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Abstract: Gut microbiome, a new organ; represent targets to alter pharmacokinetics of orally administered drugs. Recently, *in vitro* trials endorsed the idea that orally administered drugs interact and some of their quantity may be taken up by normal microbiome during transit through gut. Such transport mechanisms in microbiome may compete for drug with the host itself. Currently, no data confirms specific transport system for paracetamol uptake by gut microbiome. *In vivo* trial was conducted in normal healthy male rats (n=36). Paracetamol was administered orally in a single dose of 75mg/kg to isolate microbial mass after transit of 2, 3, 4, 5 and 6 hours post drug administration. Paracetamol absorbance by microbiome was pursued by injecting extracted microbial lysate in RP-HPLC-UV with C18 column under isocratic conditions at 207nm using acetonitrile and water (25:75 v/v) pH 2.50 as mobile phase. Paracetamol absorbance (14.10±0.75µg/mg of microbial mass) and percent dose recovery (13.16±0.55%) seen at transit of 4 hours was significantly higher (*P*<0.05) compared to other groups. Study confirms the hypothesis of homology between membrane transporters of the gut microbiome and intestinal epithelium. Orally administered drugs can be absorbed by gut microbes competitively during transit in small intestine and it varies at various transit times.

Keywords: Gut microbiome, pharmacokinetics, transit time, microbial mass, microbial lysates.

INTRODUCTION

Paracetamol (acetaminophen, N-acetyl-p-aminophenol) an OTC (over the counter) drug is administered as antipyretic and analgesic in different ailments (Jóźwiak-Bebenista and Nowak, 2014). Following the oral administration it is rapidly absorbed from the gastrointestinal tract with systemic bioavailability ranges from 70 to 90%. Its rate of oral absorption is predominantly dependent on the rate of gastric emptying which can be delayed by food (Forrest *et al*., 1982).

Intestinal microbiome harbors a diversified microbial population with more than 3 million genes that are 150 times more than human genes and regarded collectively as a microbial organ. They weigh about 1 kg that equals the weight of brain that performs many functions which the human host is unable to process individually (Qin *et al*., 2010; Dinan *et al*., 2015). Microbial cell count vary consistently along the axis of gut revealing 10^7 microbes per gram of contents in small intestine and 10^{11} to 10^{12} cells per gram in the colon (O'Hara and Shanahan, 2006). Small intestine is major absorptive site for the nutrients presents Bacilli, Actinobactria, Actinomycinacae and Streptococcaceae in intestinal lumen, attached or embedded in intestinal mucus layer and epithelial crypts as major inhabitant (Swidsinski *et al*., 2005).

These unique populations of microbes are not only vital for the distinctive care of health, but also in dispensation of exogenous compounds (medicines) intended to rectify homeostatic inequities. The comprehension of this latter action of the microbiota has changed the idea of pharmaceutical-microbiota connections, shifting the novel role of only medicines to an obligation of microbiome-medicine interaction. The microbiota and in particular microbiome-encoded enzymes, now represents probable intermediate targets to alter drug pharmacokinetics (absorption, distribution, metabolism and elimination) to subsequently enhance the clinical response. Previously, it was considered that the drugs which are having foremost absorption from the duodenal portion of the small intestine are less likely to be influenced by the gut microbes (Gill *et al.,* 2006). Advancement of drug discoveries lead to the development of drugs which have prolonged stay in the gut and slow release, so these drugs are supposed to be more effected by the gut microbiome (Sousa *et al.,* 2008). Some researchers have reported the alteration in drug absorbance by the gut epithelial cells in the presence of gut microbiota (Forsythe and Bienenstock, 2010; Furrie *et al*., 2005). However, they didn't discuss the possibility of drug uptake by the gut micro biomes itself.

