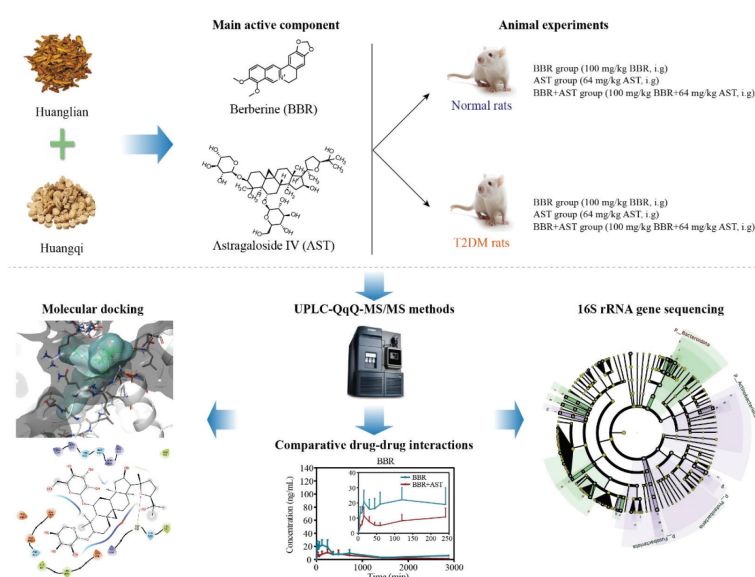


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Comparative drug-drug interactions of berberine and astragaloside IV in normal and type 2 diabetes mellitus rats based on UPLC-QqQ-MS/MS

Graphical abstract



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In brief

We investigated the drug-drug interactions of berberine and astragaloside IV both in normal and T2DM rats based on UPLC-QqQ-MS/MS and found that gut microbiota participates in the drug-drug interactions of berberine and astragaloside IV.

Highlights

- A UPLC-QqQ-MS/MS method for quantitative analysis of berberine, astragaloside IV, and their main metabolites was established.
- The drug-drug interactions of berberine and astragaloside IV both in normal and T2DM rats were investigated.
- Gut microbiota participates in the drug-drug interactions of berberine and astragaloside IV.

Comparative drug-drug interactions of berberine and astragaloside IV in normal and type 2 diabetes mellitus rats based on UPLC-QqQ-MS/MS

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ABSTRACT

The UPLC-QqQ-MS/MS method was established, validated, and used for the simultaneous detection of berberine (BBR), astragaloside IV (AST), and the main metabolites to demonstrate the comparative pharmacokinetics of BBR and AST in normal and T2DM rats. BBR and AST had reduced the internal exposure of each other and their main metabolites in normal rats. However, AST had few significant effects on the pharmacokinetic parameters of BBR and the main metabolites in T2DM rats. Similarly, BBR had no significant effect on the pharmacokinetic parameters of AST but significantly increased the exposure to cycloastragenol (CAG) *in vivo*. Molecular docking of BBR and AST with the P-glycoprotein (P-gp) was performed, which indicated that both BBR and AST are potential substrates for P-gp. The differences in gut microbiota between normal and T2DM rats were compared by 16S rRNA sequencing. Gut microbiota that could produce β -glucosidase and β -xylosidase were highly abundant in T2DM rats. The current study indicated that BBR and AST had reduced oral bioavailability. The gut microbiota was enriched in the intestines of T2DM rats and promoted the hydrolysis of AST to produce CAG, while the drug-drug interaction between AST and BBR was blocked.

Keywords: Drug-drug interactions, Berberine, Astragaloside IV, Type 2 diabetes mellitus, UPLC-QqQ-MS/MS

1. INTRODUCTION

Diabetes mellitus is a long-lasting, progressive disease with numerous etiologies and a protracted course [1]. One in 10 persons between 20 and 79 years of age had diabetes mellitus in 2021 and this number may reach 643 million by 2030 and 783 million by 2045 [2]. Type 2 diabetes mellitus (T2DM) accounts for the vast majority of diabetes cases. T2DM and associated complications, such as diabetic nephropathy (DN) and diabetic retinopathy (DR), severely endanger human health [3, 4].

The treatment goal clinicians and T2DM patients strive for is T2DM remission but there is currently no clear treatment strategy to achieve this goal [5, 6]. T2DM patients must control blood glucose levels using hypoglycemic drugs, a healthy diet, persistent exercise, and continuous blood glucose monitoring to alleviate the symptoms and stop complications from developing. Biguanides, GLP-1 agonists, sulfonylureas, and DPP-4

inhibitors are the currently used hypoglycemic agents [7-10]. Although these medications can effectively control the blood glucose level, adverse effects, such as hypoglycemia and damage to multiple organs (the retina, pancreas, liver, kidneys, and gastrointestinal tract), frequently occur during the prevention and treatment of chronic diabetic complications [11]. It is occasionally necessary to combine multiple drugs to better control the blood glucose level [12, 13]. There is a search for medications to prevent T2DM progression and complications that can successfully manage blood glucose levels and protect organs with few side effects.

Traditional Chinese medicine (TCM) has been used to treat T2DM for over two thousand years, which falls under the category of *Xiaokezhen* in Chinese. *Coptis chinensis* Franch. (Huanglian in Chinese) is a key TCM frequently included in many effective prescriptions for treating T2DM, such as Jinqi Jiangtang tablets, Xiaokeping tablets, and Tangmaikang [14-18]. Modern

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pharmacologic research and clinical trials have shown that *Coptis chinensis* Franch. has multiple pharmacologic effects, such as a hypoglycemic effect, hypolipidemic effect, and improving insulin resistance [19, 20]. Data mining and network pharmacology revealed that *Coptis chinensis* Franch. is often used in combination with *Astragalus membranaceus* (Fisch.) Bunge (Huangqi in Chinese) to treat T2DM [21]. However, the scientific connotation of compatibility between *Coptis chinensis* Franch. and *Astragalus membranaceus* (Fisch.) Bunge is unknown. Revealing the drug-drug interactions between the two drugs is crucial in explaining the synergistic mechanisms.

Coptis chinensis Franch. mainly contains alkaloids, such as berberine (BBR), epiberberine, and jatrorrhizine with the highest concentration of BBR up to 10% [22]. BBR is more closely related to the effects of *Coptis chinensis* Franch. or its alkaloids on hypoglycemia and a hypoglycemic mechanism study of *Coptis chinensis* Franch. or its alkaloids also focuses on BBR [23-25]. *Astragalus membranaceus* (Fisch.) Bunge contains complex chemical compounds that include saponins, polysaccharides, flavonoids, and amino acids. Astragaloside IV (AST) has the highest content in *Astragalus membranaceus* (Fisch.) Bunge and has been the representative component for studying the pharmacologic effects of *Astragalus membranaceus* (Fisch.) Bunge.

