetamol (▩) and paracetamol–PEG solid dispersion (▨) ($n = 3$).

The results from the study suggest that the level of differentially expressed genes varies with the type of drug formulation and time course. The results showed that a total of 15 ABC genes changed their expression, 13 of which were significantly over expressed and two remained unaltered upon exposure to paracetamol and its solid dispersion over the course of 30 min time period (Fig. 7). However, the level of expression varied significantly for paracetamol alone and its solid dispersion counterpart which suggests that formulation has an influence on level of expression of ABC transporters. Majority of the over-expressed genes were metabolic and toxic biomarkers and only few mediate the transportation of therapeutic agents (Fig. 8).

ABCC6 (MRP6) was among the expressed genes and the results suggest that paracetamol alone formulations had increased the expression of MRP6 from -0.59 ± 0.1 at the basal state to 0.1 ± 0.16 upon drug treatment (Fig. 8). MRP6 is reported to play an important role in the transportation of glutathione conjugates. Paracetamol detoxification takes place by conjugation of drug with glutathione (Ketterer et al., 1983) and was reported to occur in Caco-2 cell lines (Oude Elferink et al., 1993). It can be concluded from our results that paracetamol was metabolised and resulted in the formation of glutathione conjugate which eventually stimulated the over-expression of MRP6 for its transportation. On the other hand, paracetamol–PEG solid dispersion did not significantly affect the expression of MRP6 from the basal state. This could be attributed to the transport and metabolic inhibitory effect of PEG which was early reported by Johnson et al. (2002).

Interestingly, two hepatotoxic biomarkers (ABCC13 and ABCB4) were over-expressed upon exposure of Caco-2 cells to paracetamol alone. ABCC13 is believed to be highly expressed in fatal liver; 20 times more than normal adult liver (Yabuuchi et al., 2002). ABCC13 was significantly over-expressed from -0.63 ± 0.02 at the basal state to 2.01 ± 0.18 when Caco-2 cells were exposed to paracetamol only. Yet, paracetamol–PEG solid dispersion resulted in a slight increase of ABCC13 gene expression (0.77 ± 0.39). Similar pattern of expression was observed for ABCB4 whose expression had increased to (2.36 ± 1.5) and (0.93 ± 0.61) for paracetamol and paracetamol–PEG dispersion respectively (Fig. 8). ABCB4 is suggested to play a role in intra hepatic gallstone disease (Fracchia et al., 2001) and type 3 intra hepatic cholestasis (Jacquemin et al., 2001).

The high increase of hepatotoxic biomarker reflects the liver toxicity of paracetamol which was previously reported by Eriksson et al. (1992). However, the use of PEG 8000 in the solid dispersion induced a reduction in the toxic effects as suggested by the toxic biomarker expression level. The mechanism of action of PEG is not yet known, but it could be related to the ability of PEG to modify membrane fluidity and mitochondrial toxicity as previously reported by Johnson et al. (2002). As reported earlier in our previous work, the use of DNA microarrays is advantageous to deduce any possible side effects linked to therapeutic agent or pharmaceutical formulation (Khan et al., in press). In this study, the expression of ABCC12 changed significantly (3.52 ± 0.02) when exposed to paracetamol containing formulations and remained unaffected (0.57 ± 0.99) when the solid dispersion formulation was used (Fig. 8). No physiological function is known for ABCC12 but it is believed to play a role in a genetic disease in infancy known as paroxysmal kinesigenic choreoathetosis PKC (Shimizu et al., 2003).

Solute carrier transporters (SLC) are membrane bound proteins that play a major role in regulating the uptake of various materials such as nutrients, inorganic ions, nucleosides, amino acids and sugars. Organic anion transporter peptide (OATP), organic anion

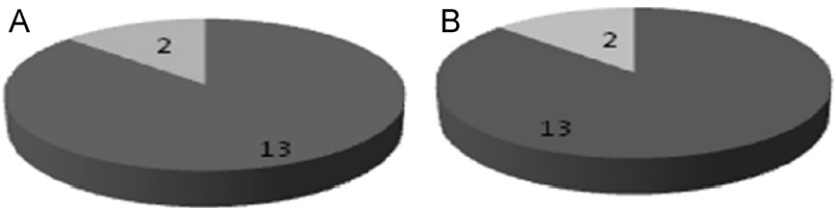

Fig. 7. Total number of ABC genes over-expressed (13 genes) (■) and unchanged (2 genes) (■) after 30 min of exposure to paracetamol (A), total number of ABC genes over-expressed (13 genes) (■) and unchanged (2 genes) (■) after 30 min of exposure to solid dispersion of paracetamol (B).

