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Pharmacokinetic interactions between metformin and berberine in rats: Role of oral administration sequences and microbiota

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ABSTRACT

Aims: Considering the potential oral administration sequences and role of microbiota for metformin (MET) and berberine (BBR) during anti-diabetic treatments, the current study aimed to investigate the pharmacokinetic interactions between MET and BBR in rats after oral administration at different sequences and impacts of microbiota on such interactions.

Main methods: Sprague-Dawley rats were divided into five groups as per what was orally administered to them: MET (G1)/BBR (G2) at 200 mg/kg, BBR 2-hour (h) after dosing MET (G3), MET 2-h after dosing BBR (G4) or MET with BBR at the same time (G5) followed by monitoring their pharmacokinetic profiles. Further *in vitro* incubations mimicking the above five treatments in rat intestinal content (G1R-G5R), human fecalase (G1H-G5H) and selected bacteria (G1B-G5B) were conducted for both MET and BBR (10 μg/ml for G1R/H-G5R/H and 50 μM for G1B-G5B) up to 24-h. Concentrations of MET and BBR were analyzed by LC/MS/MS.

Key findings: Although BBR was barely measurable *in vivo*, it significantly increased systemic exposure of MET in G3/G4. Consistent with pharmacokinetic findings, sequential *in vitro* incubations of MET and BBR in both rat intestinal content and human fecalase demonstrated significant increase on MET persisted after 24-h incubation in G3R/H & G4R/H. Moreover, post-dose (G3B) and pre-dose (G4B) of BBR decreased the MET degradation significantly in most selected bacteria.

Significance: Our finding for the first time demonstrated the significant effect of sequential co-administration of BBR and MET on their pharmacokinetic interactions, which could be related to their microbiota mediated metabolisms in gastrointestinal tract (GI).

1. Introduction

As an insulin-sensitizer, metformin (MET) has been marketed in over 100 countries. After its oral administration in human, peak plasma concentrations could be reached within three hours [1–3] followed by urine excretion with limited metabolism [4]. Recent studies indicated that MET could alter the gut microbial metabolism, leading to the increased production of butyrate by gut microbiota which had mutually beneficial relationship with colonocyte [5]. Other antihyperglycemic agent may be added in diabetes patients whose glycemic goals are not reached after three-month maximal dose of MET [6,7].

Due to increasing number of herbal medicines used for treating type 2 diabetes (T2D), they are commonly used with MET as complementary therapies [8]. Enhanced oral bioavailabilities of MET were found in diabetic rats co-administered *Musa sapientum* L. bark juice (100 ml/kg) and *Ocimum sanctum* leaf extract (150 mg/kg) [9,10]. The basal glycemic effect of 50 mg/kg MET was increased with co-administered Goutweed tincture liquid (1 ml/kg QD, 5-d) in diabetic rats [11]. Four weeks co-treatment of MET (190 mg/kg) and Jiang-Tang-Xiao-Ke Granule (1.75–3.5 g/kg) would enhance hepatic protective effect in diabetic mice [12]. In addition, coadministration of MET (500 mg/kg) with *Scutellaria baicalensis* (400 mg/kg)/*Rehmannia glutinosa* (200 mg/kg)/baicalin (120 mg/kg) could lead to enhanced anti-diabetic effect, lower risk of inflammation and reduced levels of plasma total cholesterol/triglyceride in diabetic rats [13–16]. However, reduced hypoglycemic activity of MET (100 mg/kg) was reported after its co-

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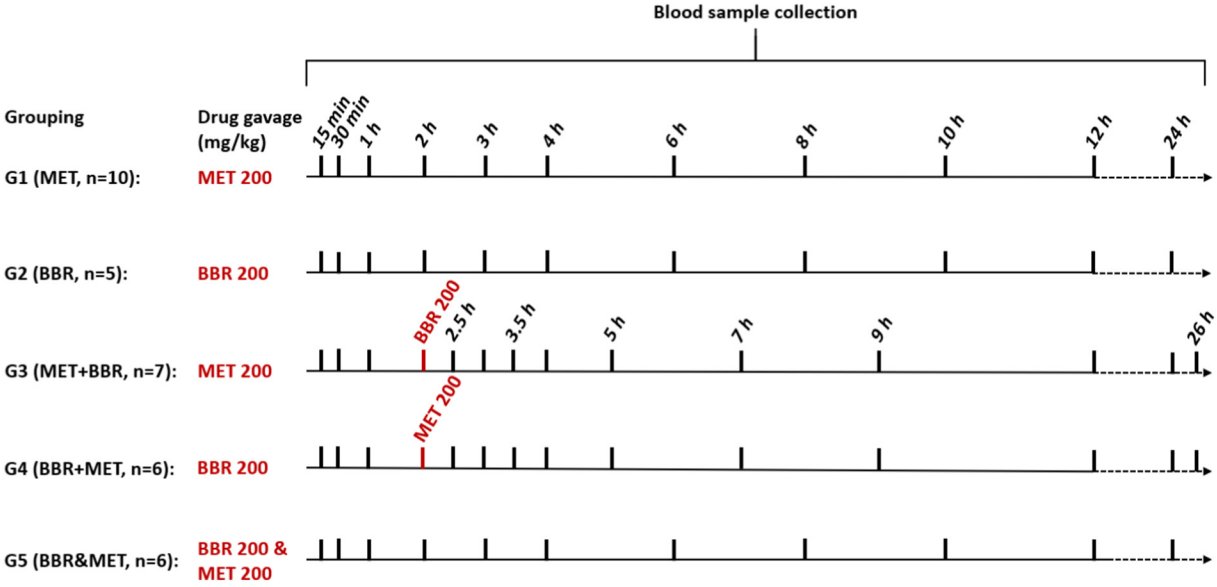


Fig. 1. Grouping, treatment and blood sampling after oral administrations of MET and/or BBR. G1 (n = 10): MET at 200 mg/kg; G2 (n = 5): BBR at 200 mg/kg; G3 (n = 7, MET + BBR): MET followed by BBR in 2-h; G4 (n = 6, BBR + MET): BBR followed by MET in 2-h; G5 (n = 6, BBR&MET): MET together with BBR at the same time.

administration with Yoyo Bitters® (15.4 ml/kg/day) in diabetic rats [17,18]. Taken together, preclinical evidence demonstrated significant herb-interaction of MET, leading to either beneficial or adverse effects depending on its dose and the type/dose of co-administered herbs.