The ultra-performance liquid chromatography-triple quadrupole-tandem mass spectrometry (UPLC-QqQ-MS/MS) method to determine the comparative drug-drug interactions of BBR and AST in normal and T2DM rats was established. The plasma concentrations of BBR and the main metabolites, such as berberrubine (M1), thalifendine (M2), demethylenoberberine (M3), and jatrorrhizine (M4), as well as AST and its metabolite, cycloastragenol (CAG), were determined in rat plasma and the pharmacokinetic parameters were calculated to characterize the patterns of change among the different constituents *in vivo*.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

BBR (#110713-202015) and nuciferine (#111566-201907) were obtained from the National Institutes for Food and Drug Control (Beijing, China). M1 (#Y-292), M3 (#Q-084), M4 (#Y-023), AST (#H-013), and CAG 9#H-051) were purchased from Chengdu Herbpurify Co., Ltd. (Chengdu, China). M2 (#B28948) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Streptozocin (#S0130) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade methanol, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

2.2 Animal experiments

Male Sprague-Dawley (SD) rats (250±20 g) were purchased from the SPF (Beijing) Biotechnology Co., Ltd.

(License No. SCXK (Beijing) 2019-0010; Beijing, China) and kept in specific pathogen-free grade conditions (22±2°C environment with 50 ± 10% relative humidity and a 12:12 h light:dark cycle). All rats were free to eat and drink water and acclimatized to the facilities for 1 week. The rats underwent a 12-h fast with access to water before the experiment. All animal experiments were evaluated and approved by the Ethics Committee of Beijing University of Chinese Medicine (Licence No. BUCM-2022060502-2132) and the animal experiments were carried out according to the guidelines from EU Directive 2010/63/EU for animal experiments.

2.3 Pharmacokinetic experiments involving BBR and AST in normal rats

Twenty-one SD rats were separated into 3 groups (n=7). Rats in the BBR and AST groups were administered oral BBR (100 mg/kg) and AST (64 mg/kg) separately and rats in the BBR+AST group were administered oral BBR (100 mg/kg) and AST (64 mg/kg) simultaneously. After the oral administration of BBR, AST, or BBR+AST, blood samples were collected into heparinized tubes through a retro-orbital puncture at 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h. Plasma samples were obtained by centrifugation at 3000 rpm for 15 min at 4°C. All plasma samples were promptly stored at -80°C.

2.4 Pharmacokinetic experiments involving BBR and AST in T2DM rats

Forty SD rats were used in this experiment with 8 rats randomly allocated to the normal control group and fed with a normal diet; the other 32 rats were fed a high-fat diet. After feeding for 4 weeks, all rats were fasted overnight. The 32 rats fed the high-fat diet were injected intraperitoneally with 35 mg/kg streptozotocin (STZ), while the 8 rats in the normal control group were injected intraperitoneally with the same volume of citrate buffer (0.1 mol/L [pH=4.5]). The fasting blood glucose level in all rats was tested with a glucometer after 3 days and the rats with a fasting blood glucose level ≥11.1 mmol/L were designated T2DM rats.

Twenty-four T2DM rats were randomly divided into the T2DM group (n=6), T2DM+BBR group (n=6), T2DM+AST group (n=6), and T2DM+BBR+AST group (n=6). Rats in the T2DM+BBR and T2DM+AST groups were administered oral BBR (100 mg/kg) and AST (64 mg/kg) separately and rats in the T2DM+BBR+AST group were administered oral BBR (100 mg/kg) and AST (64 mg/kg) simultaneously. One rat in the T2DM+AST group died due to a gavage error during the administration process. Blood samples were collected into heparinized tubes through the retro-orbital puncture at 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h after oral administration. Plasma samples were obtained by centrifugation at 3000 rpm for 15 min at 4°C. All plasma samples were promptly stored at -80°C.

2.5 UPLC-QqQ-MS/MS method

2.5.1 Plasma sample pre-treatment. Rat plasma samples were stored in a refrigerator at 4°C, then 100 µL was precisely transferred to a 1.5-mL centrifuge tube and 10 µL of internal standard solution (nuciferine [400 ng/mL]) and 290 µL of acetonitrile were added. The 400-µL liquid was vortexed and mixed for 2 min, then allowed to stand for 5 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was blown dry with nitrogen (N₂) at 40°C and 100 µL of acetonitrile-water (1:1, v/v) solution was added to the centrifuge tube to redissolve the residue. The mixture was then vortexed and mixed for 2 min, centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was transferred into sample vials for UPLC-QqQ-MS/MS analysis.

2.5.2 Preparation of standard solutions. BBR, M1, M2, M3, M4, AST, CAG, and nuciferine (internal standard) were precisely weighed and dissolved in methanol to prepare the reserve solution with a concentration of 1 mg/kg. Then, different concentrations (1, 5, 10, 50, 100, 250, 500, 1000, 2500, 5000, and 8000 ng/mL) of standard working solutions for BBR, M1, M2, M3, M4, AST, and CAG, and a nuciferine solution [400 ng/mL] (internal standard) were prepared. All the standard solutions were stored in the refrigerator at 4°C.

2.5.3 Preparation of quality control samples. Three concentrations (low, medium, and high) of quality control (QC) samples were prepared with blank rat plasma and the reserve solution of different compounds in parallel with the plasma samples for quality monitoring and method validation. Specifically, the low, medium, and high QC sample concentrations were as follows: BBR, 10.00, 100.00, and 450.00 ng/mL; M1, 10.00, 60.00, and 420.00 ng/mL; M2, 8.00, 80.00, and 347.00 ng/mL; M3, 5.00, 56.00, and 244.00 ng/mL; M4, 7.00, 70.00, and 312.00 ng/mL; AST, 5.00, 50.00, and 300.00 ng/mL; and CAG, 80.00, 150.00, and 400.00 ng/mL.

2.5.4 Chromatographic conditions. The UPLC-QqQ-MS/MS system includes an Acquity UPLC system, a triple quadrupole Xevo TQ-S micro-instrument equipped with an electrospray ionization (ESI) source, and a Masslynx 4.1 mass spectrometry workstation (Waters, Milford, MA, USA). An Acquity UPLC BEH C₁₈ column (2.1 mm×100 mm, 1.7 µm; Waters) was used for chromatographic separation at a column temperature of 40°C. Mobile phase A was 0.1% (v/v) formic acid aqueous solution and mobile phase B was acetonitrile. The gradient elution method was as follows: 0–1 min, 90%–80% A; 1–3 min, 80%–77.5% A; 3–3.5 min, 77.5%–67.5% A; 3.5–4.5 min, 67.5%–55% A; 4.5–5 min, 55%–10% A; 5–5.51 min, 10%–90% A; 5.51–8 min, 80% A; and 0.30 mL/min. A 2-µL sample was injected for analysis.

2.5.5 Mass spectrometry conditions. The ESI source was operated in the positive ion mode. The collision gas

was argon (Ar), the purge gas was nitrogen (N₂), the capillary ionization voltage was 3.9 kV, the cone-bore voltage was 109 V, the temperature of the ionization source was 150°C, the temperature of the desolventizing gas was 350°C, and the flow rate of the desolventizing gas was 650 L/h. The measurements were carried out using the multi-reaction monitoring (MRM) mode and the mass spectrometry data were acquired and processed with the Masslynx 4.1 mass spectrometric workstation.

2.5.6 UPLC-QqQ-MS/MS method validation. Method validation was carried out according to the *Pharmacopoeia of the People's Republic of China* (2020 edition).

The MRM chromatograms of blank plasma, blank plasma spiked with nuciferine (internal standard) and standard solutions, and plasma samples of rats in the BBR+AST group were contrasted to evaluate the specificity of the method.