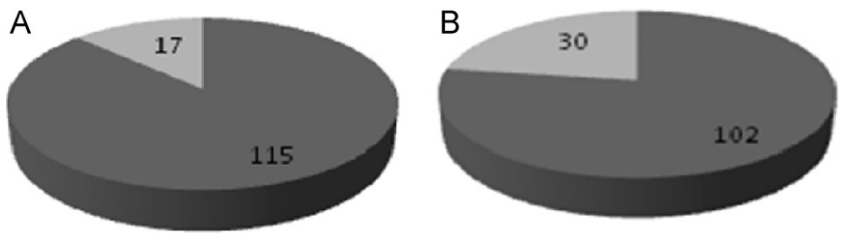

Fig. 9. Total number of SLC genes over-expressed (■) and unchanged (■) after 30 min of exposure to paracetamol (A), total number of SLC genes over-expressed (■) and unchanged (■) after 30 min of exposure to solid dispersion of paracetamol (B).

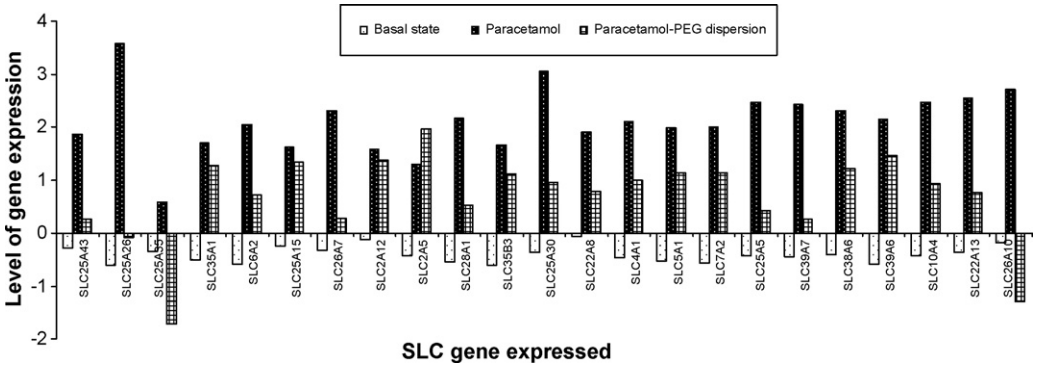

Fig. 10. Gene expression of SLC transporters on Caco-2 cells basal state (□), after 30 min of exposure to paracetamol (■) and paracetamol–PEG solid dispersion (⊞) ($n = 3$).

transporters (OATs), organic cation transporter (OCT) and peptide cotransporters (PEPTs) are mainly involved in transportation of xenobiotics (Prakash and Vaz, 2009). Therefore, the gene expression changes of SLC family were investigated in order to understand their role in transportation of paracetamol or its solid dispersion. Up to date 360 SLC genes have been identified that are grouped into 46 SLC families in the human genome (Hediger et al., 2004).

The expression changes of 132 SLC transporter genes were identified in our study (Fig. 9), among them 115 genes were significantly over-expressed and 17 remained unchanged upon exposure to paracetamol alone for 30 min. On the other hand, using paracetamol solid dispersion resulted in over-expression of 102 SLC transporter genes only and 30 transporter genes remained unchanged (Fig. 9).

Interestingly, many members of SLC25 family were over-expressed upon exposure to paracetamol only formulation. For instance, the expression of SLC25A26 increased from -0.6 ± 0.2 at the basal state to 3.58 ± 0.36 upon exposure to paracetamol for 30 min. However, the expression remained unaltered at -0.071 ± 0.43 when PEG-solid dispersion was used (Fig. 10). Haitina et al. (2006) and Robinson et al. (2008) have concluded that SLC25 are mitochondrial carriers that mediate the absorption of amino acids, malate and ATP which are essential for mitochondrial activity. The results suggest that solid dispersion formulation inhibited the over-expression of SLC25 transporter genes which in turn impair the mitochondrial function and could possibly reduce mitochondrial toxicity as suggested by Johnson et al. (2002).

The expression level of sugar and polyol transporters was affected to the same degree (e.g. SLC2A12 and SLC2A5) when paracetamol and its solid dispersion were studied suggesting inclusion of PEG 8000 or conversion of crystalline form to amorphous state has no effect on genes from sugar transporter family (Fig. 10).

