AJAB

Asian J Agric & Biol. xxxx(x).
DOI: 10.35495/ajab.2024.121

Original Article

Unlocking the potential: exploring the gut microbiome's ability to absorb the antihypertensive Enalapril

Saima Malik[1], Imran Mukhtar[1], Humaira Muzaffar[1], Laaraib Nawaz[1], Haseeb Anwar[1*]
[1]Health Biology Research Lab (HBRL), Department of Physiology, Government College University, Faisalabad, Pakistan

Received:
June 20, 2024
Accepted:
October 14, 2024
Published Online:
November 11, 2024

Abstract

The gut microbiome potentially modulates pharmacokinetics of orally administered drugs. Homologous transporting proteins in epical membrane of the enterocytes and cell membrane of the residing microbial cells of the host may compete for absorption of the orally administered drugs. Microbial cells residing the small intestine of the host may uptake/bio-accumulate some of the quantity of the dose of orally administered drug. This project is aimed to observe absorption/bio-accumulation behavior of enalapril by the gut microbiome when enalapril was administered orally in pure form and in the presence of excipients (Tablet; commercial preparation). Currently, no data confirms specific transport system for enalapril uptake by gut microbiome in absence and presence of excipients as well. Two *in-vivo* trials, enalapril pure drug treated trial and enalapril commercial tablet treated trial were conducted in parallel. Each trial was conducted in adult *Wistar albino* rats (n=42) divided into seven groups having same number of rats in each group (n=6); one control group and six drug treated groups administered orally with single dose of enalapril 10mg/kgbwt. Rats (n=6) were subsequently sacrificed at different intestinal transit times of 1, 2, 3, 4, 5 and 6 hours post drug administration to harvest microbial mass pellet from digesta. Pellet was lysed to expose microbial lysate and pursued through HPLC. The microbiome absorbed enalapril at 4hour transit time (103±7.31µg) significantly ($p \leq 0.05$) higher as compared to 5hour transit time (73.2±5.17µg). Percent dose recovery from microbiome was significantly ($p \leq 0.05$) higher at 4hour transit time (4.15±0.05%) as compared to 5hour transit time (3.14±0.18%) post drug administration. Independent of presence of excipients, from both formulations enalapril was absorbed in equal amount competitively by the intestinal microbiome through the homologous transport mechanism present in the enterocytes of the host. Conclusively, enalapril serves as a substrate of gut microbiome independent of dosage form when administered orally.

Keywords: Enalapril, Microbiome, Microbial lysate, Percent drug recovery

How to cite this:

Malik S, Mukhtar I, Muzaffar H, Nawaz L and Anwar H. Unlocking the potential: exploring the gut microbiome's ability to absorb the antihypertensive Enalapril. Asian J. Agric. Biol. xxxx(x): 2024121. DOI: https://doi.org/10.35495/ajab.2024.121

**Corresponding author email:*
drhaseebanwar@gcuf.edu.pk

Introduction

Enalapril has a chemical formula of $C_{20}H_{28}N_2O_5$, and its structure is depicted in Figure 1. Normal human gut flora consists of 100 trillion of microbial cells to contribute in different physiological functions in nutrient rich microenvironment. Breakdown of indigestible food is one of the major role, which cannot be accomplished in the absence of members of gut microbiome (Colella et al., 2023). In composition, more than 90% microbiome consists of anaerobic *Firmicutes* (60-80%) as Gram-positive and *Bacteriodetes* (20-30%) as Gram-negative while viruses, fungi archea are present less than 1% (Di Pierro, 2021; Coker, 2023). Living beings and surrounding environmental factors influence each other (Ekici et al., 2023). Moreover, supplementation of probiotics improves the gut inhabitant as health biomarkers and promotes fermentation leading to enhanced immunity against diseases (Averina et al., 2021; Coniglio et al., 2023).

Microbiome is a potential target to improve drug efficacy and safety leading to emergence of pharmacobiomics (Doestzada et al., 2018). Intra and inter-individual variability markedly impact the shape of microbiome helping in better understanding to develop new chemical entities (NCE's) according to individual's biome typing (Mussap et al., 2020). Recently found that 24% of 1000 tested drugs can inhibit one of the microbial population residing the gut (Maier et al., 2018). Resident microbes of the host should be considered while considering the pharmacodynamics and pharmacokinetics of a drug (Torres-Carrillo et al., 2023).

Protein transporters in the cell membrane exist in all kingdoms of life to play a key role in transport of different nutrients through primary as well as secondary mechanisms (Prabhala et al., 2017). Absorption and metabolism of orally administered are also regulated by drug transporters (Rubio-Aliaga and Daniel, 2008). So, transporting proteins found in cell membrane are responsible for uptake as well as efflux of various solutes by the cell (Pizzagalli et al., 2021). Categorically, such transport proteins are classified as ATP binding cassettes (ABC) and solute carrier (SLC) that mediate the transport of different substrate across cell membrane (Dobson and Kell, 2008).

Human PepT1 are oligopeptide transporters found in apical membrane of the brush border present in intestinal epithelium and functionally involved in the active transport of both various dipeptides, tripeptides and peptidomimetic drugs (Nielsen et al., 2002). Human PepT1 (hPepT1) mechanistically couples the transport of different substrate uphill with downhill movement of protons in electrochemical gradient (Brandsch et al., 2008). Human PepT1 exhibits a high degree of homology with its orthologs present in the mouse with an amino acid identity of 83% (Urttiac et al., 2001; Fei et al., 2000). Transporter YdgR and its orthologous proteins present in bacterial genome are homologous to hPepT1 responsible for uptake of peptidomimetic drugs (Prabhala et al., 2017).

Physiochemical properties of drugs define the fate of its influxed or efluxed amount (Biegel et al., 2006). Acetaminophen (Paracetamol, N-acetyl-p-aminophenol) is used as over the counter (OTC) worldwide as analgesic and antipyretic drug (Jóźwiak-Bebenista and Nowak, 2014). When administered orally, paracetamol in its pure form is readily absorbed with systemic bioavailability (70-90%). Rate of gastric emptying time majorly determines its rate of oral absorption that can be delayed by ingestion of food (Effinger et al., 2019). In recent trial, it has been proven that 13.64% of oral dose of paracetamol was absorbed by gut resident microbiome during its transit through small intestine (Mukhtar et al., 2019). Likewise, 3.91% of oral dose of pure sulpiride was also found inside the microbiome (Mukhtar et al., 2021a). Recently, the microbiome is declared as a new body organ system due to different functions it performs as group behavior of various species as well as microbiome-drug interaction (Anwar et al., 2019; Anwar et al., 2024).

Oral drug administration and microbiome bidirectional interactions outline clear motif to control pharmacobiomics of hypertension (Chen et al., 2022). Excipients contribute more than 90% as binder, coloring agents, sweetener and filler in the final dosage form (Subramaniam et al., 2023). Role of active ingredients in pharmaceutical dosage form have been investigated but interaction between inactive excipients and microbiome is still overlooked.

The gut microbiota is associated with hypertension as primary risk contributing to cardiovascular diseases (Gao et al., 2024). Enalapril is extensively metabolized in the liver to inhibit angiotensin-converting enzyme (ACE) and lowers blood pressure (Jackson and Bellamy, 2015). The current study investigated the absorption kinetics of enalapril by

the gut microbiome of rodent model in the absence and presence of excipients (Tablet, commercial preparation).