In the absorption of an orally administered drug it must be released from the dosage form, dissolved in the GI fluids,

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remain intact in the intestinal lumen and cross the intestinal epithelial membrane (Sousa *et al.,* 2008). Different anatomical, physiological and biochemical factors affect the dissolution, stability absorption and presystemic elimination of drugs vary greatly throughout the GIT (Lennernas, 2000). Membrane transporters have been recognized recently to be important determinants in regulating drug pharmacokinetics and pharmacodynamics (Rubio-Aliaga and Daniel, 2008). Therefore, Membrane transporters (primary and secondary) play key roles in the influx and efflux of various nutrients and drugs in gut epithelial cells (Kim, 2006). In Gram negative bacteria abundantly found in gut, outer membrane OmpC and OmpF channels in *Escherichia coli* (Mortimer and Piddok, 1993) OmpC, OmpF and OmpD in *Salmonella typhi* (Toro *et al*., 1990) and Omp P1 and P2 from *Haemophilus influenza* (Burns and Smith, 1987; Srikumar *et al*., 1997) are involved in protein mediated transport of drugs. Passive diffusion and secondary transport mechanisms in bacteria may involve uptake of drug into cytoplasm (Lewinson *et al*., 2003; Abdel-Sayed, 1987).

Furthermore, In *E. coli*, four protein transporters (PTR) family members have been characterized: dipeptide and tripeptide YdgR or permease A (DtpA), YhiP, YjdL and YbgH (Harder *et al.,* 2008) for their proven role in the drug pharmacokinetics and pharmacodynamics. All these findings emphasize on potentials of modifying the human physiological state by "drugging the microbiome" (Garber, 2015). These previous studies strongly support our hypothesis however the literature is completely lacking in any report regarding the drug absorbance by these gut microbes in *in vivo* by targeting the gut microbiome with orally administered drug. Results presented by Prabhala (Prabhala *et al.,* 2017) showed commonly used drugs like bestatin, sulpiride, oseltamivir, valacyclovir, ampicillin and levodopa enter the bacterial cell by bacterial POTs in *E. coli in vitro* trials, thereby; suggesting similar transporting mechanisms in gut microbes can be used for uptake of orally administered drug molecules. In the present study, we established that the orally administered drug paracetamol (fig. 1) is a substrate of gut microbiome due to the fact that several gut microbes contain POT (proton-coupled oligopeptide transporter) gene and porins in their genome.

MATERIALS AND METHODS

Animals and housing
Thirty six normal healthy male rats aged 8 to 10 weeks, weighing 150-200gm were selected for the current study from the animal rearing nursery of Department of Physiology, GCUF. The rats were shifted to isolated cages with 25±2°C temperature with 40-60% humidity maintained in the animal station. One week was given to the rats for acclimatization on chow maintenance diet (CMD) averagely consumed at 48g/kg body weight daily

(table 1). All the experiments were conducted after the due permission from the Ethical Review Board for the use of animals in research, Government College University, Faisalabad (Ref. No. GCUF/ERC/130).
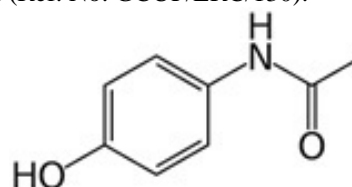

Fig. 1: Paracetamol

Table 1: Composition of diet

Dietary Contents	CMD (Chow Maintenance Diet)
Starch	76%
Protein	10%
Oil	10%
Vitamin and Mineral Mixture *	4%