Linearity and lower limit of quantitation (LLOQ). Nine concentrations of standard solutions were tested to investigate the linearity and LLOQ of the method. Standard curves were drawn using the least square method with the peak area ratio of each analyte to internal standard versus the corresponding concentration in plasma. The LLOQ is the term used to describe the lowest concentration on the standard curve.

The sample at the concentration of the upper limit of quantification (ULOQ) was tested three times to perform the carryover effects of the method, then the blank sample was detected.

Six replicate analyses of quality control (QC) samples at different concentrations (BBR: 10.00, 100.00, and 450.00 ng/mL; M1: 10.00, 60.00, and 420.00 ng/mL; M2: 8.00, 80.00, and 347.00 ng/mL; M3: 5.00, 56.00, and 244.00 ng/mL; M4: 7.00, 70.00, and 312.00 ng/mL; AST: 5.00, 50.00, and 300.00 ng/mL; and CAG: 80.00, 150.00, and 400.00 ng/mL) were detected 3 times on 1 day to evaluate the intra-day accuracy and precision of the method. Two replicate analyses of QC samples at the same concentration were detected three times on three consecutive analytical days to evaluate the inter-day accuracy and precision of the method. The ratios of the peak area of different compounds to the peak area of the internal standard were calculated and the corresponding concentrations were calculated with the standard curves. The accuracy of the method was defined as the percentage of the calculated concentration to the theoretical concentration, which should be within a range of 85%–115%. The precision was defined as the relative standard deviation (RSD) and the RSD values were <15%.

Six replicate analyses of QC samples at different concentrations were used to evaluate the extraction recovery and matrix effect of the method. Rat blank plasma (80 µL) was obtained, then 10 µL of standard solutions and 10 µL of internal standard solutions (nuciferine) were added to prepare solution A. Rat blank plasma (100 µL) was obtained, then 300 µL of acetonitrile was

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added to prepare solution B. The 400 μL liquid was vortexed and mixed for 2 min, then allowed to stand for 5 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was blown dry by N_2 at 40°C, then 10 μL of standard solutions and 10 μL of internal standard solutions (nuciferine) were added, then the solution was blown dry by N_2 at 40°C and 100 μL of acetonitrile-water (1:1, v/v) solution was added to the centrifuge tube to redissolve the residue. The mixture was vortexed, then centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was transferred into sample vials. The rat blank plasma was replaced with acetonitrile and the above extraction steps were repeated to prepare solution C. The peak area ratio of different compounds and the internal standard in solutions A, B, and C were calculated separately after the UPLC-QqQ-MS/MS analysis. The extraction recovery rate was calculated based on the ratio of solution B divided by the ratio of solution A and the matrix effect was calculated based on the ratio of solution B divided by the ratio of solution C. The extraction recovery rate and matrix effect should be within a range of 85%–115%.

Six replicate analyses of QC samples at low, medium, and high concentrations were used to evaluate the short-term, long-term, and repeated freeze-thaw stability of each compound. The concentrations were measured 6 times for each sample after placing the QC sample at 10°C for 24 h and the short-term stability was evaluated by calculating the RSD. The concentrations were measured 6 times for each sample after storing the QC samples at -80°C for 10 d and the long-term stability was evaluated by calculating the RSD. The QC samples under -80°C and room temperature conditions were freeze-thawed 3 times, the concentrations were measured 6 times for each sample, and the stability of the repeated freeze-thaw process was evaluated by calculating the RSD. The RSD values did not exceed 15%.

2.6 Pharmacokinetic study and data processing

The DAS 2.1 program (Shanghai, China) was used to determine pharmacokinetic parameters, including the area under the concentration-time curve (AUC_{0-t} and $\text{AUC}_{0-\infty}$), maximum concentration (C_{max}), time to reach the maximum concentration (T_{max}), half-time ($t_{1/2}$), and mean residence time (MRT). Pharmacokinetic data are expressed as the mean $\pm$ standard deviation (SD). An unpaired *t*-test was used to analyze statistical differences and a $P < 0.05$ was regarded as a statistically significant difference.

2.7 Molecular docking

Molecular docking was performed in autodock 4, as previously described with modifications [26]. Specifically, the 3D structures of BBR and AST were obtained from PubChem and imported into AutoDockTools-1.5.6 to calculate the rotatable bond number. The 3D structure of the human ATP-dependent translocase/P-glycoprotein (ABCB1/P-gp) was obtained from the AlphaFold Protein

Structure Database (AF-P08183-F1) and imported into AutoDockTools-1.5.6 to calculate the total charge. ABC transporter 1 and 2 of ABCB1/P-gp were selected as the receptor pocket.

2.8 16S rRNA gene sequencing

Fecal samples derived from normal and T2DM rats were 16S rRNA gene sequenced to analyze the bacterial diversity (OEBiotech Co., Ltd., Shanghai, China). The experimental process included DNA extraction, V3-V4 variable regions of 16S rRNA gene amplification, PCR product sequencing, and representative reading of each amplicon sequence variant (ASV) [27, 28].

3. RESULTS

3.1 Method validation

The chemical structures of BBR, AST, and the metabolites are shown in Figure 1A. The quantitative parameters of the seven main constituents (BBR, M1, M2, M3, M4, AST, and CAG) and internal standard (nuciferine) are shown in Figure 1B and Table 1.

3.1.1 Specificity. The chromatograms of blank plasma samples (Figure 2A), the corresponding blank plasma samples with seven measured compounds, the internal standard (Figure 2B), as well as the plasma samples collected from rats after oral administration of BBR+AST (Figure 2C) are shown. There was no interaction between the seven measured compounds and the internal standard during the retention time and the endogenous matrix components did not affect the ability to distinguish between the seven measured compounds and the internal standard. This finding demonstrated that the method had high specificity.

3.1.2 Carryover effects. After injecting the ULOQ sample three times, blank plasma was injected. As shown in Figure 2D, no substance peak was detected in the blank plasma, which indicated that there was no residue of the measured compounds in this method.

3.1.3 Standard curve, linear range, and LLOQ. The independent variable (*x*) was the theoretical concentration of the measured compounds and the dependent variable (*y*) was the peak area ratio of the measured compounds to nuciferine (internal standard). The linear regression equation was calculated with a weighting coefficient of $1/x^2$. The LLOQ was calculated based on the baseline noise. The regression equations of the standard curve, correlation coefficient (*r*), linearity ranges, and LLOQ of the seven measured compounds are shown in Table 2, which showed that the linear equations used in this method can be used to calculate the content of the measured substances in the samples. The calibration curves of the seven measured compounds were in the following ranges: 1.00–800.00 (BBR); 0.10–800.00 (M1); 0.10–800.00 (M2); 0.10–800.00