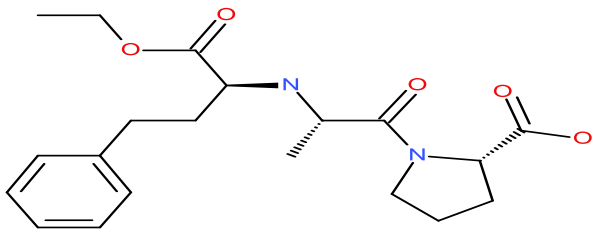

Figure-1. Enalapril 2D structure by ChemDraw.

Material and Methods

Animal and housing

Wister albino male healthy rats (n=84) aged 8 weeks and weighed 180±20 gm were randomly selected for current study. Rats were fed on rodent chow diet, reared in isolated cleaned cages in isolated room maintained at 25 ± 2 ℃, 12-hour light-dark period with maintained humidity (40-60%) in the animal nursery present in the Department of Physiology, Government College University, Faisalabad. Experimental rats were offered with autoclaved rodent diet and water observing acclimatized for a period of seven days. Experiment were proceeded under the consent of ERC (Ethical Review Committee) Refer No. GCUF/ERC/315, Government College University, Faisalabad.

Study design

Two *in vivo* experiment trials were conducted one with enalapril pure drug (HPLC grade pure enalapril maleate (reference standard, product 1235300 USP) from Sigma-Aldrich, USA and other with enalapril commercial preparation brand named as Renitec 10mg tablet (OBS Pharma, Pakistan) from local market to compared enalapril absorption by the gut microbiome at different transit time in the absence and presence of excipients. Rats (n=84) after acclimatization period of seven days, were divided into two experimental trials; enalapril pure drug treated trial (n=42) consisting of seven groups; one control group (A0, n=6) and six enalapril pure drug treated groups labelled as A1, A2, A3, A4, A5 & A6. While second experiment, enalapril commercial drug treated trial (n=42) comprised of seven groups; control group (A0C, n=6) and six commercial enalapril (tablet) treated groups designated as A1C, A2C, A3C, A4C, A5C & A6C. Each group contained same animal (n=6). Drug treated groups were categorized based upon time intervals of 1, 2, 3, 4, 5, and 6 hours at which groups were sacrificed post drug administration. Enalapril (10mg/kgbwt) https://www.ema.europa.eu/en/documents/referral/renitec-article-30-referral-summary-product-characteristics_en.pdf in sterile solution was administered to fasted rats of various subgroups orally by gastric feeding tube (16-18 gauge about 2-3 inches in length). Enalapril treated groups in both pure drug and commercial drug treated trials were sacrificed subsequently to collect the small intestine at drug transit time of 1, 2, 3, 4, 5 and 6 hours, respectively while control groups (A0 & A0C) were killed at the start of the experiment. Control groups in both trials were orally administered with sterile water (1mL) only without any drug and sacrificed at the very start of the experiment to collect small intestine at 0 hour intestine transit time. Microbial mass pellet was harvested from gut digesta by following procedure.

Microbial lysate for detection of drugs from normal microbiome

Microbial mass pellet was harvested described previously (Mukhtar et al., 2019). Briefly, Small intestine was incised to small portions and shaken vigorously in cold saline water to remove the digesta present in the lumen. Solution was vortexed and filtered through two, four and eight layered cheese cloth respectively in autoclaved flask. Filtrate was filtered by mesh filter (70-micron nylon) and centrifuged at 14000 × g for 2 minutes. Supernatant was saved while pellet was discarded. Supernatant was centrifuged for 20 minutes at 6000 × g to get pellet which was dissolved in 10 ml of normal saline and again centrifuged for 20 minutes and at 6000 × g. This step was repeated thrice to finally obtain the microbial mass pellet. Acetonitrile 2ml was finally added in the microbial mass pellet to prepare microbial lysate and stored overnight at -4℃ then again centrifuged for 20 minutes at 14000 × g. Collected supernatant was dried by the help of nitrogen gas and mixed with mobile phase (1000µl) and then after filtering through the membrane filters (pore size, 0.45 µm, Milli Pore, USA). This filtrate was preserved at -20℃ for further analysis.

HPLC system and conditions

Method for HPLC suggested previously (Sultana et

al., 2013) was used under isocratic conditions with some modifications. Mobile phase used in this procedure was methanol and acetonitrile and water (40: 50: 10 v/v). Liquid chromatography consisted of HPLC system (Perkin Elmer, USA.) which is attached with the Flexer Binary LC pump, column of reverse phase C18 (5µm, 250 × 4.6mm), detector of UV/VIS LC (Shelton CT, 06484 USA) and accompanying oven which was set at 30℃ temperature. For the data analysis software chromera, version 4. 1. 2. 6410 PerkinElmer, Waltham, MA, USA was used. To measure the concentration of enalapril, standard curve was constructed by injecting10µl of standard solution in HPLC system at the rate of flow 0.8/ml/min at wave length of 230nm (Figure 2A). Standard concentrations of enalapril (0.5, 1, 5, 10, 20, 50 and 100µg/ml) were prepared by using the pure enalapril (HPLC grade) purchased from Sigma Aldrich, USA. The conditions for HPLC wave length was set at 230nm at retention time 3.81±0.02 with run time of 10min. Range of linearity 0.5-100µg/mL, regression equation Y=5972X-873.4, coefficient of correlation (R2) 0.997 and % age recovery 96.4% were calculated. Representative chromatograms of enalapril 20µg/mL (Figure 2B), sample harvested at 4hour post oral administration enalapril pure drug (Figure 2C), sample harvested at 5hour post oral administration enalapril pure drug (Figure 2D), sample harvested at 4hour post oral administration enalapril tablet commercial preparation (Figure 2E) and sample harvested at 5hour post oral administration enalapril tablet commercial preparation (Figure 2F) are shown in Figure 2 respectively.