Coptidis Rhizoma is the most commonly used anti-diabetic Chinese medicine [19]. Although its primary active ingredient Berberine (BBR) depicted poor oral bioavailability (< 1%) due to its low gastrointestinal (GI) absorption [20,21], Berberine Hydrochloride Tablets® at 500–2000 mg/person/day was used for T2D treatment. In diabetic rats, BBR could modulate oxidative stress and lower glial fibrillary acidic protein immunoreactivity [22]. In retinas of T2D patients with diabetic retinopathy, the leukocyte-mediated killing of retinal endothelial cells was inhibited by BBR [23]. Due to its alteration on the gut microbiota composition, BBR and BBR-containing herbal medicine have been used in the treatment of bacterial diarrhea [24].

Considering their potential combination treatment in T2D and impact on gut microbiota, data on interactions between MET and BBR are essential to provide clinical guidance. Limited report on pharmacokinetic interactions after their intravenous [25]/oral [26] simultaneous administrations in rats revealed enhanced/decreased systemic exposure of MET. Such discrepancy in findings could be due to their non-clinically relevant route of administrations and dosing regimen. Although two-hour interval is usually recommended during integrative medicine practice to avoid the physical-chemical interactions between the two orally administered drugs in GI tract [27], there is no specific guidance on the sequence of drug administrations. To address these knowledge gaps, we designed the current study aiming to 1) investigate the pharmacokinetic interactions of MET and BBR in rats at their clinically relevant doses with various oral administration sequences adopted in clinical practice, and 2) further evaluate the potential microbiota mediated interactions between MET and BBR in the GI tract in different species. Nine common gut bacterial strains from both rat and human were included in this study [28,29], namely *Bacteroides fragilis*, *Bifidobacterium pseudolongum*, *Clostridium perfringens*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Enterobacter cloacae* and *Lactobacillus fermentum*.

2. Materials and methods

2.1. Chemicals and reagents

BBR chloride with purity over 97% was the product of Sigma-Aldrich Chem. Co. (Milwaukee, WI, USA). MET hydrochloride (purity > 99%), albuterol (internal standard for MET) and caffeine (internal standard for BBR) were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, Liaoning, China). Acetonitrile (ACN) and methanol (RCI Labscan Limited, Bangkok, Thailand) were in HPLC grade and used without further purification. Distilled and deionized water was provided by Millipore water purification system (Millipore, Milford, MA, USA).

2.2. Animals

Male Sprague–Dawley (SD) rats (200–220 g) were provided by Laboratory Animal Services Center of The Chinese University of Hong Kong (Hong Kong SAR, China). Animals were individually housed in standard cages on a 12-h cycle of light and darkness at an average room temperature range from 23 to 27 °C, fed with standard animal chow and tap water daily. The rats were fasted overnight before administration of MET and/or BBR. All experimental procedures were approved by the Department of Health of Hong Kong (Reference No.: (18/12) in DH/SHS/8/2/1 Pt.7) and Animal Ethics Committee (Reference No.: 18-022-MIS) at the Chinese University of Hong Kong.

2.3. Pharmacokinetic interactions between MET and BBR

2.3.1. Grouping and administrations

Male SD rats were randomly divided into five groups, as shown in Fig. 1. Based on the human equivalent doses (1000–2500 mg/day/person) of MET and BBR, 200 mg/kg of each drug were investigated in the current study. MET and BBR were dissolved in the distilled water and suspended in 0.5% sodium carboxyl methyl cellulose (CMC-Na), respectively. The doses were adjusted according to the rat body weight. In order to mimic the potential co-administration pattern of the two drugs, rats were divided into five groups (G1 to G5). Rats from G1 (n = 10) and G2 (n = 5) were orally administered with MET and BBR single dose at 200 mg/kg, respectively. For rats in G3 (n = 7), BBR

(200 mg/kg) was orally administered 2-h after dosing MET (200 mg/kg), while rats in G4 ($n = 6$) received MET 2-h after dosing BBR. In G5 ($n = 6$), rats were administered MET (200 mg/kg, p.o.) together with BBR (200 mg/kg, p.o.) at the same time.

For rats from all treatment groups, cannula-insertion surgery was performed one day before the MET and/or BBR dose of the day, in preparation for blood sampling after the last administration. A polyethylene catheter (1.00-mm OD, 0.50-mm ID, Portex Limited, Kent, England) was inserted into the right jugular vein under anesthesia (intramuscular injection of a mixture containing ketamine and xylazine in ratio 2:1). After cannulation, the rats were placed individually into metabolic cages and allowed to recover overnight, with fasting and free access to water.

2.3.2. Blood sampling and sample treatment

On the day of dosing, blood samples (0.2 ml each) were obtained *via* the catheter before and post-dose at specific time points as shown in Fig. 1. The catheter was back-flushed with 200 μl of heparinized saline (50 IU/ml) after each blood draw and capped until next sampling time. All blood samples were immediately placed into heparinized tubes to obtain the plasma after centrifugation at 8000 rpm for 3 min under the room temperature. All plasma samples were stored at −80 °C until LC/MS/MS analysis for content of MET and BBR.

To 100 μl rat plasma sample, 50 μl albuterol (ALB, IS for MET, 250 ng/ml), 50 μl caffeine (CAF, IS for BBR, 250 ng/ml) and 200 μl methanol were added for protein precipitation. 300 μl supernatant was then collected for N_2 drying. Samples were reconstituted with 50 μl 0.2% formic acid and 50 μl ACN after N_2 drying. The mixture was vortex mixed for 20 s. After centrifugation at 13,200 rpm for 5 min, the supernatants were collected. Concentrations of MET and BBR in rat plasma were analyzed by validated LC/MS/MS methods.