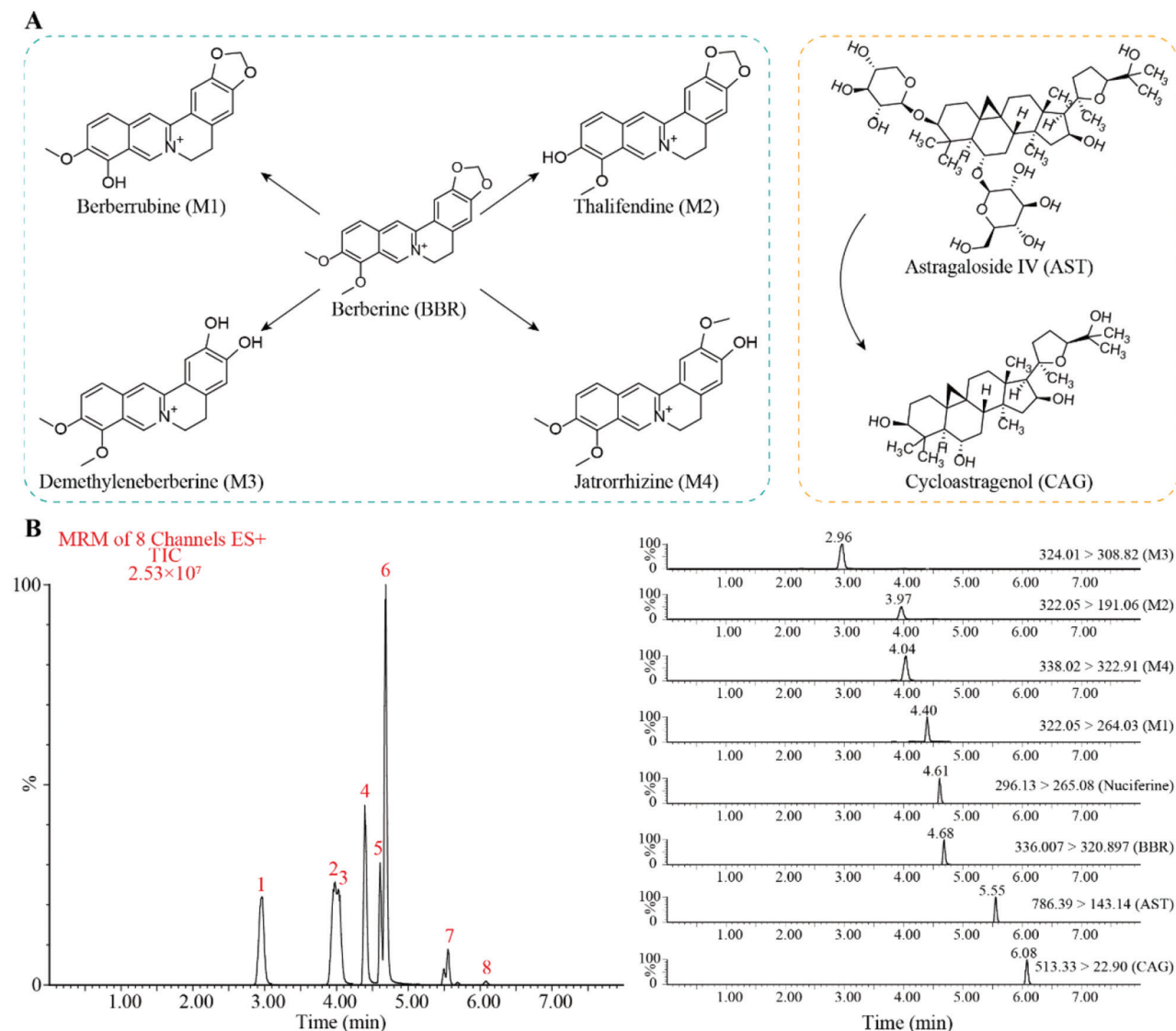


Figure 1 | Multi-reaction detection ion chromatograms of investigated compounds and internal standards.

(A) Chemical structures of berberine, astragaloside IV, and the metabolites. (B) Multi-reaction detection ion chromatograms of 1: demethyleneberberine (M3), 2: thalifendine (M2), 3: jatrorrhizine (M4), 4: berberrubine (M1), 5: nuciferine (internal standard), 6: berberine (BBR), 7: astragaloside A (AST), and 8: cycloastragenol (CAG).

(M3); 0.10–800.00 (M4); 5.00–800.00 (AST); and 5.00–800.00 ng/mL (CAG). The LLOQs of the seven measured compounds were 1.00, 0.10, 0.10, 0.10, 0.10, 5.00, and 5.00 ng/mL, respectively.

3.1.4 Precision and accuracy. The precision and accuracy of the method were evaluated according to the procedures described in the Methods section. The calculated concentration percentage to the theoretical concentration was calculated to determine accuracy, while RSD was calculated to define the intra- and inter-day precision. The RSD was <14.4% and the accuracy of intra- and

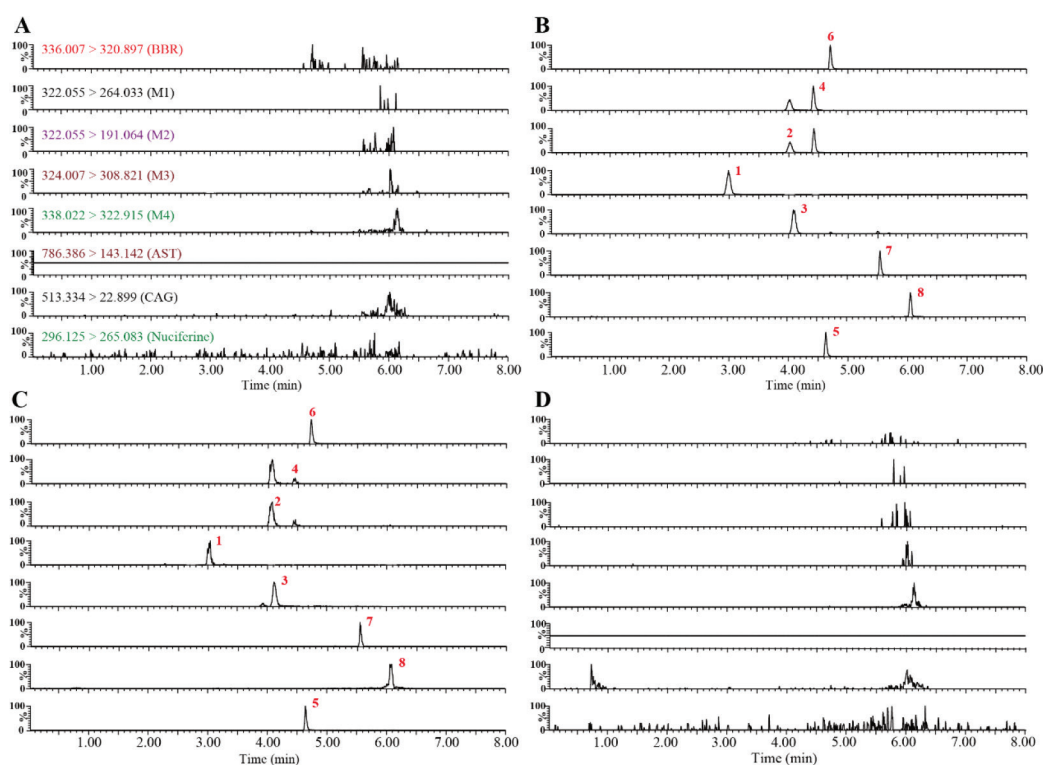
inter-day were 85.8–105.4% and 85.3–112.4%, respectively, as shown in Table 3. These results indicate that the precision and accuracy of the UPLC-QqQ-MS/MS method met the quantitative requirements.