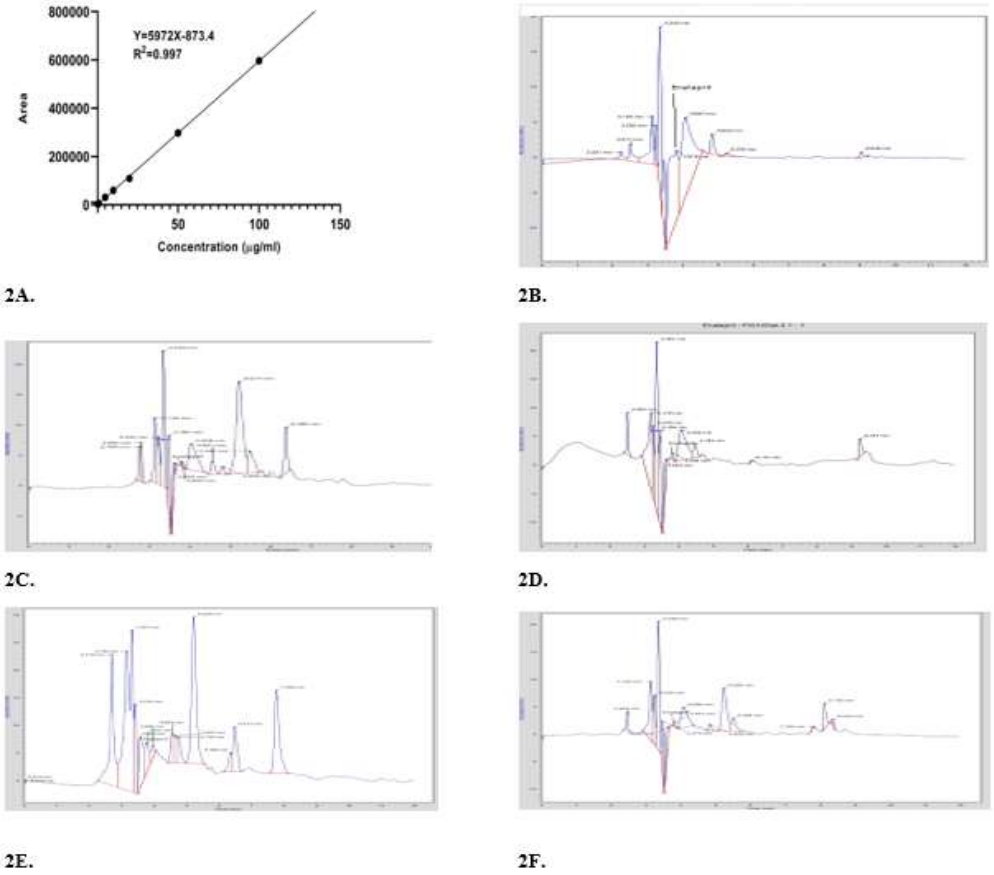

Figure-2. 2A. Enalapril calibration curve seven point (0.5-100µg/mL), 2B. Chromatogram of enalapril (20µg/mL), 2C. Chromatogram of sample harvested at 4 hour post oral administration enalapril pure drug (10mg/kgbwt). 2D. Chromatogram of sample harvested at 5 hour post oral administration enalapril pure drug (10mg/kgbwt). 2E. Chromatogram of sample harvested at 4 hour post oral administration enalapril commercial tablet (10mg/kgbwt). 2F. Chromatogram of sample harvested at 5 hour post oral administration commercial enalapril tablet (10mg/kgbwt).

Molecular docking

In molecular docking, we used Autodock4, Autogrid4, and MGLtools software (Morris et al., 2009; Zentgraf et al., 2009). We obtained the crystal structure of the peptide transporter DtpA-nanobody membrane protein *Escherichia coli* K-12, Lama glama as 6GS4 is crystal structure for DtpA (YdgR) dipeptide transporter in cell membrane of *E. coli*. PDB ID: 6GS4 from the RCSB protein data bank https//www.rcsb.org/6GS4 and prepared it using BIOVIA's Discovery Studio Visualizer (Visualizer DS, 2005) and Autodock tools (Morris et al., 2009). We removed heteroatoms, co-crystal ligand and solvent molecules, and optimized the protein for docking. Ligands were drawn in ChemDraw Ultra, energy minimized in Chem3D Pro (CambridgeSoft, 2009) and transformed into pdbqt files using OpenBabel GUI (Ferreira et al., 2015). The ligand was docked into the protein's active site using the Autodock vina (Yusuf et al., 2008; Eberhardt et al., 2021). Finally, BIOVIA's Discovery Studio Visualizer was used to analyze ligand-protein interactions.

Statistical analysis

The collected data were first tested for normality using the Shapiro-Wilk test to ensure they followed a normal distribution. The collected data were presented as Mean ± SEM, and statistical analyses were performed using two-way analysis of variance (ANOVA) to assess the interaction effects between treatment type (pure enalapril vs. commercial preparation; Tablet of enalapril) and time (1 to 6 hours post-administration). Post-hoc Dunnett's test was applied to compare the drug-treated groups to the control group. Principal Component Analysis (PCA) was conducted to reduce the dimensionality of the dataset and identify key contributing variables across experimental conditions, with the first two principal components explaining 60.53% of the variance (F1: 37.22%, F2: 23.31%). The factors considered in the analyses included microbial mass, enalapril absorption, and other physiological parameters. A p-value of ≤ 0.05 was considered statistically significant, revealing a significant increase in enalapril absorption by the microbiome at the 4hour transit time compared to the 5hour time point ($p \leq 0.05$). These results highlight the time-dependent absorption pattern of enalapril, independent of the formulation type. Analyses were performed using Graph Pad Prism 10.2.3 (San Diego, CA, USA).

Results

Absorbance of enalapril by the microbiome

The microbiome residing the small intestine maximally absorbed enalapril from pure drug treated group (103±7.31µg) and commercial tablet treated group (97.20±9.37µg) at 4hour transit time was found significantly ($p \leq 0.05$) higher as compared to the quantity of the enalapril absorbed by the microbiome from pure drug treated group (73.2±5.17µg) and commercial drug treated group (74.8±3.27µg) at 5hour transit time post drug administration. None of the microbiome samples absorbed the enalapril isolated from control, 1, 2, 3 and 6hour post drug administration in pure enalapril treated group and commercial enalapril treated groups. Maximum enalapril in pure drug treated group and commercial enalapril drug treated group was bio-accumulated by the microbiome at 4hours sampling time post drug administration however enalapril absorbance in the microbiome was continued till 5 hours post drug administration in both enalapril pure drug treated group and commercial enalapril drug treated group. Enalapril absorbance by the intestinal microbiome continued maximally at 4hours transit time but continued till 5hour transit time post drug administration (Figure 3). Enalapril absorbance per mg of microbial mass of intestinal microbiome harvested from pure enalapril treated group (2.84±0.05 µg) and commercial drug treated group (2.79±0.04 µg) was significantly ($p \leq 0.05$) higher at 4hour transit time as compared to pure drug treated group (1.48±0.18 µg) and commercial drug treated group (1.14±0.18 µg) at 5hour transit time post drug administration (Figure 4). Percent dose recovery of enalapril from the intestinal microbiome harvested from pure enalapril treated group (4.15±0.05%) and commercial drug treated group (3.83±0.04%) was significantly ($p \leq 0.05$) higher at 4hour transit time as compared to pure drug treated group (3.20±0.18%) and commercial drug treated group (3.14±0.18%) at 5hour transit time post drug administration (Figure 5). Enalapril bio-accumulation in the intestinal microbiome at group behavior initiated at 4 hours and continued till 5hour transit time post oral administration in both enalapril pure drug treated group and commercial enalapril tablet oral formulation treated group. None of the microbiome samples absorbed the enalapril in both pure and commercial enalapril treated group harvested at 6hour transit time post drug administration.

Mean body weight, small intestine length, small intestine weight, wet content weight and microbial mass weight (Table 1) were found non-significant among control and various treatment groups.

Table-1. Different physical parameters measured in control and enalapril pure treated groups (P) and enalapril tablet as commercial preparation treated group (C) at different transit times post drug administration.