2.3.3. Plasma sample analyses using LC/MS/MS (liquid chromatography–tandem mass spectrometry)

Agilent 6430 LC/MS/MS was used for analyzing rat plasma and incubation samples. Concentrations of MET and BBR were analyzed by validated LC/MS/MS methods. The fragmentations for MET, BBR, ALB, CAF were set at 90 V, 143 V, 90 V and 110 V, respectively, and the collision energy set at 15 eV, 32 eV, 15 eV and 16 eV, respectively. The liquid chromatographic separation was carried out on a SunFire™ C8 (4.6 × 250 mm, 5 μm) analytical column. The column was equilibrated for approximately 30 min before the commencement of run. For detecting MET concentration, quantification was carried out using selected reaction monitoring m/z 130.1 → 60.1 for MET and m/z 240.0 → 148.0 for ALB, IS for MET. The mobile phase consisted of ACN and water (20:80, *v*/v) at a flow rate of 0.8 ml/min. For detecting BBR concentration, quantification was carried out using selected reaction monitoring m/z 336.1 → 320.1 for BBR and 195.1 → 138.0 for CAF, IS for BBR. The total analysis time was 11 min and a gradient condition was used as follows (time, % of ACN): 0.00–3.00 min, 10–30% ACN; 3.00–7.50 min, 30–10% ACN; 7.50–8.00 min, 10% ACN; 8.00–11.00 min, 10% ACN.

For MET/BBR pharmacokinetic study, calibration curve was prepared by adding 100 μl MET standard solution (97.66–50,000 ng/ml in MeOH), 100 μl BBR standard solution (0.98–31.25 ng/ml in MeOH), 50 μl ALB (IS, 250 ng/ml in MeOH) and 50 μl CAF (IS, 250 ng/ml in MeOH) into 100 μl rat blank plasma.

For quality control (QC) samples preparation, 100 μl of each MET (100, 2000 and 40,000 ng/ml) and BBR (1, 5 and 25 ng/ml) nominal concentration solution, 50 μl ALB (IS for MET) and 50 μl CAF (IS for BBR) were added into 100 μl rat blank plasma. After centrifugation at 13,200 rpm for 15 min, 300 μl supernatant was dried with N_2 and reconstituted with 50 μl 0.2% formic acid and 50 μl ACN. QC samples were applied for the validation according the FDA guideline [30]. Linearity, accuracy (percentage), precision (R.S.D., relative standard deviation) and stability (room temperature for 4-h, auto-sampler for 12-h, −80 °C for 30 days as well as three and thaw cycles) were determined by three analyses of low, medium and high concentrations of MET and BBR in each calibration curve on separated five days.

The separated analytical methods for MET and BBR were evaluated for its selectivity, linearity, recovery, stability, accuracy and precision of intra-day and inter-day with regard to the U.S. Food and Drug Administration guidance for industry bioanalytical method validation. Three concentrations (low, medium, high) of MET and BBR in the range of expected concentrations were used. Five determinations per concentration were tested for the precision of methods. The intra-day and inter-day accuracy and precision for MET/BBR were calculated by relative error (R.E.) as well as relative standard deviation (R.S.D.). The intra-day and inter-day accuracy were within the range of −7.41%–1.81% for MET and from −9.36% to 10.46% for BBR. The precision of both intra-day and inter-day were below 11.60% and 7.51% for MET and BBR respectively.

2.4. Further investigations on the impact of the incubation sequences on the stabilities of MET and BBR in rat intestinal content, human fecalase and selected bacteria

2.4.1. Preparation of rat intestinal content suspensions

SD rats (200–220 g) were sacrificed by excessive anesthesia. The rat intestine was removed and placed in the 0.9% cold saline solution immediately. The intestinal contents were separated within 2-h after the death. The intestinal contents (2 g for each rat) were diluted and homogenized by 18 ml 0.9% cold saline to give a mixture of content suspensions. All preparation procedures were maintained at 4 °C to keep the survival of bacteria for the incubation.

2.4.2. Preparation of human fecalase

Human fecalase was prepared as described in previous literature [31]. Briefly, the human fecal samples were processed by suspending 12.5 g sample into 50 ml cold saline supplemented with 10% glycerol, followed by centrifuging at 500 rpm for 5 min. The supernatant was sonicated on ice for 10 min before centrifugation at 10,000 rpm for 20 min. The supernatants were stored in the −80 °C freezer until further analyses.

2.4.3. Preparation of selected bacteria

All the bacteria were cultured for 24–48 h in advance by using modified Gifu anaerobic medium (mGAM) agar plates under anaerobic conditions. Then, bacteria were mixed with mGAM broth to make a suspension of 0.50 McFarland turbidity. The suspension was 10 times diluted and then 10 μl was transferred to each well for further drug incubation.

2.4.4. Incubation of MET with BBR at different sequences in rat intestinal content suspensions, human fecalase and selected bacteria

To 950 μl rat intestinal content suspensions and human fecalase, 50 μl 200 μg/ml MET (in purified water) and 50 μl 200 μg/ml BBR (in purified water) were spiked. To imitate the oral administration order sequences, two-hour interval between the two drugs spiking was performed during the incubation. Grouping set was demonstrated as follows ($n = 5$ in each group); G1R/H and G2R/H were incubated with MET and BBR at a final concentration of 10 μg/ml respectively. For G3R/H, to 950 μl rat intestinal content, 50 μl MET (200 μg/ml) was spiked advanced and started incubation, then after a 2-h interval, 50 μl BBR at 200 μg/ml (final concentration: 9.52 μg/ml) was spiked. G4R/H changed the spiking sequences of MET and BBR. For G5R/H, two drugs were spiked together before the incubation start. For incubation with the selected bacteria (G1B to G5B), MET and/or BBR at the final concentration of 50 μM in mGAM medium were incubated followed the same grouping as above.