The above data strongly suggests that paracetamol is passively transported across Caco-2 cells as no transporter genes were over-expressed throughout the permeability study of paracetamol and its solid dispersion. Therefore, the concentration gradient of the drug in the donor chamber is the driving force for the drug permeability. The higher permeability achieved by solid dispersion could be attributed to the indirect effects of PEG on the membrane fluidity and buffer osmolarity which in turn results in disturbing membrane integrity and enhances paracetamol partitioning across the membrane. Furthermore, the solid dispersion formulation had converted the crystalline form of paracetamol into the amorphous one resulting in higher solubility and higher concentration of the drug in the donor chamber.

Nevertheless, our current study suggests that coupling microarrays with drug permeability across Caco-2 cells could provide a large set of information about drug transportation, metabolism and possible toxicity. The use of different drug formulations was found to have a great impact on the level of expression of the corresponding transporter genes or biomarkers.

4. Conclusions

Solid dispersion in PEG 8000 is regarded as highly effective means of increasing the bioavailability of model drug paracetamol. The study demonstrated a chemical interaction between the drug and the carrier in the formulation through hydrogen bonding. Permeability data analysis showed that drug permeability was higher from solid dispersion when compared to drug alone, while the DNA microarrays data has shown that neither transporter carriers nor efflux proteins were involved in the absorption process of paracetamol or its PEG solid dispersion. The higher permeability exhibited by the solid dispersion is possibly due to conversion of crystalline paracetamol to an amorphous form and the presence of hydrophilic carrier.

Importantly, gene expression analysis has established that paracetamol metabolism could possibly take place in Caco-2 cells and that the solid dispersion inhibited the metabolism indirectly by down-regulating SLC25 transporter gene expression (which reduces mitochondrial toxicity and ATP depletion). One of the reasons behind failure of most new chemical entities (NCEs) is its low *in vivo* efficacy, undesired side effects, unfavorable metabolism and toxicity, the introduction of microarrays in drug screening cycle would be helpful in early detection and reduction of these attritions.

Conflict of interest

The authors declare no conflict of interest.

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References

Alwine, J.C., Kemp, D.J., Stark, G.R., 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. 74, 5350–5354.

Artursson, P., Borchardt, R., 1997. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. Pharm. Res. 14, 1655–1658.

Badens, E., Majerik, V., Horvath, G., Szokonya, L., Bosc, N., Teillaud, E., Charbit, G., 2009. Comparison of solid dispersions produced by supercritical antisolvent and spray-freezing technologies. Int. J. Pharm. 377 (1–2), 25–34.

Bagnall, W.E., Kelleher, J., Walker, B.E., Losowsky, M.S., 1979. The gastrointestinal absorption of paracetamol in the rat. J. Pharm. Pharmacol. 31, 157–160.

Cornaire, G., Woodley, J., Hermann, P., Cloarec, A., Arellano, C., Houin, G., 2004. Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo. Int. J. Pharm. 278, 119–131.

Damian, F., Blaton, N., Naesens, L., Balzarini, J., Kinget, R., Augustijns, P., Van den Mooter, G., 2000. Physicochemical characterization of solid dispersions of the antiviral agent UC-781 with polyethylene glycol 6000 and Gelucire 44/14. Eur. J. Pharm. Sci. 10, 311–322.

Dubois, J., Ford, J.L., 1985. Similarities in the release rates of different drugs from polyethylene glycol 6000 solid dispersions. J. Pharm. Pharmaco. 37, 494–496.

Eisen, M.B., Spellman, P.T., Brown, P.O., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A. 95, 14863–14868.

Eriksson, L.S., Broome, U., Kalin, M., Lindholm, M., 1992. Hepatotoxicity due to repeated intake of low doses of paracetamol. J. Int. Med. 231, 567–570.

Evans, A.M., 2007. Membrane transport as determinant of hepatic elimination of drugs and metabolites. Clin. Exp. Pharmacol. Physiol. 23, 970–974.

Fracchia, M., Pellegrino, S., Secreto, P., Gallo, L., Masoero, G., Pera, A., Galatola, G., 2001. Biliary lipid composition in cholesterol microlithiasis. Gut 48, 702–706.

Getz, G., Levine, E., Domany, E., 2000. Coupled two-way clustering analysis of gene microarray data. Proc. Natl. Acad. Sci. 97, 12079–12084.