Groups; Transit time (hours)	Body weight (g)		Small intestine length (cm)		Small intestine weight (g)		Wet content weight (g)		Microbial mass (mg)	
	P	C	P	C	P	C	P	C	P	C
A0** (control)**	166.31 ±2.10	172.80 ±1.15	102.32 ±2.04	107.34 ±1.73	5.01 ±0.36	4.52 ±0.12	2.11 ±0.21	2.24 ±0.11	105.13 ±7.18	117.11 ±9.88
A1; 1hour	183 ±10.12	187.52 ±14.23	110 ±1.47	106 ±1.20	5.34 ±0.06	6.51 ±0.38	1.99 ±0.11	1.28 ±0.11	115 ±0.32	121 ±0.63
A2; 2 hours	168.72 ±5.65	172.20 ±3.10	104.41 ±1.30	101.52 ±3.40	5.04 ±0.45	6.21 ±0.37	1.94 ±0.28	1.89 ±0.43	109 ±17.03	116 ±24.04
A3; 3 hours	170.34 ±5.23	169.41 ±5.23	107.15 ±4.24	104.41 ±2.61	4.16 ±0.53	5.01 ±0.41	2.40 ±0.16	2.25 ±0.40	125.33 ±11.60	117.61 ±12.73
A4; 4 hours	169.56 ±3.76	164.73 ±3.76	106.70 ±1.83	102.34 ±1.83	4.85 ±0.17	6.01 ±0.23	1.95 ±0.11	1.95 ±0.39	129 ±14.87	118 ±9.86
A5; 5 hours	165.41 ±2.66	174.80 ±2.66	99.66 ±2.52	105.14 ±2.52	6.24 ±0.19	5.78 ±0.34	2.14 ±0.47	2.56 ±0.51	114.45 ±6.04	136.32 ±4.84
A6; 6 hours	170 ±5.51	176.10 ±4.19	103.24 ±1.95	107.22 ±1.46	6.20 ±0.32	5.72 ±0.70	2.13 ±0.24	2.27 ±0.51	135.52 ±7.43	121.54 ±5.14

P*= Pure enalapril treated group, C**= Commercial enalapril treated group, ***N=6

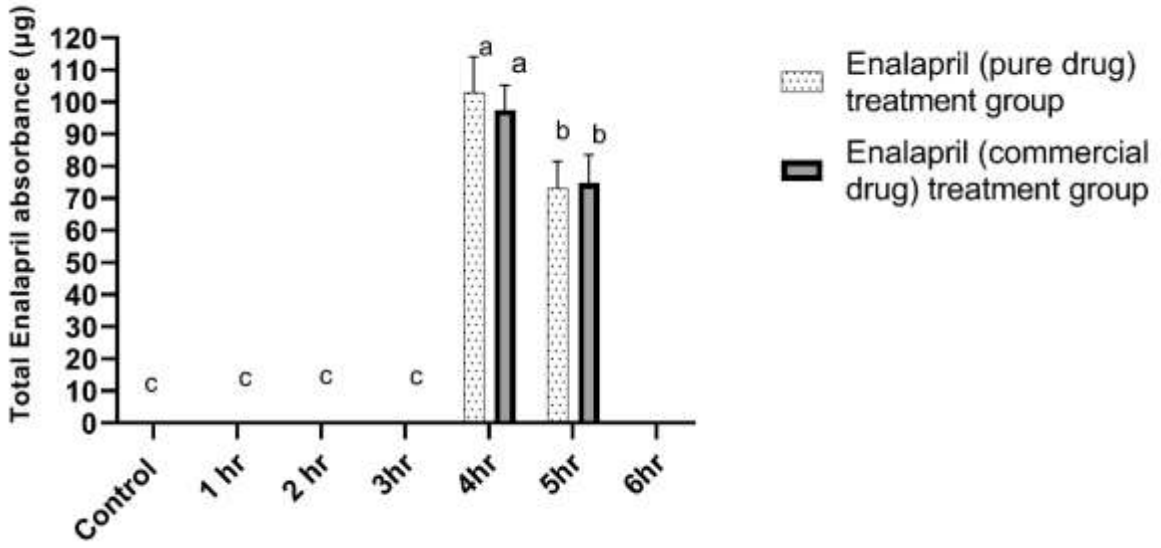

Figure-3. Total enalapril absorbance (µg±SEM) of rats (n=6) in control group (hour=0) and different treatment groups at sampling time of hour=1, hour=2, hour=3, hour=4, hour=5 and hour=6 post drug administration (10mg/kgbwt).

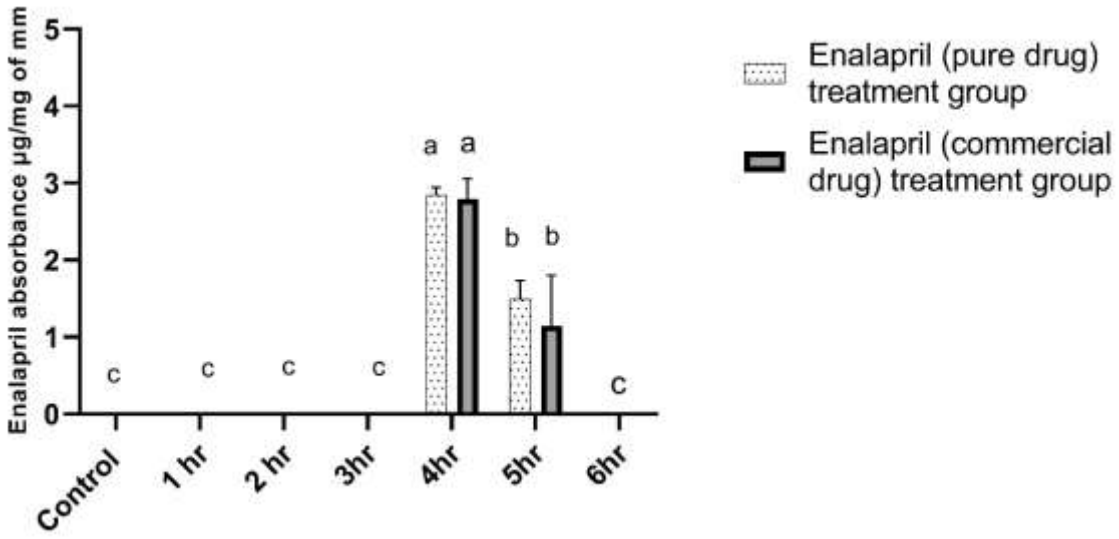

Figure-4. Mean enalapril absorbance (µg/mg of microbial mass ±SEM) of rats (n=6) in control (hour=0) and different treatment groups at sampling time of hour=1, hour=2, hour=3, hour=4, hour=5 and hour=6 post drug administration (10mg/kgbwt).

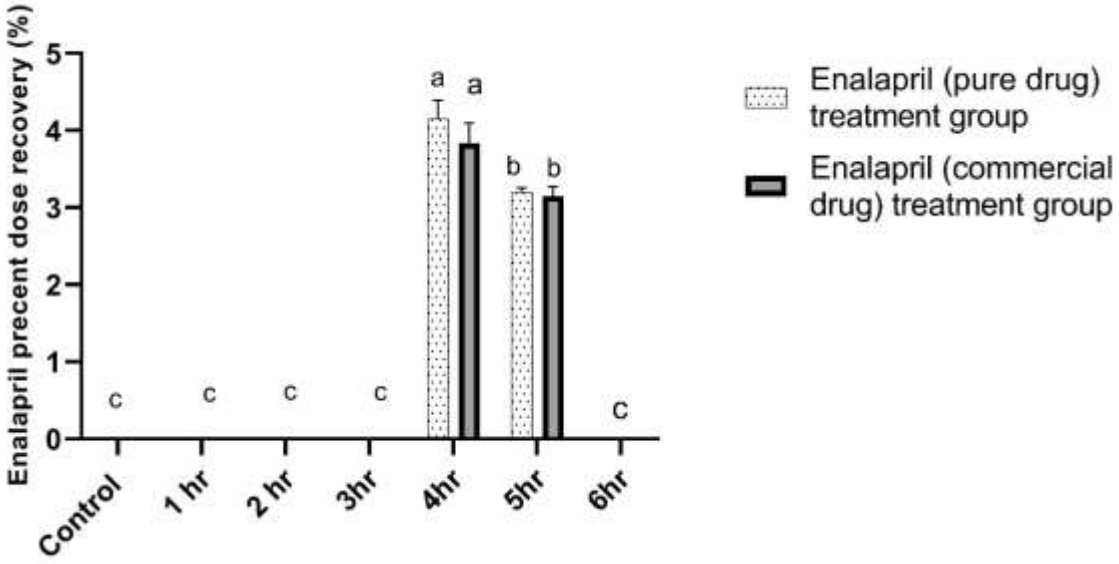

Figure-5. Percent dose recovery of enalapril (%±SEM) of rats (n=6) in control (hour=0) and different treatment groups at sampling time of hour=1, hour=2, hour=3, hour=4, hour=5 and hour=6 post drug administration (10mg/kgbwt).