3.1.5 Extraction recovery rate and matrix effect. The extraction recovery rates of the seven measured compounds ranged from 87.4%–114.5% (RSD<13.1) and the matrix effects ranged from 85.1%–112.9% (RSD<12.7), as shown in Table 4. The results indicated that the endogenous elements in rat plasma only marginally interfered with the components being evaluated, the

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Table 1 | MRM parameters of seven measured compounds and internal standard.

Compound	Molecular formula	t_R (min)	Parent ion m/z	Daughter ion m/z	Cone voltage (V)	Collision energy (eV)
BBR	$C_{20}H_{18}NO_4$	4.68	336.007	320.897	78	78
M1	$C_{19}H_{16}NO_4$	4.40	322.05	264.03	20	42
M2	$C_{19}H_{16}NO_4$	3.97	322.05	191.06	20	70
M3	$C_{19}H_{18}NO_4$	2.96	324.01	308.82	36	18
M4	$C_{20}H_{20}NO_4$	4.04	338.02	322.91	32	18
AST	$C_{41}H_{68}O_{14}$	5.55	786.39	143.14	14	8
CAG	$C_{30}H_{50}O_5$	6.08	513.33	22.90	94	38
Nuciferine	$C_{19}H_{21}NO_2$	4.61	296.13	265.08	2	12

**Figure 2** | Specificity and carryover effects of the UPLC-QqQ-MS/MS method.

MRM chromatograms of investigated components and internal standards in rat blank plasma (A), blank plasma samples added with seven measured compounds and the internal standard (B), and rat plasma after administration of BBR+AST for 45 min (C). MRM chromatograms of investigated components and internal standards in rat blank plasma after injecting the upper limit of the quantification sample three times (D). 1: demethylenberberine (M3), 2: thalifendine (M2), 3: jatrorrhizine (M4), 4: berberrubine (M1), 5: nuciferine (internal standard), 6: berberine (BBR), 7: astragaloside A (AST), 8: cycloastragenol (CAG).

sample pre-treatment method was practical, and the recovery rate of samples in plasma was high, all of which met the requirements for determination.

3.1.6 Stability. The short-term, long-term, and freeze-thaw stability were assessed according to the procedures described in the Methods section. As shown in [Table 5](#),

all 7 measured compounds were stable under the above conditions with an $RSD \leq 14.2\%$.

3.2 BBR and AST drug-drug interactions in normal rats

The validated UPLC-QqQ-MS/MS method was applied to study the drug-drug interactions of BBR and AST in

Table 2 | Investigation of the linear relationship.

Compound	Linear range (ng/mL)	Linear equation	Correlation coefficient (r)	LLOQ (ng/mL)
BBR	1.00–800.00	$y=0.2252x+0.9928$	0.9968	1.00
M1	0.10–800.00	$y=0.3151x+0.2467$	0.9979	0.10
M2	0.10–800.00	$y=0.2977x+0.2167$	0.9974	0.10
M3	0.10–800.00	$y=0.6591x+1.6806$	0.9985	0.10
M4	0.10–800.00	$y=1.3569x+0.0856$	0.9979	0.10
AST	5.00–800.00	$y=0.0372x+0.3673$	0.9955	5.00
CAG	5.00–800.00	$y=0.1733x+1.3466$	0.9956	5.00

Table 3 | Accuracy and precision of seven investigated compounds in rat plasma.

Compound	Theory concentration (ng/mL)	Intra-day (n=6)		Inter-day (n=6)	
		Accuracy (%)	Precision (RSD/%)	Accuracy (%)	Precision (RSD/%)
BBR	10.00	103.7	5.4	103.4	3.5
	100.00	99.8	8.4	96.6	8.6
	450.00	98.7	4.1	97.6	7.0
M1	10.00	95.2	9.6	85.8	11.7
	60.00	96.9	9.0	92.6	10.4
	420.00	96.2	7.2	88.1	10.2
M2	8.00	88.1	10.7	85.3	5.1
	80.00	95.7	9.2	87.1	11.2
	347.00	96.5	5.2	88.9	11.4
M3	5.00	89.9	6.1	91.7	8.5
	56.00	94.2	13.9	94.6	11.6
	244.00	97.5	12.0	98.3	12.1
M4	7.00	86.8	6.1	90.2	6.0
	70.00	93.6	13.8	92.1	10.8
	312.00	91.4	6.2	92.7	6.0
AST	5.00	89.5	14.3	112.4	9.7
	50.00	105.4	11.3	90.9	14.4
	300.00	89.8	4.3	100.2	14.3
CAG	80.00	103.9	14.4	99.6	13.9
	150.00	85.8	11.0	90.0	13.4
	400.00	90.1	11.8	87.9	12.5

normal rats after the administration of BBR (100 mg/kg) AST (64 mg/kg) and BBR+AST (100 mg/kg BBR and 64 mg/kg AST) at the single dosages. The mean concentration-time curves of the seven measured compounds are illustrated in [Figure 3](#). The pharmacokinetic parameters,

including AUC_{0-t} , $AUC_{0-\infty}$, C_{max} , T_{max} , $t_{1/2}$, MRT_{0-t} and $MRT_{0-\infty}$ are presented in [Table 6](#).

After oral administration of 100 mg/kg of BBR, the C_{max} of BBR, M1, M2, M3, and M4 in the rat plasma were 489.99 ± 463.66 , 3.67 ± 2.04 , 8.13 ± 4.73 , 3.76 ± 2.24 ,

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Table 4 | Extraction recovery rate and matrix effect of seven compounds.

Compound	Theory concentration (ng/mL)	Extraction recovery rate		Matrix effect	
		$\bar{x} \pm s$ (%)	RSD (%)	$\bar{x} \pm s$ (%)	RSD (%)
BBR	10.00	114.5±3.3	2.9	90.5±6.9	7.7
	100.00	87.4±5.5	6.3	93.5±10.5	11.2
	450.00	114.1±0.3	0.2	85.1±1.0	1.2
M1	10.00	114.3±14.3	12.5	109.1±11.0	10.0
	60.00	101.1±10.3	10.2	93.3±10.7	11.5
	420.00	104.2±10.5	10.0	104.0±11.9	11.4
M2	8.00	110.4±8.2	7.4	98.6±8.3	8.4
	80.00	112.5±5.8	5.2	105.5±8.0	8.0
	347.00	111.5±7.2	6.5	100.7±12.8	12.7
M3	5.00	95.5±6.0	6.2	112.9±5.8	5.1
	56.00	97.6±11.4	11.7	104.6±7.8	7.4
	244.00	100.5±7.9	7.9	85.9±2.9	3.3
M4	7.00	112.4±5.8	5.2	99.5±6.6	6.6
	70.00	112.3±2.7	2.4	105.3±8.2	7.8
	312.00	107.4±7.6	7.1	100.2±4.3	4.3
AST	5.00	97.3±12.8	13.1	112.8±4.7	4.2
	50.00	101.3±7.9	7.8	105.7±11.8	11.1
	300.00	88.2±8.3	9.4	104.9±7.6	7.3
CAG	80.00	101.8±10.2	10.0	98.9±10.0	10.1
	150.00	92.6±9.8	10.6	95.1±11.0	11.5
	400.00	99.5±12.8	12.9	88.3±4.8	5.4