*Calcium 35%, Folic Acid 0.2%, Copper Sulphate 0.03%, Vitamin A 200000i.u, Phosphorus 32% Iron 0.89%, Selenium 0.08%, Vitamin D 96000i.u, Sodium 9.44, Manganese 0.39%, Cobalt 0.39%, Vitamin E 350i.u, Magnesium 8.64, Zinc 0.22%, Potassium Iodide 0.87%, Vitamin B 0.6% (Vit. B1 350i.u, Vit. B2 85000i.u, Vit. B6 67000i.u, Vit. B12 350i.u).
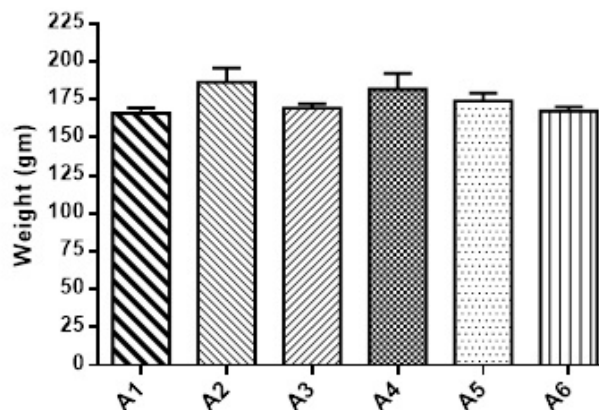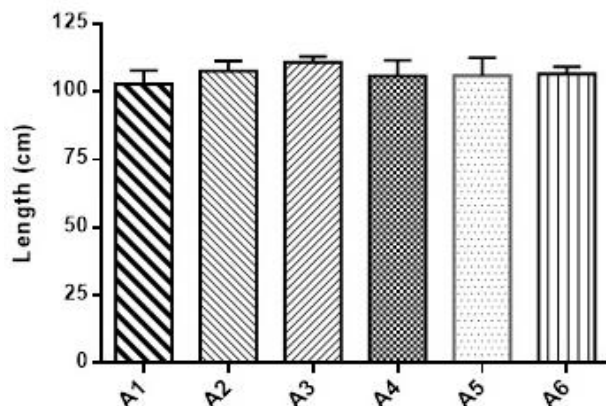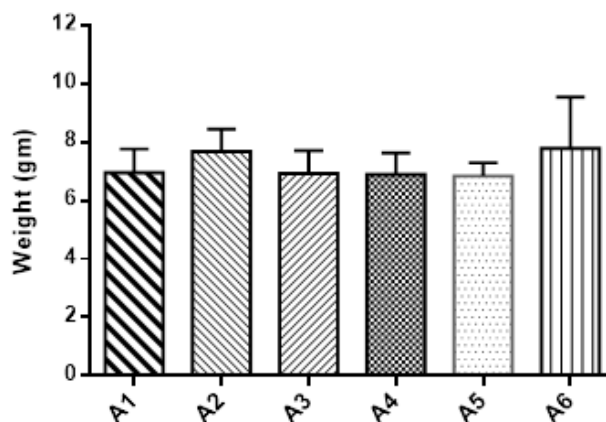

Fig. 2: Body weight (gm ± SEM) measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours.

In vivo trial was conducted by differentiating the rats into the following groups; control A1 (n=6) without any treatment and Paracetamol treated groups (n=30) A2, A3, A4, A5 and A6 with single oral dose (75mg/kg body weight). All the doses were given orally by 16-18 gauge feeding tube about 2-3 inches in length. Six rats in each treated group A2, A3, A4, A5 and A6 were sacrificed at 2, 3, 4, 5 and 6hours, respectively and their microbial mass from small intestine was collected to separate the

pure microbial lysate. Control group (A1) was sacrificed at the start of trial to collect small intestine for isolation of microbial mass.

Fig. 3: Small intestine length (cm ± SEM) measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours.

Fig. 4: Small intestine weight (gm ± SEM) measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours.

Microbial lysate

Methods described by (Upadhaya *et al*., 2012) and (Tong *et al*., 2014) for isolation of gut microbiota were employed with some modification. Immediately after sacrificing the rats, small intestine was incised from end of pyloric sphincter to illeo-caecal junction with sterile scalpel and placed in sterile petri dish. Mean small intestine length (fig. 3) and weight (fig. 4) was measured. Intestine was cut into 5-6cm long segments which were incised longitudinally with a sharp sterile scalpel and shaken vigorously with forceps in petri dish to remove the luminal contents (digesta). Intestinal digesta was shifted to 100ml conical flask and weight of the wet content was measured (fig. 5). Ten ml of ice cold normal saline was added with digesta and vortexed for 5min. To remove the mucosally attached microbes, intestinal segments were gripped with forceps and were washed by pipetting