Molecular docking and binding afinity

The Table 2 shows the binding affinity, hydrogen-binding, hydrophobic, and electrostatic interactions of enalapril with the membrane protein *Escherichia coli K-12*, Lama glama PDB ID: 6GS4. The binding affinity is reported as the change in free energy (ΔG) in units of kcal/mol. Enalapril has a binding affinity of -7.2 kcal/mol, which is more negative. A more negative binding affinity indicates a stronger binding between the ligand and the protein. Enalapril shows several hydrogen-binding interactions with the protein as shown in Figure 6, including THR427, ILE399, PHE289, TYR71, TYR292, and ALA285, located at distances ranging from 3.06 to 5.41

Angstroms. Hydrogen bonds are strong, directional interactions that can contribute significantly to ligand binding, and thus, these interactions may contribute to Enalapril's strong binding affinity. Enalapril also interacts with the protein through hydrophobic interactions with several residues, including PHE288, VAL74, and LEU402, located at distances ranging from 4.35 to 5.83 Angstroms. Hydrophobic interactions are non-polar interactions between hydrophobic groups and can also contribute to ligand binding. Finally, Enalapril also shows electrostatic interactions with LYS130, located at a distance of 4.86 Angstroms. Electrostatic interactions are attractive interactions between oppositely charged groups and can also contribute to ligand binding nd suggset enalapril as a suitable substrate of bacterial transporer YdgR. These results provide insights into the molecular mechanisms of ligand binding to the membrane protein *Escherichia coli K-12*, Lama glama PDB ID: 6GS4, and can inform the design of new ligands with improved binding affinity and selectivity. Statistically, Extracted component F1 contributed (37.22%) followed by F2 (23.31%) with total contribution of 60.53%. However, body weight, small intestine weight, microbial mass and wet content weight negatively correlates with given dose, total drug absorption and percent dose recovery as shown in Figure 7.

Table- 2. Binding affinity (kcal/mol), hydrogen binding, hydrophobic and electrostatic interactions with distances in Angstrom for investigated ligands Enalapril and protein co crystalline ligand as standard Valganciclovir using membrane protein *Escherichia coli K-12*, Lama glama PDB ID: 6GS4.

Ligands with 6GS4	Binding Affinity, ΔG (kcal/mol)	Hydrogen-Binding Interaction, Residue (Distance Å)	Hydrophobic Interaction, Residue (Distance Å)	Electrostatic Interaction, Residue (Distance Å)
Enalapril	-7.2	THR427(3.06)	ILE399(3.87) PHE289(4.16) VAL74(4.35) LYS130(4.86)	PHE288(5.83) TYR71(5.37) TYR292(5.41) ALA285(5.17) LEU402(5.18)

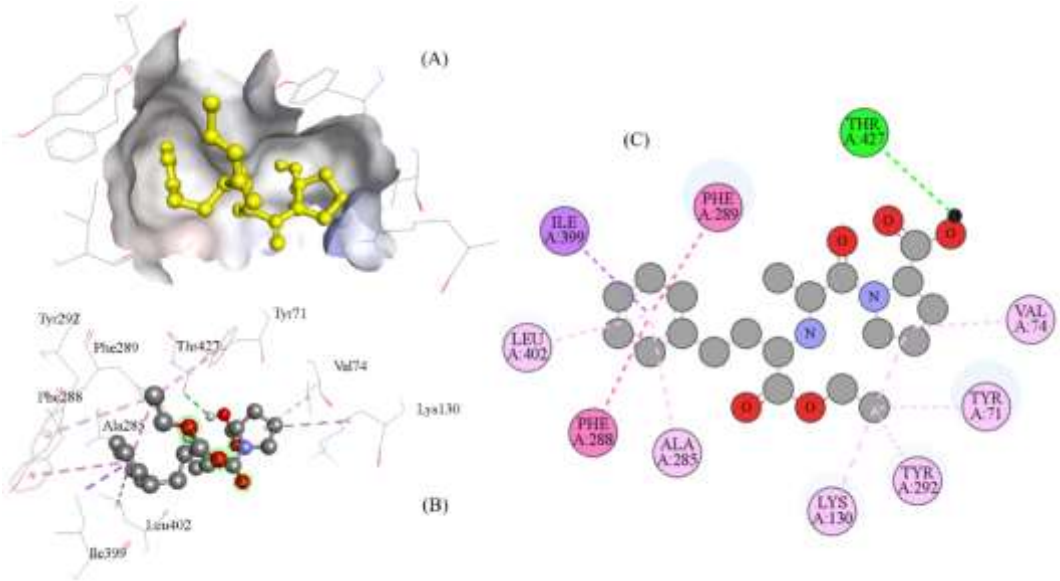

Figure-6. Different interactions of enalapril (A) Binding pocket (B) amino acid interactions with surface and (C) 2D interaction diagram after molecular docking using 6GS4 target prote.