All above mixture were incubated at 37 °C, while the incubations with bacteria were conducted under anaerobic conditions. Rat and

human incubation samples were removed before (starting point) and 1, 2, 3 and 24-h after the post-spike. Bacterial samples were removed by centrifugation after 24-h incubation. All samples were frozen right away to stop further metabolism. To 50 μl incubation sample, 25 μl ALB, 50 μl CAF, and 100 μl methanol were added to precipitate protein and then vortexed for 20 s at room temperature. After centrifugation at 13,200 rpm for 10 min, the supernatants were collected and injected to LC/MS/MS system for further analyses.

2.4.5. Sample analyses using LC/MS/MS

Similar LC/MS/MS as plasma samples was used for analyzing samples from rat intestinal content incubation, human fecalase incubation and bacterial incubation except for that the calibration curves were prepared by adding 50 μl of each MET/BBR standard solution (625–20,000 ng/ml in MeOH) and 25 μl of each IS (ALB/CAF, 250 ng/ml in MeOH) into 50 μl blank rat intestinal content suspensions, human fecalase or bacterial incubation buffer.

2.5. Data analyses

The pharmacokinetic profile (plasma concentration *vs* time) of MET was processed by Phoenix 64 (Version 8.0.0.3176, Certara USA, Inc., Princeton, NJ, USA). Pharmacokinetic parameters including $AUC_{0-\infty}$ (area under the concentration-time curve), CL/F (oral clearance), $t_{1/2}$ (elimination half-life) and MRT (mean residence time) were calculated by applying the non-compartmental model. C_{max} (peak plasma concentration) and T_{max} (time to reach C_{max}) were obtained from the experimental data directly. Data statistical analysis was done by one-way analysis of variance (ANOVA) (GraphPad Prism, Version 7.00). $p < 0.05$ was considered significant statistically. Data were given as the mean ± standard deviation (SD).

3. Results

3.1. LC/MS/MS method development and validation

The peak concentrations for MET and BBR in rat plasma reached approximately 30,000 ng/ml and 12 ng/ml, respectively. Due to such a huge difference in concentration levels of MET and BBR, we applied two separate analytical methods for detecting the MET and BBR concentrations after sample treatment in current pharmacokinetics studies. Chromatographic spectra for blank rat plasma spiked with MET/ALB/BBR/CAF working solution depicted peak of each compound at 3.030 min, 3.026 min, 5.637 min and 6.610 min respectively (Fig. 2 Middle) under the LC/MS/MS analytical conditions. For rat blank plasma, there were no peaks observed at each compound retention time (Fig. 2 Left). Fig. 2 Right showed the rat plasma sample after oral administration of MET and BBR. Plasma concentration of MET and BBR provided satisfied linearity ($r^2 > 0.99$). The typical regression equations were $y = 1.770764x + 0.684100$ ($r^2 = 0.9998$) for MET by using a weight factor of 1/y and $y = 0.234177x + 1.407724$ ($r^2 = 0.9997$) for BBR by using a weight factor of 1/x. The lower limits of quantification (LLOQ) were 50 ng/ml and 0.98 ng/ml for MET and BBR respectively. As depicted in Table 1, the recoveries of plasma extraction at three concentrations of quality control (QC) samples were all above 85% for MET and from 89.76 ± 4.97% to 92.71 ± 1.81% for BBR [30]. The stability for MET and BBR in rat plasma were tested under four different conditions. As shown in Table 1, MET and BBR were stable at room temperature for 4-h and in auto-sampler for 12-h. Both compounds were stable at −80 °C for 30 days, with the accuracy mean value in the range of 98.62 ± 2.33%–109.01 ± 2.16%. Besides, concentrations after three freeze-thaw cycles were all within 15%. Both accuracy and precision of intra-day and inter-day for the developed assay methods are demonstrated in Table 1.

3.2. Effect of MET co-administration sequences on the pharmacokinetics of BBR

Due to the low bioavailability of BBR, the plasma concentrations at different time points between different groups were all below 5.69 ng/ml with large standard deviation (Table 3). Comparing with BBR alone group (G2), no significant difference was found when MET was co-administered at 2-h before (G3) and after (G4) BBR was dosed or when MET was dosed with BBR at the same time (G5). Overall, comparing with G2, when MET was co-administered with BBR at different sequences, it had no obvious alteration on the pharmacokinetics profiles of BBR.

3.3. Effect of co-administered BBR sequences on the pharmacokinetics of MET

After oral administration of MET and BBR at 200 mg/kg to rats, MET plasma concentration *versus* time pharmacokinetic profile as well as pharmacokinetic parameters of different treatment groups were shown in Fig. 3. It was found that BBR had a significant impact on the pharmacokinetics of MET. Although the T_{max} of MET did not suggest any significant difference between MET single-dose group (G1) and co-administration group (G3–G5), the $AUC_{0-\infty}$ (from 7708 ± 2897 to 11,919 ± 4256 min·μg/ml), $t_{1/2}$ (from 257 ± 67 to 498 ± 190 min) and MRT (from 411 ± 93 to 711 ± 198 min) of MET were significantly increased in G4, representing a longer maintaining time of MET in the body when BBR was orally administrated at 2-h before MET was dosed. In addition, CL/F of MET in rats was reduced by 50% from 32 ± 11 ml/min/kg (G1) to 18 ± 6 ml/min/kg (G4) ($p < 0.05$). Although the MET pharmacokinetics parameters in rats from G3 and G5 lacked significant difference from that in G1, rats in G3 depicted a tendency of a prolonged $t_{1/2}$ and MRT and reduced CL/F in comparison to that from G1.

3.4. Role of microbiota-mediated metabolism of MET/BBR during their coadministration at different sequences

As shown in Table 2, about 61.32 ± 7.83% and 52.25 ± 5.21% of MET remained after 2-h and 24-h incubation in rat intestinal content (G1R), respectively. When BBR was added at 2-h before the addition of MET (G4R), the degradation of MET reduced significantly with an increased percentage of MET remaining of 71.83 ± 6.17% and 63.06 ± 9.05% after 2-h and 24-h incubations, respectively. However, when MET and BBR were incubated at the same time (G5R), no significant changes in MET remaining was observed at any incubation period. In summary, based on our *in vitro* incubation results, 2-h pretreatment of BBR with MET could significantly inhibit the degradation of MET in rat intestinal contents.