Glavinas, H., Krajcsi, P., Cserepes, J., Sarkadi, B., 2004. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr. Drug Dev. 1, 27–42.

Guyot, M., Fawaz, F., Bildet, J., Bonini, F., Lagueny, A.M., 1995. Physicochemical characterization and dissolution of norfloxacin:cyclodextrin inclusion compounds and PEG solid dispersions. Int. J. Pharm. 123, 53–63.

Haitina, T., Lindblom, J., Renström, T., Fredriksson, R., 2006. Fourteen novel human members of mitochondrial solute carrier family 25 (SLC25) widely expressed in the central nervous system. Genomics 88, 779–790.

Hal, N.L.W., Vorst, O., Houwelingen, A.M., Kok, E.J., Peijnenburg, A., Aharoni, A., Tunen, A.J., Keijer, J., 2000. The application of DNA microarrays in gene expression analysis. J. Biotechnol. 78, 271–280.

Hediger, M.A., Romero, M.F., Peng, J.B., Rolfs, A., Takanaga, H., Bruford, E.A., 2004. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Introduction. Pflugers Arch. 447, 465–468.

Jacquemin, E., De Vree, M., Cresteil, D., Sokal, E., Sturm, E., Dumont, M., Scheffer, G., Paul, M., Burdelski, M., Bosma, P., Bernard, O., Hadchouel, M., Oude Elferink, R., 2001. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 120, 1448–1458.

Johnson, B.M., Charman, W.N., Porter, C.J.H., 2002. An in vitro examination of the impact of polyethylene glycol 400, pluronic p85, and vitamin E D-α-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. AAPS PharmSci 4, 4.

Ketterer, B., Coles, B., Meyer, D.J., 1983. The role of glutathione in detoxication. Environ. Health Perspect. 49, 59–69.

Kalantzi, L., Reppas, C., Dressman, J.B., Amidon, G.L., Junginger, H.E., Midha, K.K., Shah, V.P., 2006. Biowaiver monographs for immediate release solid oral dosage forms: acetaminophen (paracetamol). J. Pharm. Sci. 95, 1.

Khan, S., ElShaer, A., Rahman, A.S., Hanson, P., Perrie, Y., Mohammed, A.R., Genomic evaluation during permeability of indomethacin and its solid dispersion. J. Drug Target., doi:10.3109/1061186X.2010.531730, in press.

Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.

Oude Elferink, R.P.J., Bakker, C.T.M., Jansen, P.L.M., 1993. Glutathione-conjugate transport by human colon adenocarcinoma cells (Caco-2 cells). Biochem. J. 290, 759–764.

Prabhu, S., Ortega, M., Ma, C., 2005. Novel lipid-based formulations enhancing the in vitro dissolution and permeability characteristics of a poorly water-soluble model drug, piroxicam. Int. J. Pharm. 301, 209–216.

Prakash, C., Vaz, A.D.N., 2009. Drug metabolism: significance and challenges. In: Xie, W. (Ed.), Nuclear Receptors in Drug Metabolism. John Wiley & Sons, Hoboken, NJ, USA, pp. 1–42.

Robinson, A.J., Overy, C., Kunji, E.R.S., 2008. The mechanism of transport by mitochondrial carriers based on analysis of symmetry. PNAS 105, 46.

Saeed, A., Bhagabati, N.K., Braisted, J.C., Liang, W., Sharov, V., Howe, E.A., Li, J., Thiagarajan, M., White, J.A., Quackenbush, J., 2006. TM4 microarray software suite. Methods in Enzymology 411, 134–193.

Shimizu, H., Taniguchi, K., Hippo, Y., Hayashizaki, Y., Aburatani, H., Ishikawa, T., 2003. Characterisation of the mouse Abcc12 gene and its transcript encoding an'ATP-binding transporter, an orthologue of human ABCC12. Gene 310, 17–28.

Tibshirani, R., Hastie, T., Eisen, M., Ross, D., Botstein, D., Brown, P., 1999. Clustering Methods for the Analysis of DNA Microarray Data. Technical Report. Department of Statistics, Stanford University, Stanford.

Yabuuchi, H., Takayanagi, S., Yoshinaga, K., Taniguchi, N., Aburatani, H., Ishikawa, T., 2002. ABCC13, an unusual truncated ABC transporter, is highly expressed in fetal human liver. Biochem. Biophys. Res. Commun. 299, 410–417.

Yee, S., 1997. In vitro permeability across caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharm. Res. 14, 763–766.