and 27.01±19.60 ng/mL, the T_{max} of BBR, M1, M2, M3, and M4 in rat plasma were 0.18±0.15, 12.94±11.08, 9.18±10.41, 6.98±11.63, and 1.00±2.21 h, the $t_{1/2}$ of BBR, M1, M2, M3, and M4 were 15.61±13.28, 10.94±8.49, 6.52±3.85, 6.86±3.95, and 22.68±26.13 h, the $AUC_{(0-t)}$ of BBR, M1, M2, M3, and M4 were 461.51±187.91, 59.76±36.57, 79.07±88.78, 36.33±45.98, and 42.42±27.60 h·ng·mL⁻¹, the $AUC_{(0-\infty)}$ of BBR, M1, M2, M3, and M4 were 494.25±196.81, 489.33±718.05, 214.41±412.85, 306.24±532.45, and 61.07±60.74 h·ng·mL⁻¹, the $MRT_{(0-t)}$ of BBR, M1, M2, M3 and M4 were 7.06±6.01, 19.78±7.95, 13.45±8.56, 15.20±10.29, and 11.93±11.36 h, the $MRT_{(0-\infty)}$ of BBR, M1, M2, M3, and M4 were 11.14±8.45, 17.47±17.35, 12.42±9.83, 11.84±12.42, and 26.05±24.97 h, respectively, as shown in **Figure 3** and **Table 6**. There was no significant change in the BBR C_{max} , $t_{1/2}$, and $MRT_{(0-t)}$ and the main metabolites in rat plasma and there was no significant change in the BBR $MRT_{(0-\infty)}$ and the main metabolites except for M1 in rat plasma after administration of BBR and AST. However, the combination of BBR and AST significantly increased

the BBR T_{max} ($P<0.05$) but simultaneously reduced the M1 T_{max} ($P<0.05$). The BBR and AST combination significantly reduced the BBR $AUC_{(0-t)}$ by 43.0% ($P<0.05$) and M1 by 73.1% ($P<0.01$). The BBR $AUC_{(0-\infty)}$ and the main metabolites in rat plasma had different reductions but difference between the BBR and the BBR+AST group was not statistically significant. These results indicate that AST significantly inhibited the absorption of BBR in normal rats.

With respect to the pharmacokinetic characteristics of AST, after oral administration of AST (64 mg/kg), the AST and CAG C_{max} in rat plasma was 176.51±59.13 and 429.63±108.51 ng/mL, respectively, the AST and CAG T_{max} in rat plasma was 3.68±3.13 and 0.75±0.14 h, respectively, the AST and CAG $t_{1/2}$ was 11.75±9.89 and 15.78±11.45 h, respectively, the AST and CAG $AUC_{(0-t)}$ was 2354.48±1177.20 and 4152.30±940.11 h·ng·mL⁻¹, respectively, the AST and CAG $AUC_{(0-\infty)}$ was 3028.27±1628.81 and 5943.53±2425.53 h·ng·mL⁻¹, respectively, the AST and CAG $MRT_{(0-t)}$ was 11.67±5.17 and 14.08±3.38 h, respectively, and the AST and CAG $MRT_{(0-\infty)}$ was 22.45±17.99

Table 5 | Stability of sever investigated components in rat plasma.

Compound	Theory concentration (ng/mL)	Short-term stability		Long-term stability		Freeze-thaw stability	
		Accuracy (%)	Precision (RSD/%)	Accuracy (%)	Precision (RSD/%)	Accuracy (%)	Precision (RSD/%)
BBR	10.00	99.8	2.5	104.7	7.4	85.0	2.3
	100.00	99.6	11.7	87.9	4.5	108.6	1.0
	450.00	100.2	3.0	99.3	14.2	94.4	13.2
M1	10.00	101.1	3.6	100.8	8.7	101.0	8.2
	60.00	100.1	4.7	101.2	8.6	101.6	11.5
	420.00	100.8	5.1	100.8	7.5	101.9	8.9
M2	8.00	98.2	1.2	100.8	7.5	100.8	11.2
	80.00	100.5	2.0	100.5	6.4	101.2	10.3
	347.00	100.2	2.2	100.3	2.6	101.3	5.0
M3	5.00	100.6	2.6	100.3	8.4	100.7	7.0
	56.00	99.8	1.6	100.0	9.5	100.9	11.1
	244.00	100.9	4.3	99.7	7.4	100.1	5.1
M4	7.00	99.4	2.6	100.6	3.9	99.6	5.1
	70.00	101.0	3.8	100.2	4.8	100.8	6.1
	312.00	100.4	4.3	100.0	3.1	100.8	3.1
AST	5.00	102.9	9.9	86.4	10.5	102.9	9.5
	50.00	101.6	5.5	87.5	3.9	92.4	7.9
	300.00	100.7	3.5	94.3	9.5	93.3	8.9
CAG	80.00	101.6	5.3	99.4	12.2	102.4	11.5
	150.00	102.0	5.3	98.8	9.6	98.7	6.0
	400.00	101.2	4.2	96.8	13.3	101.6	6.2

and 27.36 ± 13.75 h, respectively, as shown in [Figure 3](#) and [Table 6](#). There was no significant change in the AST and CAG T_{max} , $t_{1/2}$, and $MRT_{(0-\infty)}$ in rat plasma after administration of BBR and AST. However, the BBR and AST combination significantly increased the CAG C_{max} ($P < 0.05$) but reduced the AST $AUC_{(0-t)}$ ($P < 0.05$) and CAG $AUC_{(0-t)}$ ($P < 0.001$) and the CAG $AUC_{(0-\infty)}$ ($P < 0.001$). It was obvious that the BBR and AST combination significantly reduced the AST $AUC_{(0-t)}$ by 38.5% and CAG $AUC_{(0-t)}$ by 70.8% and the CAG $AUC_{(0-\infty)}$ by 64.5%. Although there was no statistical difference in the AST $AUC_{(0-\infty)}$ between the AST and BBR+AST groups, the BBR and AST combination reduced the AST $AUC_{(0-\infty)}$ in rat plasma. The above results indicated that BBR significantly inhibited AST absorption in normal rats.

3.3 BBR and AST drug-drug interactions in T2DM rats

There was a significant increase in the fasting blood glucose level in rats after 4 weeks of feeding a high-fat diet

and intraperitoneal injection of STZ. Rats with a fasting blood glucose level ≥ 11.1 mmol/L were designated as T2DM rats for follow-up experiments. The fasting blood glucose levels are shown in [Figure 4A](#).

The validated UPLC-QqQ-MS/MS method was successfully applied to study the BBR and AST drug-drug interactions in T2DM rats after administration of BBR (100 mg/kg), AST (64 mg/kg), BBR+AST (BBR [100 mg/kg] and AST [64 mg/kg]) at the single dosages. The mean concentration-time curves of BBR, AST, and the main metabolites are illustrated in [Figure 4B-H](#) and [Table 7](#).