On the other side, after the 24-h incubation in absence of MET (G2R), the percentage of BBR remaining was found to be 59.17 ± 10.41%, which was consistent with previous findings [32]. Comparison of BBR remaining during 24-h incubation in G2R with that from G3R to G5R (Table 2) showed no significant difference, suggesting lack of significant impact from MET on the degradation of BBR in intestinal content.

The corresponding incubations of MET and BBR with human fecalase (G1H to G5H) depicted a similar trend in percentage remaining as that from rat intestinal content (G1R to G5R) (Fig. 4). Moreover, significant impacts of the selected bacteria on MET and/or BBR metabolism were also found after 24-h *in vitro* incubation (Fig. 5). Compared with G1B, post-dose (G3B) and pre-dose (G4B) of BBR decreased the MET degradation significantly (1.26%–25.67%) in most selected of bacteria. Meanwhile, MET decreased the BBR degradation (1.32%–25.36%) only in certain bacteria after 24-h incubation.

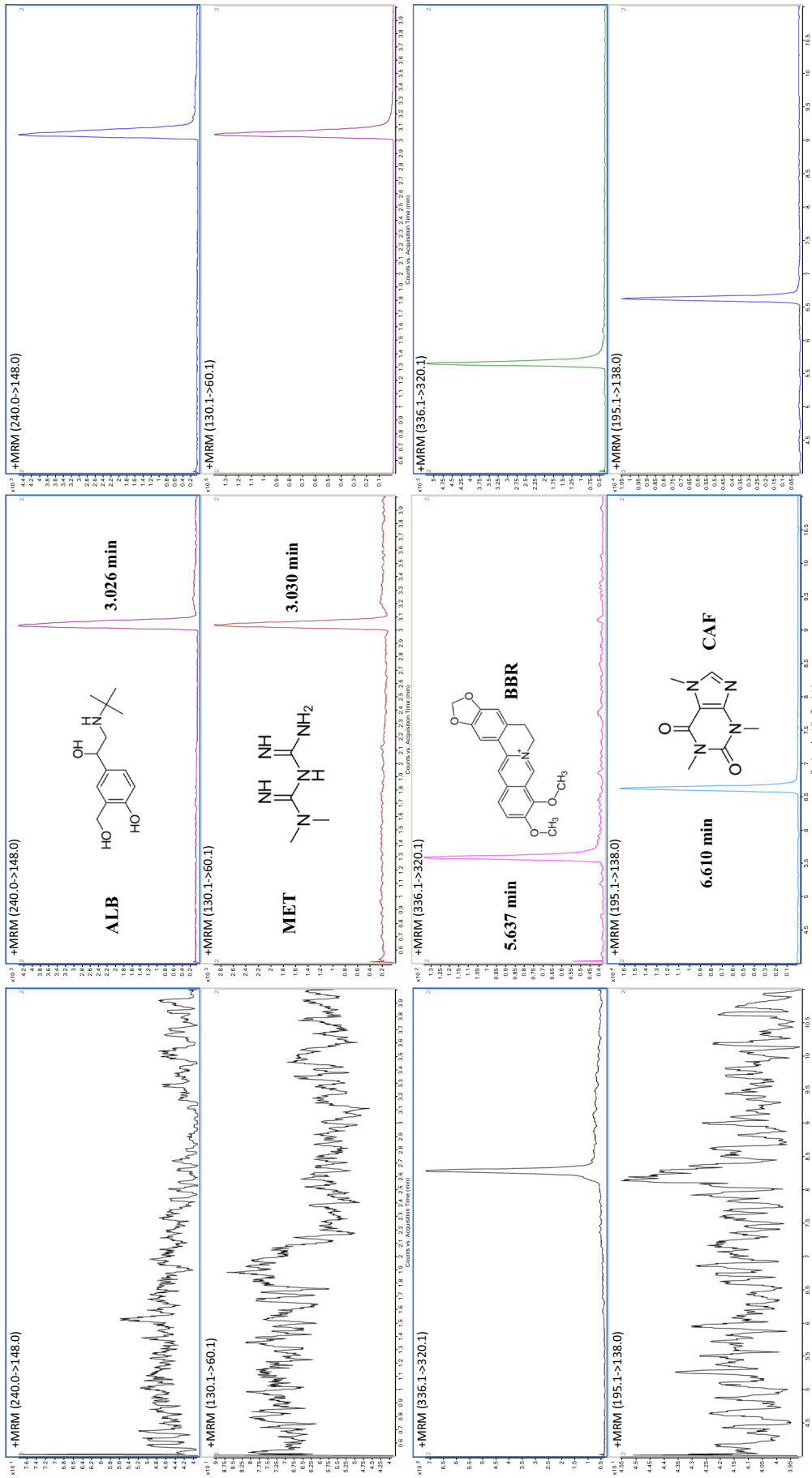
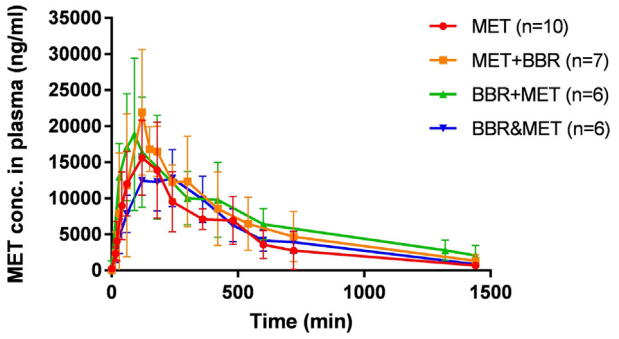


Fig. 2. LC/MS/MS spectra of MET, BBR, ALB, CAF in blank rat plasma (left), spiked with the working solution (middle) and plasma sample collected 30 min after oral administration of MET (200 mg/kg) and BBR (200 mg/kg) at the same time (right).