normal saline in petri dish to remove digesta if present. Intestinal pieces were returned to petri dish and added with normal saline until tissue was submerged and shaken vigorously by forceps. Solution was added in conical flask while intestinal sections were placed in 25ml falcon tubes already filled with 10ml Normal saline and 1ml of 1mM dithiothreitol (DTT) pre-warmed at 37°C. Mixture was placed horizontally in orbital shaker at $180 \times g$ for 40min. Mixture was vortexed for 1min and solution was added in conical flask. Now final solution in original conical flask was vortexed for 5min and filtered through two, four and eight-layer cheesecloth, respectively in clean sterile conical flask. Filtrate was vortexed for 1min and passed through a 70 micron nylon mesh filter that was centrifuged at $14000 \times g$ for 2min. The supernatant was saved and centrifuged at $6000 \times g$ for 20min, supernatant was discarded while sediment was suspended with normal saline to a volume of 10ml and centrifuged at $6000 \times g$ for 20min. Supernatant was discarded, and sediment was resuspended with normal saline to a volume of 10ml. This step was repeated twice to get pellet which was dissolved in 1ml ddH$_2$O and centrifuged at $14000 \times g$ for 20min at 4°C to get the pallet of pure microbial mass (fig. 6). Finally, microbial mass was added with 2ml acetonitrile to lyse the microbes left overnight at -4°C then centrifuged at $14000 \times g$ for 20min. Supernatant was taken and dried with nitrogen gas, it was then dissolved in 800µl of mobile phase and filtered through membrane filters (pore size, 0.45µm, Milli Pore, USA).The filtrate was stored at -20°C till further analysis.
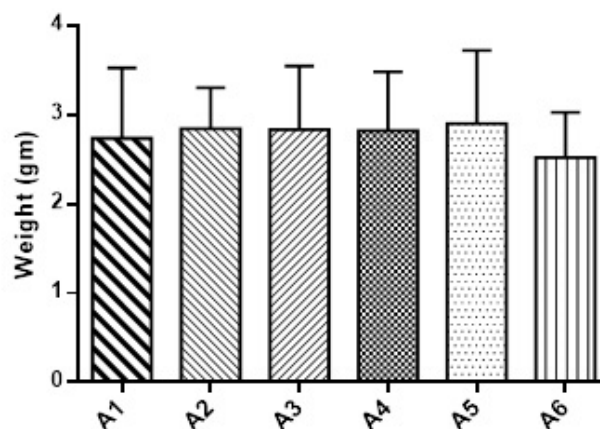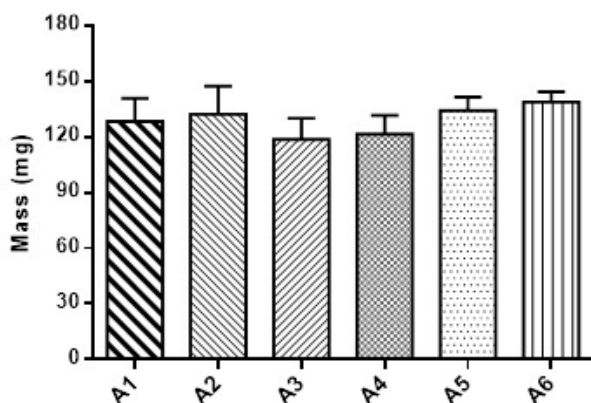

Fig. 5: Wet content (gm ± SEM) measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours.

HPLC system and conditions

Method for HPLC was adopted from Franeta *et al*., (2002) with some amendments. Mobile phase was prepared by dissolving acetonitrile and water (25: 75 v/v) adjusted to pH 2.50 with phosphoric acid. Liquid chromatography consisted of HPLC system (Perkin Elmer, USA.) attached with Flexer Binary LC pump,

UV/VIS LC Detector (Shelton CT, 06484 USA) and reverse phase C18 column (5μm, 250 × 4.6mm) accompanying oven set at 30°C. Chromera software version. 4. 1. 2. 6410 was used to analyze data.

Fig. 6: Microbial mass (mg ± SEM) measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours.
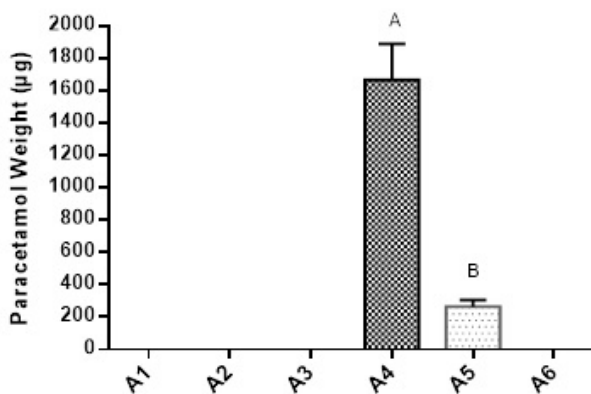

Fig. 7: Total paracetamol absorbance (μg ± SEM) by whole small intestine microbiome measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours. AB alphabets shows significant difference between different groups (*P*<0.05).

Volume of 10μl was injected at a flow rate of 1.5mL/min to measure the drug concentration from calibration curve (fig. 10) prepared using pure paracetamol (HPLC grade) standards (1, 5, 10, 20, 30, 40 and 50μg/ml) purchased from Sigma Aldrich, USA, at given HPLC conditions that is 207nm at retention time of 2.16±0.02min with in run time of 8 min.