After oral administration of BBR (100 mg/kg), the BBR, M1, M2, M3, and M4 C_{max} in T2DM rat plasma was 32.27 ± 25.47 , 0.68 ± 0.12 , 3.10 ± 2.20 , 4.03 ± 2.18 , and 0.68 ± 0.36 ng/mL, respectively the BBR, M1, M2, M3, and M4 T_{max} in rat plasma was 2.71 ± 2.90 , 5.11 ± 9.38 , 12.13 ± 17.84 , 0.26 ± 0.37 , and 5.68 ± 9.21 h, respectively, the BBR, M1, M2, M3, and M4 $t_{1/2}$ was 17.41 ± 8.16 , 11.83 ± 9.54 , 8.63 ± 2.61 , 27.00 ± 22.59 , and 19.41 ± 11.64 h, respectively, the BBR, M1, M2, M3, and M4 $AUC_{(0-t)}$ was

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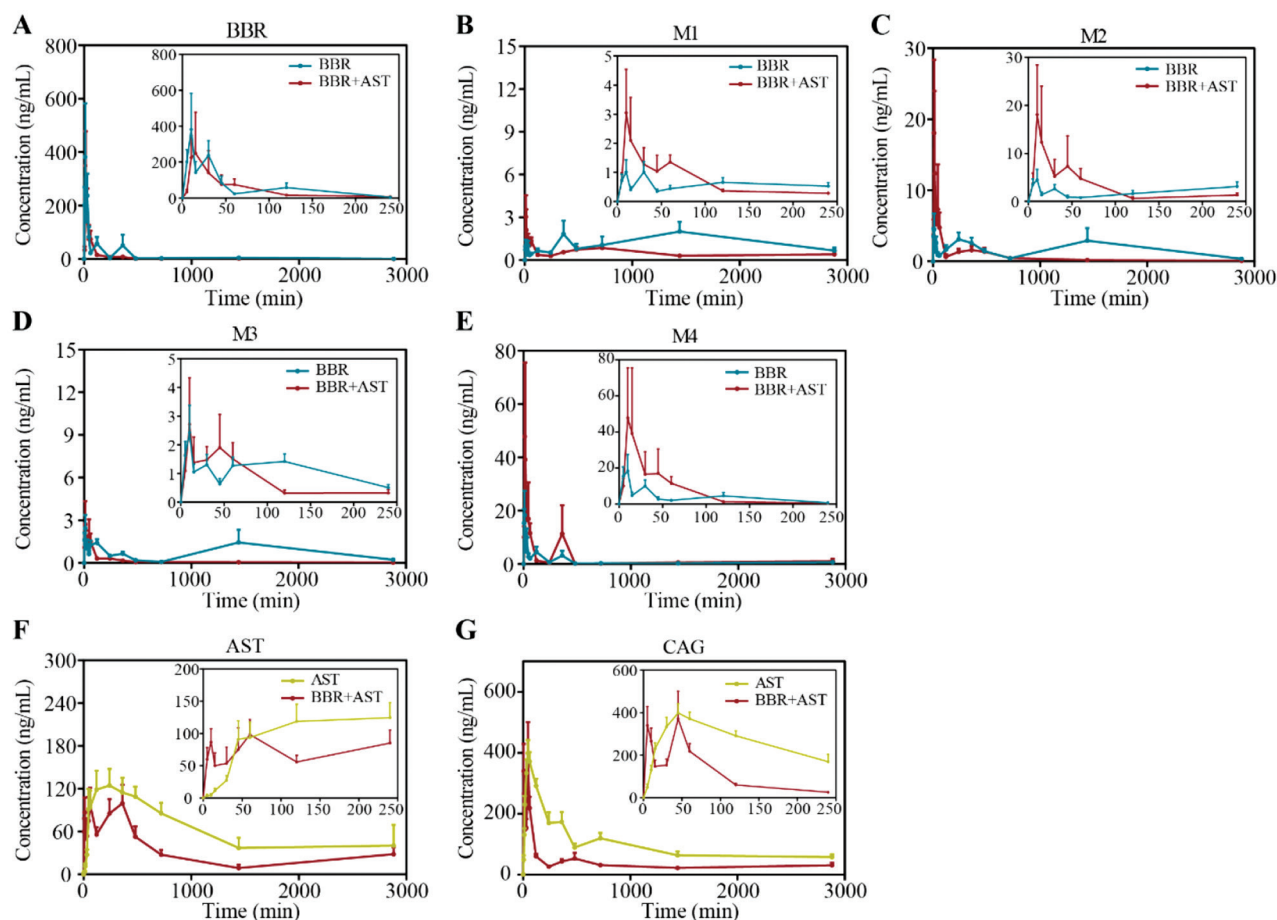


Figure 3 | Plasma concentration-time profiles of BBR, AST, and the main metabolites in normal rats.

Mean plasma concentration-time profiles of BBR (A) and its main metabolite M1 (B), M2 (C), M3 (D), and M4 (E) in normal rats after oral administration of BBR or BBR+AST ($n=7$); mean plasma concentration-time profiles of AST (F) and its main metabolite, CAG (G) in normal rats after oral administration of AST or BBR+AST ($n=7$).

297.01±247.80, 10.72±5.84, 41.45±26.96, 36.47±14.58, and 8.97±4.19 h·ng·mL⁻¹, respectively, the BBR, M1, M2, M3, and M4 AUC_(0-∞) was 376.12±219.74, 20.98±23.19, 42.77±28.04, 71.99±32.65, and 11.32±5.21 h·ng·mL⁻¹, respectively, the BBR, M1, M2, M3, and M4 MRT_(0-t) was 15.19±8.21, 20.07±7.29, 19.42±4.47, 24.47±3.92, and 16.09±5.65 h, respectively, and the BBR, M1, M2, M3, and M4 MRT_(0-∞) was 36.71±20.71, 26.78±13.69, 31.43±14.56, 53.16±31.66, and 31.39±12.54 h, respectively (Figure 4B-H and Table 7). There was no significant change in the BBR and the main metabolites (except for M1) C_{max}, AUC_(0-t), and AUC_(0-∞) in T2DM rat plasma after administration of BBR and AST. However, the combination of BBR and AST significantly increased the M3 T_{max} ($P<0.01$) and the M2 t_{1/2} ($P<0.05$). The BBR and AST combination significantly reduced the M3 MRT_(0-t) and MRT_(0-∞) ($P<0.01$ and $P<0.05$, respectively). These results indicated that AST had little significant effect on the pharmacokinetic parameters of BBR in T2DM rats.

With respect to the pharmacokinetic characteristics of AST, the AST and CAG C_{max} in the rat plasma was 156.17±62.36 and 197.50±143.17 ng/mL, respectively, the AST and CAG T_{max} in rat plasma was 3.60±1.67 and 3.55±2.58 h, respectively, the AST and CAG t_{1/2} was 14.33±6.11 and 18.06±12.52 h, respectively, the AST and CAG AUC_(0-t) was 2923.84±977.75 and 3416.75±1479.31 h·ng·mL⁻¹, respectively, the AST and CAG AUC_(0-∞) was 3517.81±867.43 and 5194.69±1534.69 h·ng·mL⁻¹, respectively, the AST and CAG MRT_(0-t) was 17.98±7.14 and 17.84±6.19 h, respectively, the AST and CAG MRT_(0-∞) was 42.70±20.28 and 38.85±16.55 h, respectively, after oral administration of 64 mg/kg of AST (as shown in Figure 4B-H and Table 7). There was no significant change in the pharmacokinetic parameters of AST in T2DM rat plasma after administration of BBR and AST. However, it was obvious that the BBR and AST combination significantly increased the CAG C_{max} 3.8-fold ($P<0.01$), reduced the CAG T_{max} by 75.2% ($P<0.05$), increased the CAG

Table 6 | Pharmacokinetic parameters of seven components in normal rats after administration of BBR, AST, and BBR+AST (n=7).