Table 1
Extraction recoveries, stabilities and intra-day, inter-day accuracy and precision of MET and BBR analyses *via* LC/MS/MS.

Compounds	Nominal conc. (ng/ml)	Recoveries (%, n = 5)		Intra-day (n = 5)		Inter-day (n = 5)		Stability (n = 5)			
		Mean ± SD	R.S.D. (%)	Accuracy (% R.E.)	Precision (% R.S.D.)	Accuracy (% R.E.)	Precision (% R.S.D.)	Room temperature for 4-h	Auto-sampler for 12-h	−80 °C for 30 days	Three freeze and thaw cycles
								Accuracy (%)	Accuracy (%)	Accuracy (%)	Accuracy (%)
MET	100	86.19 ± 1.05	1.22	−5.38	6.87	0.04	8.74	96.54 ± 7.13	94.69 ± 9.49	105.78 ± 4.50	105.47 ± 2.98
	2000	90.61 ± 4.26	4.70	−7.41	8.23	−3.34	11.60	94.24 ± 6.32	92.40 ± 5.55	98.62 ± 2.33	100.15 ± 0.62
	40,000	88.36 ± 1.09	1.23	1.81	4.26	0.99	4.86	102.36 ± 2.83	103.27 ± 3.48	100.91 ± 2.62	98.96 ± 0.73
BBR	1	92.71 ± 1.81	1.96	10.25	3.51	10.46	3.88	109.65 ± 3.99	107.38 ± 2.29	109.01 ± 2.16	109.31 ± 3.78
	5	89.76 ± 4.97	5.53	−7.71	7.51	−9.36	3.71	95.85 ± 5.84	97.87 ± 4.29	101.62 ± 5.80	101.00 ± 3.11
	25	91.73 ± 4.08	4.45	0.02	3.40	1.32	4.17	99.69 ± 2.46	98.92 ± 2.88	99.26 ± 1.47	101.51 ± 3.18



Pharmacokinetic parameters	MET (G1, n=10)	MET+BBR (G3, n=7)	BBR+MET (G4, n=6)	BBR&MET (G5, n=6)
T_{max} (min)	138±40	150±75	150±139	180±93
C_{max} (μg/ml)	17.08±5.7	22.46±8.2	20.17±8.9	14.47±3.0
$AUC_{0-\infty}$ (min·μg/ml)	7708±2897	10227±4119	11919±4256*	7876±2044
CL/F (ml/min/kg)	32±11	22±7	18±6*	27±7
$t_{1/2}$ (min)	257±67	382±147	498±190**	323±52
MRT (min)	411±93	566±174	711±198**	512±52

Notes: Data are given as the mean ± standard deviation (SD); *Significantly different ($p < 0.05$) from the G1; **Significantly different ($p < 0.01$) from the G1;

Fig. 3. Effects of BBR (200 mg/kg, p.o.) co-administration on plasma concentration *vs.* time profiles (upper) and the pharmacokinetic parameters (bottom) of MET (200 mg/kg, p.o.) in the different treatment groups.

Table 2
Percentage of MET and BBR remaining after various incubations with the rat intestinal contents at different time intervals.

Group	Incubation time (h)	Drug remaining percentage (%)	
		MET	BBR
G1R (MET, n = 5)	1	72.19 ± 14.18	N.A.
	2	61.32 ± 7.83	
	3	57.35 ± 4.74	
	24	52.25 ± 5.21	
G2R (BBR, n = 5)	1	N.A.	79.22 ± 3.75
	2		69.13 ± 9.99
	3		63.60 ± 13.73
	24		59.17 ± 10.41
G3R (MET + BBR, n = 5)	1	N.A.	74.00 ± 1.55
	2	N.A.	66.22 ± 10.90
	3	71.54 ± 3.77**	69.11 ± 9.66
	24	62.84 ± 4.54	53.99 ± 11.43
G4R (BBR + MET, n = 5)	1	76.38 ± 7.45	N.A.
	2	71.83 ± 6.17*	N.A.
	3	69.84 ± 5.86**	77.49 ± 6.80
	24	63.06 ± 9.05*	55.94 ± 9.43
G5R (BBR&MET, n = 5)	1	69.59 ± 7.89	69.59 ± 7.89
	2	64.75 ± 7.88	64.75 ± 7.88
	3	64.63 ± 5.68	64.63 ± 5.68
	24	58.04 ± 7.77	56.61 ± 9.12

Notes: Data are given as the mean ± standard deviation (SD); *Significantly different ($p < 0.05$) from the MET (%) in G1R; **Significantly different ($p < 0.01$) from the MET (%) in G1R; N.A.: not applicable.

4. Discussion

Although the combination therapy of MET with another class of anti-diabetic medication (*e.g.* thiazolidinediones, sulfonylureas, non-sulfonylurea, alpha-glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, sodium glucose co-transporter-2 (SGLT2) inhibitors) for treating T2D patients improved the glycemic control [33–35], promoted weight loss [35,36], and slowed the progression of renal disease [37], the potential drug-drug especially herb-drug interactions of MET warrant further investigations. To our knowledge, the results of this study was the first to demonstrate that co-administration of BBR with MET *via* oral route led to increased $AUC_{0-\infty}$, $t_{1/2}$ and MRT value with decreased CL/F value of MET. Comparing the pharmacokinetic impacts of both drugs after their co-administration at different dosing

Table 3
BBR plasma concentration (ng/ml) after oral administrations of BBR (200 mg/kg) in absence and presence of MET (200 mg/kg).