STATISTICAL ANALYSIS

For statistical analysis one way analysis of variance (ANOVA) was employed by Graph Pad Prism. 6, setting level of significance at *P*<0.05.

RESULTS

Mean body weight (fig. 2), small intestine length (fig. 3), small intestine weight (fig. 4), wet content weight (fig. 5) and total microbial mass (fig. 6) showed no significant difference (*P*>0.05) among control and treated groups. In the initial screening no drug was detected in group A1 (control), A2, A3 and A6 at 0, 2, 3 and 6hours intestinal transit time of sampling, respectively. However, the drug absorbance was seen only in group A4 at 4hours and A5 at 5hours after sampling. Total paracetamol absorbance was significantly higher (*P*<0.05) in group A4 at 4hours transit time as compared to group A5 at 5hours transit time (fig. 7). Maximum paracetamol absorbance (μg/mg) of microbial mass (fig. 8) was seen in group A4 as compared to the rest of the groups that was significant higher (*P*<0.05). Percent dose recovery was significantly higher (*P*<0.05) in group A4 at 4hours transit time as compared to group A5 at 5hours transit time (fig. 9). Maximum drug absorption was seen in group A4 at 4hours of transit time while maximum transit time was 5hours at which drug was detected. No drug was detected at 6 hours transit time.
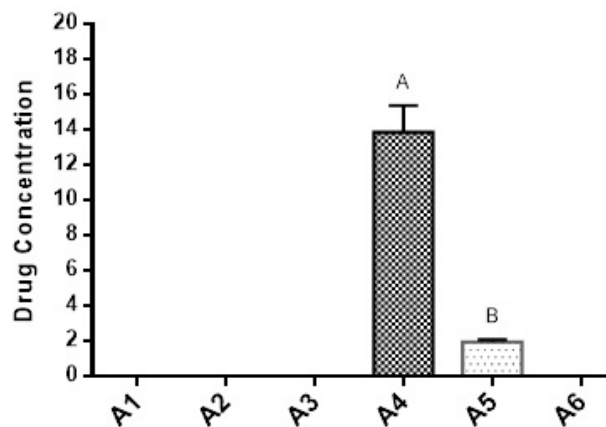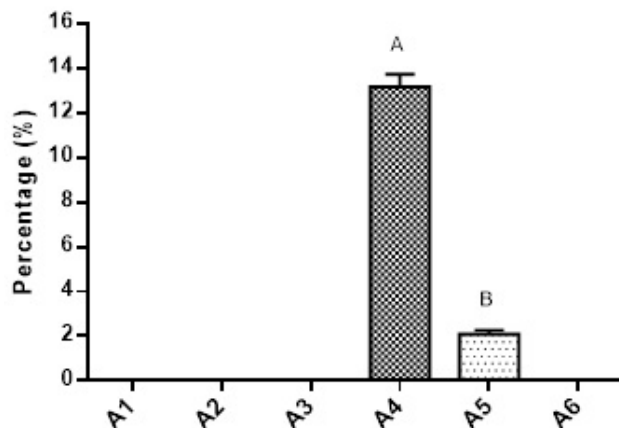

Fig. 8: Paracetamol absorbance (μg/mg ± SEM) of microbial mass measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours. AB alphabets shows significant difference between different groups (*P*<0.05).

DISCUSSION

In the start of the trial, paracetamol was administered to rats by gastric tube to ensure homogeneous solution in the stomach. Gastric emptying time of paracetamol solution is comparable to water. In next 10 min after oral administration about 70% of the paracetamol was available for absorption in small intestine as gastric emptying time of paracetamol is about 10-15 min in solution form, mixed with low caloric diet and high caloric diet (Bartholomé, 2015) with systemic bioavailability ranging from 70 to 90% (Forrest *et al.,* 1982).

Grouping and sampling time was based on intestinal transit time 3.5 ± 1 hour for solution form of drugs that is the maximum time of solution retained in small intestine for absorption in the systemic circulation. Intestinal transit time is an independent parameter irrespective of fed and non-fed state (Davis, 1986). It was hypothesized that during its passage through small intestine paracetamol also interact with resident microbes either present in lumen or attached to mucosal walls of small intestine.