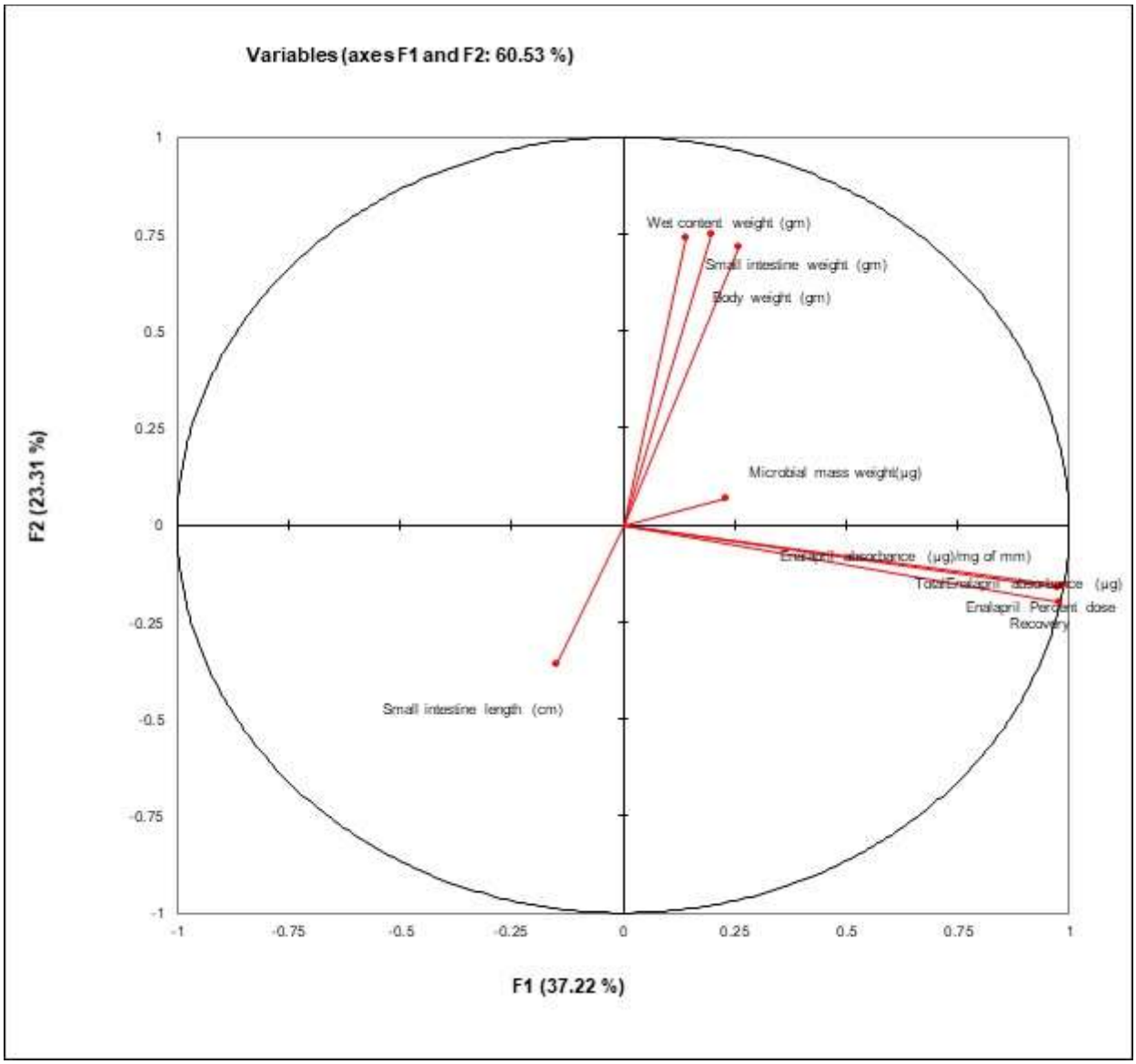

Figure-7. PCA showing various parameters for a single orally administered dose of enalapril (pure) measured in untreated (control) and treated groups (10 mg/kg of body weight) at different transit times. MM=Microbial mass.

Discussion

Previously, it has been established that the gut microbiome actively bio-transform many orally administered drugs as it secretes microbial enzymes in the gut lumen thereby affecting both pharmacokinetic and pharmacodynamics resulting in altered clinical response (Stojančević et al., 2014). Moreover, slow release drug formulations stay for longer time in gut, so metabolized to greater extent (El Aidy et al., 2015).

In current study, we determined the quantity of enalapril, angiotensin converting enzyme inhibitor, bio-accumulated in the microbiome during different transit time in the small intestine after oral administration of pure enalapril drug and commercially available tablet as Renitec.

In recent studies, the gut microbiome absorbed paracetamol (Mukhtar et al., 2019) Sulpiride (Mukhtar et al., 2021a) caffeine (Mukhtar et al., 2021b) and phenobarbital (Mukhtar et al., 2022) were proven substrate and absorbed by the gut microbiome as dose recoveries varies at varied transit time in small intestine when administered in their pure form.

Microbial diversity in gut depends upon, age, gender, geography, nutrition and racial characteristics (Hollister et al., 2014). In current study we observed the strict protocol ensuring same gender, feed and breed raise locally.

The intestinal microbiome bio-accumulated maximum and same quantity of enalapril from both pure enalapril group (103±7.31µg) and commercial enalapril (Renitec) treated group (97.20±9.37µg) at same 4hour transit time post drug administration. However, enalapril bio-accumulation in the intestinal microbiome was observed to decline at 5hour transit time post drug administration in both trials. Likewise, orally administered drugs during their transit through gut are being absorbed by enterocyte the epithelial cells of the small intestine and remain in direct contact with residing microbial cells in complex micro environment in different regions of the gut (Stojančević et al., 2014). In both groups, the intestinal microbiome bio-accumulated same quantity of enalapril from pure enalapril group (73.2±5.17µg) as well as commercial enalapril (Renitec) treated group (74.8±3.27µg) at 5hour transit time post drug administration. Principal component analysis (PCA) analysis of various parameters measured (Figure 7) during the study is inductive of major contribution of components F1and F2. Extracted component F1 contributed (37.22%) followed by F2 (23.31%) with total contribution of 60.53%. Total enalapril absorption showed a significant positive correlation with drug absorption per mg of microbial mass (0.987***) and administered dose of enalapril (0.993***).

Previously, enalapril was absorbed by passive diffusion by Caco-2 cells (Morrison et al., 1996). Such transport mechanism is also present in the form of porins present in outer membrane of microbes that mediates primary transport of solute inside the microbial cell (Nikaido, 2003).

Molar mass of enalapril is 376.44 g/mol and contain a peptide bond in its structure (Figure 1) having the size of the dipeptide and strong hydrogen bonding (Figure 6) make it suitable substrate of YdgR which mediate transport of di and tripeptide in microbes residing the small intestine. Enalapril seems to absorb through Proton-dependent oligopeptide transporters (POTs) present in gram-positive *lactobacilli* dominating the microbes residing the gut (Lorca et al., 2007).

Such absorbance/bio-accumulation of enalapril by the intestinal microbiome suggests uptake of enalapril by intestinal microbe presents a state of competition with transporters present on apical side of enterocytes. Homology studies confirms the presence of transporting protein YdgR along with three additional orthologos in the bacterial genome of microbes residing the gut. Transporter YdgR are promiscuous in nature and resembles in conserved domains with transporter hPept1 present in epical membrane of the enterocyte with low affinity and high capacity characteristics (Harder et al., 2008). Such state of competition for absorbance of same substrate exists between enterocyte and resident microbes in small bowl which is major site of absorption of most of the drugs and nutrients. Microbial diversity is rich in stomach and small intestine due to transient microbiome as compared to other body regions (Gu et al., 2013) revealing 16 microbial phyla dwelling small intestine which increases in composition and functions moving along the length of the gut reaching maximum in large intestine (Li et al., 2017). The POTs from several bacteria have been expressed and characterized. But very little is known about the regulation of different POTs except for the YdgR which is known to be regulated by two components the OmpR/EnzZ system in response to environmental stress (Goh et al., 2004).

Renetic tablet is a commercial preparation of enalapril contains excipients lactose, magnesium stearate, sodium bicarbonate, and starch to produce hardness and disintegration characteristics of the tablet when dissolved in solution form. All such secondary ingredients are transported by LacY transporter, Mg^{2+} transporters (Demishtein et al., 2019). Bicarbonate transporter BicA (Bu et al., 2020) respectively but do not impact on enalapril uptake by the bacterial cell as they are absorbed by different transporter other than YdgR as shown in current study. Enalapril absorbance per mg of microbial mass of intestinal microbiome in enalapril pure drug treated group (2.84±0.05 µg) as well as commercial enalapril treated group (2.79±0.04 µg) at 4 hours sampling time was found high as compared to enalapril pure drug treated group (1.48±0.18 µg) and commercial drug treated group (1.14±0.18 µg) at 5hour transit post drug administration. Such amount of enalapril absorbance by microbes reduced as a function of time. While no drug was found in microbiome samples harvested at 1, 2, 3 and 6hours post drug administration.

Percent dose recovery of enalapril from the intestinal

microbiome harvested from pure enalapril treated group (4.15±0.05%) and commercial drug treated group (3.83±0.04%) was significantly ($p \leq 0.05$) higher at 4hour transit time as compared to pure drug treated group (3.20±0.18%) and commercial drug treated group (3.14±0.18%) at 5hour transit time post drug administration.