Time (h)	BBR (G2, n = 5)	MET + BBR (G3, n = 7)	BBR + MET (G4, n = 6)	BBR&MET (G5, n = 6)
0.5	2.32 ± 1.54	0.17 ± 0.45	1.94 ± 3.71	0.65 ± 1.58
1	0.96 ± 1.10	0.32 ± 0.55	2.53 ± 3.35	1.09 ± 1.73
2	N.A.	2.07 ± 3.88	5.69 ± 7.20	1.97 ± 3.31
3	1.55 ± 1.81	1.38 ± 1.18	3.06 ± 3.11	1.31 ± 2.52
4	0.48 ± 0.66	1.38 ± 0.69	1.17 ± 1.62	1.48 ± 2.33
12	0.65 ± 0.91	1.21 ± 0.99	0.32 ± 0.51	0.23 ± 0.58
24	0.61 ± 0.83	0.75 ± 1.02	0.43 ± 0.70	0.26 ± 0.63

Notes: Data are given as the mean ± standard deviation (SD); N.A.: not applicable.

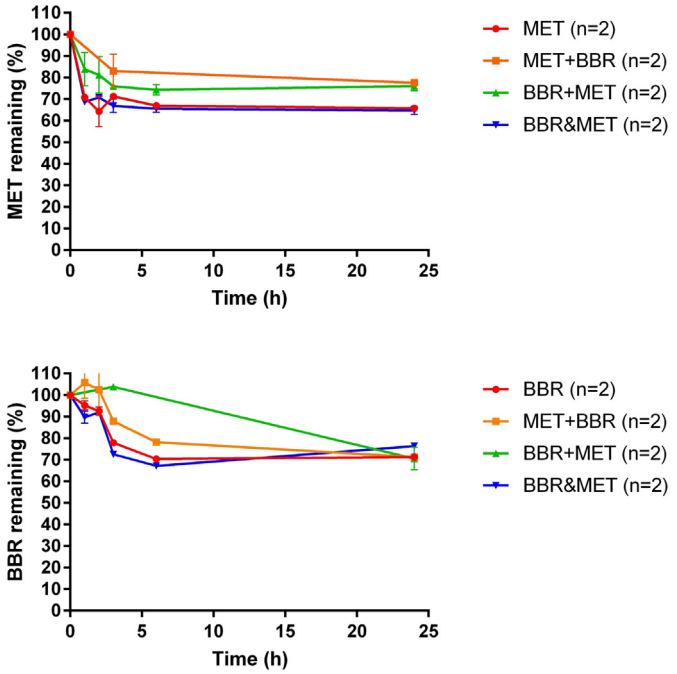


Fig. 4. The percentage remaining of MET (upper) and BBR (bottom) during 24-h incubation with 10 μg/ml MET and BBR at different administration sequences in human fecalase (n = 2 in each group).

sequences, BBR appeared to have a more significant impact on the pharmacokinetics of MET.

Several factors contributing to the impact of BBR on the pharmacokinetics of MET deserve attention. First, BBR can be metabolized by certain liver enzymes (*e.g.* CYPs) [38], as well as gut microbiota, to at least seventeen metabolites (including phase I and phase II metabolites in feces, bile and urine) [32]; while MET was reported not to be metabolized in liver of either human or experimental animals including rats and mice [4]. Therefore, chances for liver enzyme mediated interactions between MET and BBR were low. Second, considering the possible interaction during the elimination phase of the two drugs, although it was noted that BBR could decrease the hepatic uptake and/or renal elimination of MET through organic cation transporter (OCT) 1 and OCT2 transporters in previously published study [25], the chances for BBR to inhibit MET disposition *in vivo* are still low due to its extremely low *in vivo* concentrations after oral administrations at the dose up to 100 mg/kg [26]. Third, as to the potential interactions between MET and BBR at absorption stage, it is well known that absorption uptake of MET is mediated by OCT3, plasma monoamine transporter (PMAT) and serotonin transporter (SERT) [39,40], while the efflux of BBR is mediated by P-glycoprotein (P-gp) [41–43] on the apical membrane of intestinal epithelial cells. Although potential inhibition of OCT1 mediated influx of MET by BBR would lead to the decreased MET systemic concentration in first 4-h after their co-administration at the same time, the AUC_{0-24} of MET remained unaffected [26]. Therefore, impact of BBR on MET absorption is very limited.

Since the chances of interactions between BBR and MET on hepatic metabolism, hepatic and renal elimination and intestinal absorption are all very low, we focused on the role of gut microbiota on the pharmacokinetic interaction between BBR and MET in the current study. It has been proven that MET-altered gut microbiota can improve glucose tolerance by reducing hemoglobin A_{1c} (HbA_{1c}) level and lowering fasting blood glucose concentrations in human [44]. In addition, findings suggested that BBR not only inhibited insulin resistance and obesity in high-fat-diet-fed rats by modulating gut microbiota [45], but also restored gut microbiota dysbiosis [46] and regulated energy metabolism by promoting the production of butyrate in gut microbiota [47]. Our results demonstrated that co-administered BBR could significantly influence intestine tract metabolism of MET, which may lead to its altered pharmacokinetics. According to our findings from *in vitro* rat intestinal contents incubation with MET in the absence and presence of BBR, both MET and BBR could be degraded by gut microbiome to some extents. Two-hour pre-treatment with BBR could significantly reduce the MET degradation in intestinal content incubation, leading to the increase of MET remaining concentration in intestinal tract and subsequent greater *in vivo* absorption.