Fig. 9: Percentage dose recovery for given dose of Paracetamol measured in different groups: A1; Control (untreated), groups based upon post drug sampling time: A2; 2hours, A3; 3hour, A4; 4hours, A5; 5hours and A6; 6hours. AB alphabets shows significant difference between different groups ($P<0.05$).
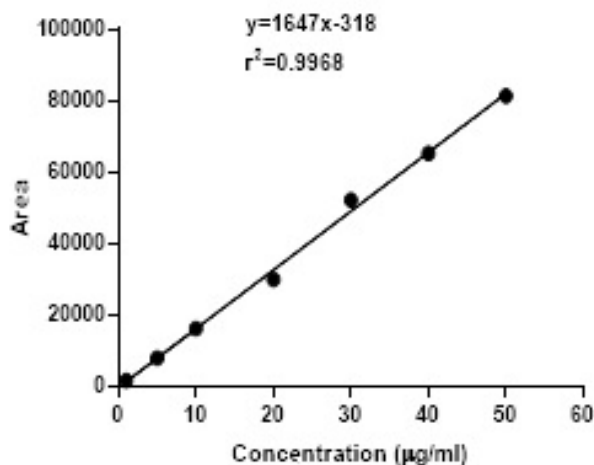

Fig. 10: Calibration curve of paracetamol is linear over the concentration range (1-50 µg/ml) studied.

No significant difference ($P>0.05$) was found among control and treated groups in mean body weight (fig. 2), small intestine length (fig. 3), small intestine weight (fig. 4), wet content weight (fig. 5) and total microbial mass (fig. 6). Non significant difference between above said parameters is a positive indicator as it strengthens our trial's results regarding microbiome absorbance in various groups thereby, minimizing experimental errors between groups.

The small intestine is the major site of absorption for nutrients in the body. Microbial density is not homogenous in small intestine as it is restricted in duodenum due to flushing, bile and pancreatic secretions (O'Hara & Shanahan, 2006) but increases abruptly in ileum (Booijink *et al.,* 2007). In rats microbial composition shows community diversity richness increases from upper to lower GIT segments. The small intestine shows richness in phylum diversification (16 out of 21 Phyla) inhabited predominantly by Firmicutes mainly Lactobacillus, Turicibacter and Streptococcus (Li *et al.,* 2017).

Table 2: Linearity data of Paracetamol

Parameter	Paracetamol
Linearity Range	1-50 µg/ml
Regression equation	$Y= 1647x-318$
Correlation Coefficient (R^2)	0.9968
% Recovery	98.12%.
Reproducibility (%RSD) <2%	0.13
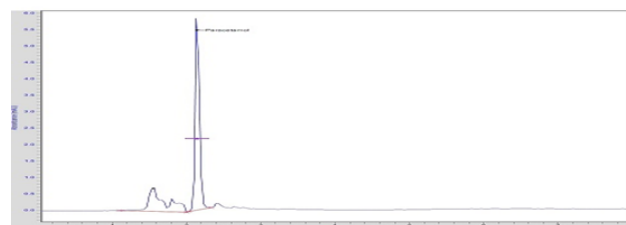
Fig. 11: Chromatogram of paracetamol (20µg/ml) standard.
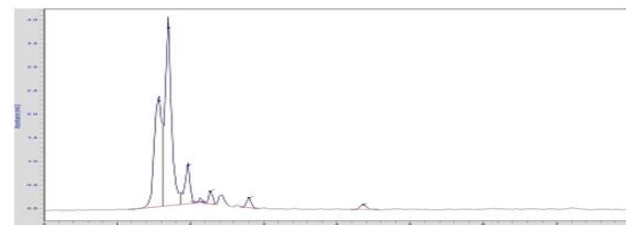

Fig. 12: Chromatogram of blank sample

Current study shows that maximum absorbance of paracetamol by gut microbes was seen at intestinal transit time of 4hours which was reduced as a function of time till transit time of 5hours may be due to competitive absorption between gut microbes and gut epithelial cells of the host. None of the samples at 6hours were detected with drug which shows that maximum transit time of paracetamol is 5hours and drug absorbance by intestinal microbiome is a time dependent response based on intestinal transit time.