Previously, varied percent dose recoveries of paracetamol 13.16±0.55% (Mukhtar et al., 2019), sulpiride 3.91% (Mukhtar et al., 2021a), caffeine 32.27±0.16% (Mukhtar et al., 2021b) and phenobarbital 5.73±0.19% (Mukhtar et al., 2022) at 3, 4, 2 and 4 hours transit time depend upon the nature, size, presence of peptide bond of each substrate. Enalapril as prodrug was not discussed because current study was limited to pre-systemic absorption kinetics of enalapril in the gut microbiome. We were concerned only with the absorptive behavior of the enalapril by the gut resident microbiome before entering in the systemic circulation.

Bacterial homologues have provided valuable insights into the mechanisms of substrate binding and transport, but their usefulness for drug transport is limited. However, a promising exception is DtpA (YdgR), a homologue from *Escherichia coli*, which exhibits ligand selectivity and drug transport profiles comparable to human PepT1 (SLC15A1) reported by Harder et al. (2008). In our study, we have elucidated the crystal structure of DtpA in its native state at a resolution of 3.30 A. We have also determined the crystal structure of DtpA in complex with enalapril showing binding affinity (-7.2) confirming enalapril a suitable substrate of DtpA. These structures provide important insights into the molecular basis of ligand recognition and transport by DtpA. Such recognition of enalpril as asuitable substrate for microbial transporter may be translated in terms of idiosyncratic side effects of the enalapril being used in long term in human during control of hypertension. Our findings have significant implications for drug development, as DtpA can potentially be used as a model system for screening and optimizing drug candidates for transport by human PepT1. We need to find the fate of bio-accumulated enalapril inside the microbial cell to translate in relation to idiosyncratic side effects. Moreover, the structural information obtained from our study can aid in the rational design of more effective and selective drugs that can be transported by YdgR.

Conclusion

The findings reveal that enalapril was maximally absorbed through both primary and secondary transport mechanisms found in microbial cells. Furthermore, the study sheds light on the homology between the mechanism of transport exciting in the epical membrane of enterocytes and microbial membranes. Enalapril absorption by the intestinal microbiome was found to occur in a time-dependent manner, highlighting the need for further investigations of individual drugs in their respective monographs through "*in vivo* microbial drug absorption assay". This study also suggests that the bioavailability of orally administered drugs can be improved by using substrates with the potential to reduce or block drug absorption by the gut microbiota in order to avoid loss of expensive drugs being used in clinical setting. In future, such investigations may be helpful to find the antibacterial effects of non-antibacterial drugs (enalapril) during their interactions with the gut microbiome. The molecular docking study provides a basis for future research on the interaction of enalapril with other transporters and membrane proteins involved in drug absorption and disposition, which may have important implications for drug development and patient care.

Acknowledgment

Authors acknowledge the services of staff at Hi-Tech Laboratory and Animal Experimental Station of Department of Physiology, GCUF.

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: The project was completed under the grant of Higher Education Commission of Pakistan, grant award No. 7548/Punjab/ NRPU/R&D/HEC/2017, project entitled “Interaction of gut microbiome with drug absorbance in *in vivo* experimental model”.

Ethics approval statement: The study was ethically approved by Ethical Review Committee, Government College University, Faisalabad. Reference No. GCUF/ERC/315.

Contribution of Authors

Malik S: Performed the experiment and collected the data.
Mukhtar I: Analyzed and interpreted the results
Muzaffar H: Finalized and proofread the manuscript.
Nawaz L: Manuscript writing
Anwar H: Conceptualized the idea, Finalized and proofread the manuscript.

References

Anwar H, Irfan S, Hussain G, Faisal MN, Muzaffar H, Mustafa I, Mukhtar I and Malik S, 2019. Gut microbiome: A new organ system in body, pp. 17-21. In: Pacheco GAB and Kamboh AA (eds) Parasitology and Microbiology Research, vol 1. IntechOpen, 67-169 Great Portland Street, London, W1W 5PF, United Kingdom.

Anwar H, Mukhtar I, Muzaffar H, Irfan S, Saleem S and Ijaz F, 2024. Host–Microbiome Interactions, pp. 25-50. In: Khurshid M and Akash MSH (eds) Human Microbiome: Techniques, Strategies, and Therapeutic Potential. Springer, Singapore.

Averina OV, Poluektova EU, Marsova MV and Danilenko VN, 2021. Biomarkers and utility of the antioxidant potential of probiotic *Lactobacilli* and *Bifidobacteria* as representatives of the human gut microbiota. Biomedicines. 9(10): 1340.

Biegel A, Knütter I, Hartrodt B, Gebauer S, Theis S and Luckner P, 2006. The renal type H+/peptide symporter PEPT2: structure-affinity relationships. Amino acids. 31: 137-56.

Brandsch M, Knütter I and Bosse-Doenecke E, 2008. Pharmaceutical and pharmacological importance of peptide transporters. J. Pharm. Pharmacol. 60(5): 543-585.

Bu G, Parrish S, Gleason PR, Nielsen DR and Nannenga BL, 2020. Heterologous expression and purification of the bicarbonate transporter BicA from Synechocystis sp. PCC 6803. Protein Expr. Purif. 175: 105716.

CambridgeSoft, 2009. ChemDraw Ultra 12.0 0 (Copyright) 1986 to 2009; CambridgeSoft Corp. Cambridge, MA, USA.

Chen HQ, Gong JY, Xing K, Liu MZ, Ren H and Luo JQ, 2022. Pharmacomicrobiomics: exploiting the drug-microbiota interactions in antihypertensive treatment. Front. Med. 8: 742394.

Coker O, 2023. Non-bacteria microbiome (virus, fungi, and archaea) in gastrointestinal cancer, pp. 91-106. In Microbiome in Gastrointestinal Cancer. Springer, Singapore.

Colella M, Charitos IA, Ballini A, Cafiero C, Topi S, Palmirotta R and Santacroce L, 2023. Microbiota revolution: How gut microbes regulate our lives. WJG. 29(28): 4368.

Coniglio MV, Luna MJ, Provensal P, Watson S, Ortiz ME, Ludueña HR and Magnoli AP, 2023. The Impact of *Saccharomyces cerevisiae var. Boulardii* RC009 on productive parameters in weaned calves and cull cows. ABRs.13: 1-6.

Di Pierro F, 2021. Gut microbiota parameters potentially useful in clinical perspective. Microorganisms. 9(11): 2402.

Demishtein K, Reifen R and Shemesh M, 2019. Antimicrobial properties of magnesium open opportunities to develop healthier food. Nutrients. 11(10): 2363.

Dobson PD and Kell DB, 2008. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat. Rev. Drug Discov. 7(3): 205-20.

Doestzada M, Vila AV, Zhernakova A, Koonen DP, Weersma RK, Touw DJ, Kuipers F, Wijmenga C and Fu J, 2018. Pharmacomicrobiomics: a novel route towards personalized medicine?. Protein Cell. 9(5): 432-445.

Eberhardt J, Santos-Martins D, Tillack AF and Forli S, 2021. AutoDock Vina 1.2. 0: New docking methods, expanded force field, and python bindings. J. Chem. Inf. Model. 61(8): 3891-3898.

Effinger A, O'Driscoll CM, McAllister M and Fotaki N, 2019. Impact of gastrointestinal disease states on oral drug absorption–implications for formulation design–a PEARRL review. J. Pharm. Pharmacol. 71(4): 674-698.