It is noted that the degradations of MET and BBR in human fecalase at different administration sequences were consistent with that in rat intestinal content incubation, suggesting such degradation tendency in GI tract could also occur in T2D patients co-administered with MET and BBR. Moreover, in accordance with our findings from nine strains bacteria incubated with MET and/or BBR, BBR decreased the MET degradation in seven types of bacteria including *Bacteroides fragilis*, *Clostridium perfringens*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae* and *Lactobacillus fermentum*. Meanwhile, after 24-h incubation, MET could decrease the BBR degradation in only four types of bacteria, namely *Bacteroides fragilis*, *Bifidobacterium pseudolongum*, *Clostridium perfringens* and *Escherichia coli*. Such different impact on bacteria from MET and BBR suggested that BBR may influence the MET metabolism in GI tract more. It is well reported that changes of microbiota by one drug, such as antibiotics, may influence GI stability of its co-administered drug, leading to alteration of the pharmacokinetics for the co-administered drug [48–52]. Zhang et al. revealed that MET and BBR have similarities in gut microbial phylotypes modulations, including the enrichment of certain bacteria and reduction of microbial diversity [53]. Literatures reported the alterations of different phylum by MET or BBR in T2D related animal disease models including high-fat-diet (HFD) obesity or HFD obesity/insulin resistance rats with both drugs reducing the number of *Firmicutes* and increasing/maintaining the number of *Proteobacteria* [53–55]. In addition, BBR could *reduce/maintain* the number of *Bacteroidetes* and increase that of *Fusobacteria*, whereas MET has no impact on them [53–55]. In HFD mice models, both drugs demonstrated reduction and increase in the number of *Firmicutes* and *Verrucomicrobiota*, respectively [4656–59]. Besides, BBR could increase the number of *Bacteroidetes*, while controversial alterations in were found for MET. Among all the treatment groups, 2-h pre-treatment with BBR has the highest impact on the pharmacokinetics of MET. Similar to the microbiota mediated drug-drug interactions of antibiotics, we hypothesized that 2-h pre-treatment of BBR may reduce certain types of bacteria commonly shared by the two drugs in intestine contents leading to less degradation and more absorption of MET in the intestine when BBR was pretreated to rats. When MET was administered with BBR at the same time, absence of such antibiotic like pretreatment could result in limited microbiota mediated interactions, leading to no significant impact on the pharmacokinetics of MET. Since the current study is designed to initiate the investigation on the pharmacokinetic interactions for the co-administered MET and BBR in healthy animal model first, further

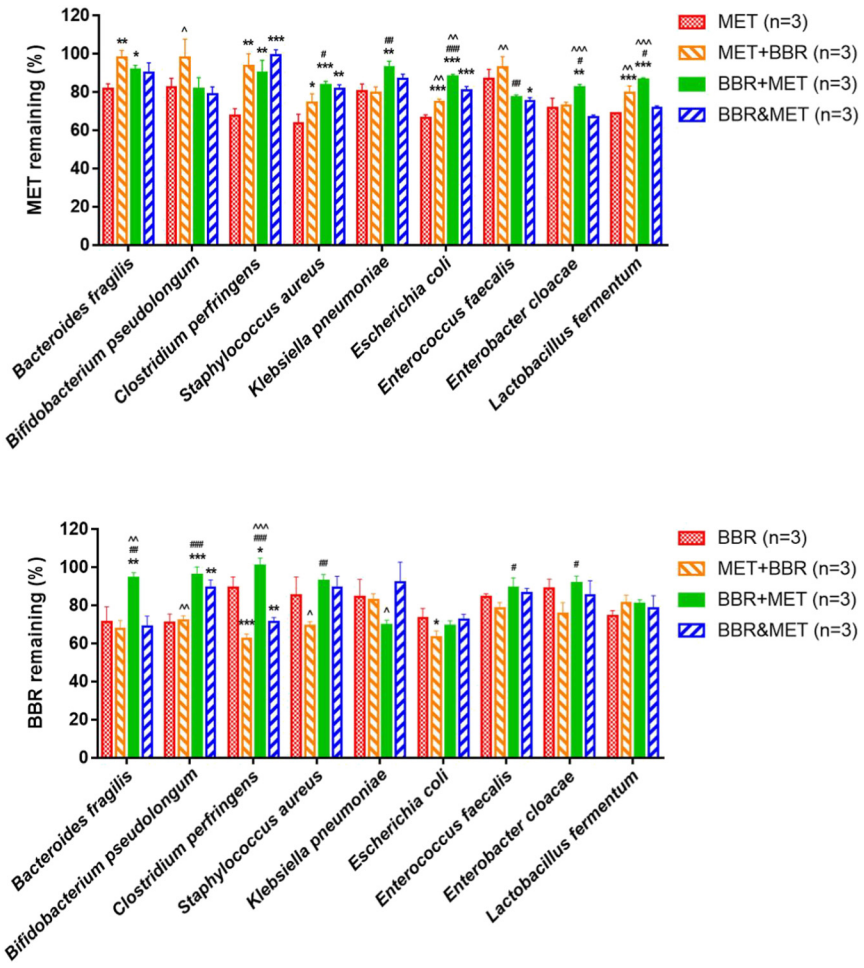


Fig. 5. The percentage remaining of MET (upper) and BBR (bottom) after 24-h incubation with 50 μM MET and BBR at different administration sequences in selected bacteria ($n = 3$ in each group). Data are given as the mean ± standard deviation (SD); *Significantly different ($p < 0.05$) from the G1B/G2B; **Significantly different ($p < 0.01$) from the G1B/G2B; ***Significantly different ($p < 0.001$) from the G1B/G2B; #Significantly different ($p < 0.05$) from G3B, ##Significantly different ($p < 0.01$) from G3B, ###Significantly different ($p < 0.001$) from G3B; ^Significantly different ($p < 0.05$) from G5B, ^^Significantly different ($p < 0.01$) from G5B, ^^^Significantly different ($p < 0.001$) from G5B.

pharmacodynamic based interaction in T2D diseased model to monitor the changes of plasma glucose levels, insulin levels, as well as related biomarkers are warranted for more clinically relevant interpretation.

5. Conclusion

In summary, co-administration of MET and BBR with different dosing sequences resulted in different extent of pharmacokinetic interactions. BBR pretreatment had significant impacts on the MET pharmacokinetics, whereas simultaneous co-administration of BBR and MET led to minimal changes in pharmacokinetics of MET. MET and BBR could be degraded in rat intestinal content, human fecalase and bacteria *in vitro* with a similar trend. Two-hour pretreatment with BBR could significantly decrease microbiota mediated degradation of MET, leading to its higher bioavailability *in vivo*. Our current study for the first time indicated that co-treatment of MET and BBR with 2-h administration interval would be potentially beneficial for T2D patients due to the increased systemic exposure of MET.

Declaration of competing interest

The authors declared no conflict of interest.

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