Results of the current study show that paracetamol is absorbed by microbial cells by some unknown mechanism either by passive transport or by secondary transport. However, paracetamol is absorbed by epithelial cells in small intestine through passive transport process

(Bagnall *et al*, 1979). The studies on structural homology confirmed the presence of porins in bacterial cell involved in the passive transport of different kind of solutes. Passive diffusion and secondary transport mechanisms in bacteria may involve uptake of drug into cytoplasm (Lewinson *et al.,* 2003; Abdel-Sayed, 1987). Recently, researchers concluded from their *in vitro* studies in *E. coli* cell line that POTs and porins found in the bacterial cells particularly in *E. coli* can transport a number of orally administered drugs to the bacterial cell. They gave a fair suggestion that similar POTs and porins are also present in the gut microbiome which will indeed present a similar type of interaction of gut microbiome with the orally administered drugs (Prabhala *et al.,* 2017).
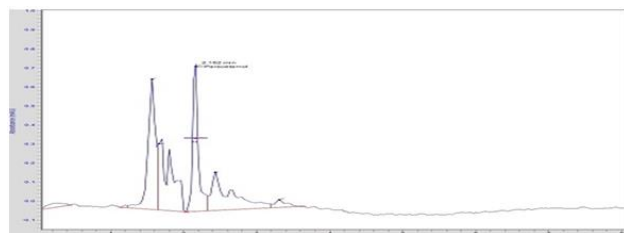
Fig. 13: Chromatogram showing paracetamol in microbial lysate extracted from microbiome after intestinal transit time of 5hours.

Moreover, their findings suggest a homology between transporting mechanism in bacterial cell membrane and epithelial cells of the gut of the host which further emerged a possibility of drug uptake competition between these two transport mechanisms which may lead to the decreased absorption of some drugs in the gut epithelium. This recent finding became a base for the current project to test this idea in the *in vivo* experimental models. The drug uptake was seen in the paracetamol fed group based upon the theory of homology between transporting mechanisms in microbial cell and epithelial cell. However, the drug uptake was detected at 4 and 5hours only which shows that this drug uptake is very well related to the drug intestinal transit time. No drug uptake was seen in control group, 2, 3 and 6hours which endorsed the findings that HPLC-UV analyses are fair enough to verify the results. Volume of 10µl for each sample was run in triplicate to find the mean peak area at retention time of 2.16±0.02min for run time of 8 min. HPLC method was validated by linearity (r^2=0.9968) assessed by linear regression analysis which was calculated by least square method. Series of working solution (7 points) ranging from 1 µg/ml to 50 µg/ml for paracetamol were injected in triplicate and mean value of peak area was obtained for calibration curve (fig. 10) that indicates good correlation exists between peak areas and paracetamol concentrations. Accuracy was studied that indicate differences between peak area of spiked microbial lysate control samples with known concentration (20µg/ml) of paracetamol and standard working solution. Mean recovery of paracetamol was

98.12% within the range of 98-100%. Reproducibility was 0.13% expressed as relative standard deviation (%RSD) <2% obtained by analyzing six replicate of 20µg/ml showing RSDs value in table 2; are in line with Franeta *et al.,* (2002). In current study, maximum dose recovery was 13.16±0.55% seen at transit time of 4hours that was significantly higher (*P*<0.05) than 2.09±0.18% at transit time of 5hours while bioavailability of paracetamol after oral administration is 70 to 90% (Forrest *et al.,* 1982). Percent dose recovery of paracetamol from gut microbial lysate in current study can be a possible answer to the missing drug amount in systemic circulation for effective therapeutic response.

CONCLUSION

Orally administered paracetamol was absorbed by gut microbes through primary as well as secondary transport mechanisms maximally at intestinal transit time of 4hours which confirms the idea of homology between transport mechanisms in microbial membranes and epical membrane of epithelial cells in small intestine. However, Maximum transit time of paracetamol in small intestine is 5hours. Rate of drug absorbance by microbes is reduced as a function of prolonged intestinal transit time after 4hours, providing a competitiveness in drug absorbance mechanisms in microbes and epithelial cells in small intestine of the host. This study led to the development of "*in vivo* microbial drug absorption assay" that has not been addressed so far in scientific work. One of the possible strategies to increase the bioavailability of orally administered drugs may be the inhibition of microbial absorbance of drugs by some substance. This also leads to the idea to explore the other orally administered drugs with a possibility of microbial absorbance in gut.

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