Ekici S, Demirci M, Dülger D and Yiğin A, 2023. The Quinolone Resistance Genes in the Bacteriophage DNA Fractions in the Healthy Calf Stool Samples Via qPCR. Kafkas Univ. Vet. Fak. Derg. 29 (1): 49-54.

El Aidy S, Van Den Bogert B and Kleerebezem M, 2015. The small intestine microbiota, nutritional modulation and relevance for health. Curr. Opin. Biotechnol. 32: 14-20.

Hollister EB, Gao C and Versalovic J, 2014. Compositional and functional features of the gastrointestinal microbiome and their effects on

human health. J. Gastroenterol. 146(6): 1449-58.
Fei YJ, Sugawara M, Liu JC, Li H., Ganapathy V, Ganapathy ME and Leibach FH, 2000. cDNA structure, genomic organization, and promoter analysis of the mouse intestinal peptide transporter PEPT1. Biochim. Biophys. Acta Gene Struct. Expression.1492(1): 145-154.
Ferreira LG, Dos Santos RN, Oliva G and Andricopulo AD, 2015. Molecular docking and structure-based drug design strategies. Molecules. 20(7): 13384-13421.
Gao K, Wang PX, Mei X, Yang T and Yu K, 2024. Untapped potential of gut microbiome for hypertension management. Gut Microbes. 16(1): 2356278.
Goh EB, Siino DF and Igo MM, 2004. The Escherichia coli tppB (ydgR) gene represents a new class of OmpR-regulated genes. J. Bacteriol. 186(12): 4019-24.
Gu S, Chen D, Zhang J-N, Lv X, Wang K and Duan L-P, 2013. Bacterial community mapping of the mouse gastrointestinal tract. PloS one. 8(10) :e74957.
Harder D, Stolz J, Casagrande F, Obrdlik P, Weitz D and Fotiadis D, 2008. DtpB (YhiP) and DtpA (TppB, YdgR) are prototypical proton-dependent peptide transporters of *Escherichia coli*. The FEBS J. 275(13): 3290-8.
Jackson R and Bellamy M, 2015. Antihypertensive drugs. BJA Education.15(6): 280-5.
Jóźwiak-Bebenista M and Nowak JZ, 2014. Paracetamol: mechanism of action, applications and safety concern. Acta Pol. Pharm. 71(1): 11-23.
Li D, Chen H, Mao B, Yang Q, Zhao J and Gu Z, 2017. Microbial biogeography and core microbiota of the rat digestive tract. Sci. Rep. 7(1): 1-16.
Lorca GL, Barabote RD, Zlotopolski V, Tran C, Winnen B, Hvorup RN, Stonestrom AJ, Nguyen
E, Huang LW, Kim DS and Saier MH Jr, 2007. Transport capabilities of eleven gram-positive bacteria: comparative genomic analyses. Biochim. Biophys. Acta Biomembr. 1768(6): 1342–66.
Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A and Anderson EE, 2018. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 555:623–8. doi: 10.1038/nature25979
Morrison RA, Chong S, Marino AM, Wasserman MA, Timmins P and Moore VA, 1996. Suitability of enalapril as a probe of the dipeptide transporter system: in vitro and in vivo studies. J. Pharm. Res. 13: 1078-82.
Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS and Olson AJ, 2009. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30(16): 2785-2791.
Mukhtar I, Anwar H, Hussain G, Rasul A, Naqvi SAR, Faisal MN, Mustafa I, Malik S, Shaukat A, Mirza OA and Sohail MU, 2019. Detection of Paracetamol as substrate of the gut microbiome. PJPS. 32(2, Supplementary): 751-757.
Mukhtar I, Anwar H, Mirza OA, Ali Q, Ijaz MU, Hume M, Prabhala BK, Iftikhar A, Hussain G, Sohail MU and Khan KU, 2021a. Sulpiride Serves, a Substrate for the Gut Microbiome. Dose-Response. 19(1): 1559325820987943.
Mukhtar I, Iftikhar A, Imran M, Ijaz MU, Irfan S and Anwar H, 2021b. The competitive absorption by the gut microbiome suggests the first-order absorption kinetics of caffeine. Dose-Response. 19(3): 15593258211033111.
Mukhtar I, Anwar H, Iftikhar A, Hashem HE, Ali Q and Siddique F, 2022. Human targeted phenobarbital presents a poor substrate of gut microbiome deciphering new drug targets beyond pharmacokinetic curbs. BMC Pharmacol. Toxicol. 23(1): 85.
Mussap M, Loddo C, Fanni C and Fanos V, 2020. Metabolomics in pharmacology-a delve into the novel field of pharmacometabolomics. Expert Rev. Clin. Pharmacol. 13(2):115-134.
Nielsen CU, Brodin B, Jørgensen FS, Frokjaer S and Steffansen B, 2002. Human peptide transporters: therapeutic applications. Expert Opin. Ther. Pat.12(9): 1329-1350.
Nikaido H, 2003. Molecular basis of bacterial outer membrane permeability revisited. J. Mol. Biol. 67(4): 593-656.
Pizzagalli MD, Bensimon A and Superti-Furga G, 2021. A guide to plasma membrane solute carrier proteins. The FEBS J. 288(9): 2784-2835.
Prabhala BK, Aduri NG, Iqbal M, Rahman M, Gajhede M and Hansen PR, 2017. Several hPepT1-transported drugs are substrates of the *Escherichia coli* proton-coupled oligopeptide transporter YdgR. Microbiol. Res. 168(5): 443-9.

Rubio-Aliaga I and Daniel H, 2008. Peptide transporters and their roles in physiological processes and drug disposition. Xenobiotica. 38(7-8): 1022-42.

Stojančević M, Bojić G, Al Salami H and Mikov M, 2014. The influence of intestinal tract and probiotics on the fate of orally administered drugs. Curr. Iss Mol. Biol. 16(1): 55-68.

Subramaniam S, Kamath S, Ariaee A, Prestidge C and Joyce P, 2023. The impact of common pharmaceutical excipients on the gut microbiota. Expert. Opin. Drug. Deliv. 20(10): 1297-1314.

Sultana N, Naveed S and Arayne MS, 2013. Monitoring of in vitro interaction studies of enalapril with hypoglycemic agents by LC-UV. R&R Med. Chem. 3: 19-29.

Torres-Carrillo N, Martínez-López E, Torres-Carrillo NM, López-Quintero A, Moreno- Ortiz JM, González-Mercado A and Gutiérrez-Hurtado IA, 2023. Pharmacomicrobiomics and Drug–Infection Interactions: The Impact of Commensal, Symbiotic and Pathogenic Microorganisms on a Host Response to Drug Therapy. Int. J. Mol. Sci. 24(23): 17100.

Urttiac A, Sadée W and Johns SJ, 2001. Genomic structure of proton-coupled oligopeptide transporter hPEPT1 and pH-sensing regulatory splice variant. AAPS PharmSciTech. 3(1): 66-79.

Visualizer DS, 2005. Discovery Studio Visualizer. Accelrys Software Inc, Boston, USA.

Yusuf D, Davis AM, Kleywegt GJ and Schmitt S, 2008. An alternative method for the evaluation of docking performance: RSR vs RMSD. J. Chem. Inf. Model. 48(7): 1411-1422.

Zentgraf M, Steuber H, Koch C, La Motta C, Sartini S, Sotriffer CA and Klebe G, 2009. How reliable are current docking approaches for structure-based drug design? Lessons from aldose reductase. Angew. Chem. 46(19): 3575-3578.