Compound	Group	C _{max} (ng·ml ⁻¹)	T _{max} (h)	t _{1/2} (h)	AUC _(0-t) (h·ng·ml ⁻¹)	AUC _(0-∞) (h·ng·ml ⁻¹)	MRT _(0-t) (h)	MRT _(0-∞) (h)
BBR	BBR	489.99±463.66	0.18±0.15	15.61±13.28	461.51±187.91	494.25±196.81	7.06±6.01	11.14±8.45
	BBR+AST	542.17±610.39	0.54±0.39 [#]	14.05±6.07	263.26±215.32 [#]	414.30±388.92	6.69±4.18	9.35±6.09
M1	BBR	3.67±2.04	12.94±11.08	10.94±8.49	59.76±36.57	489.33±718.05	19.78±7.95	17.47±17.35
	BBR+AST	4.58±4.19	3.17±4.82 [#]	22.67±18.62	16.06±7.28 ^{##}	29.33±13.79	13.06±6.35	35.89±20.74 [#]
M2	BBR	8.13±4.73	9.18±10.41	6.52±3.85	79.07±88.78	214.41±412.85	13.45±8.56	12.42±9.83
	BBR+AST	27.83±33.64	2.38±3.22	5.88±4.02	28.66±16.44	29.62±16.62	8.65±4.58	10.47±6.15
M3	BBR	3.76±2.24	6.98±11.63	6.86±3.95	36.33±45.98	306.24±532.45	15.20±10.29	11.84±12.42
	BBR+AST	4.30±4.24	0.46±0.26	10.14±9.17	5.68±3.38	7.97±4.96	10.54±6.61	13.68±10.69
M4	BBR	27.01±19.60	1.00±2.21	22.68±26.13	42.42±27.60	61.07±60.74	11.93±11.36	26.05±24.97
	BBR+AST	83.75±103.26	1.26±2.11	20.31±12.49	53.40±39.08	61.16±44.06	12.29±14.63	20.51±20.93
AST	AST	176.51±59.13	3.68±3.13	11.75±9.89	2354.48±1177.20	3028.27±1628.81	11.67±5.17	22.45±17.99
	BBR+AST	177.11±58.34	2.63±2.63	11.01±13.10	1448.42±405.96 [#]	1827.31±1172.25	17.47±6.95	29.14±18.92
CAG	AST	429.63±108.51	0.75±0.14	15.78±11.45	4152.30±940.11	5943.53±2425.53	14.08±3.38	27.36±13.75
	BBR+AST	598.25±189.23 [#]	0.50±0.40	21.68±16.63	1212.98±169.59 ^{##}	2112.08±655.24 ^{###}	9.86±3.8 [#]	26.76±21.87

[#]P < 0.05, ^{##}P < 0.01, ^{###}P < 0.001 vs. the BBR or AST groups.

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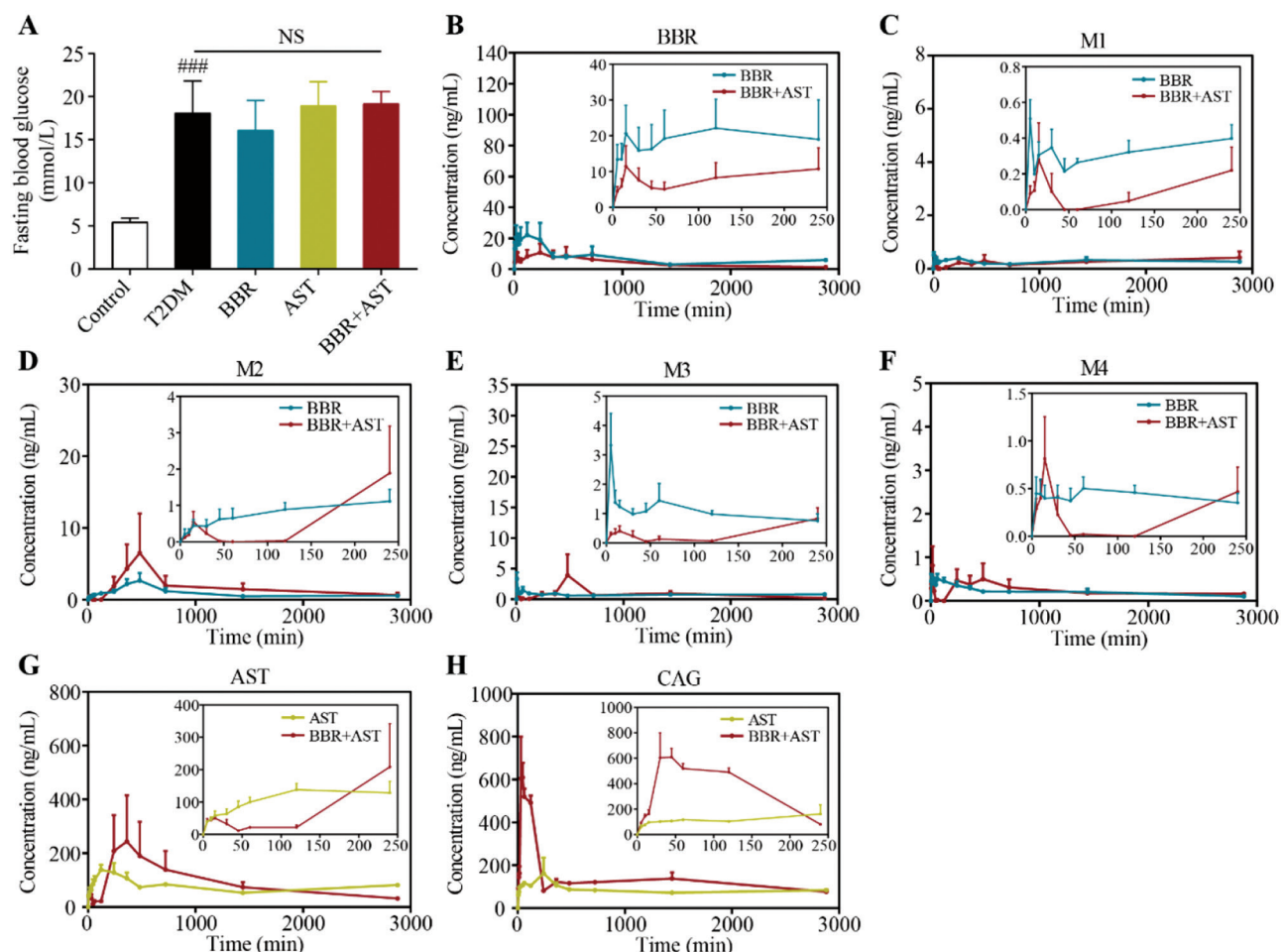


Figure 4 | Plasma concentration-time profiles of BBR, AST, and the main metabolites in T2DM rats.

Fasting blood glucose levels of rats in each group (A), ### $P < 0.001$ vs. the control group, NS: no statistical difference vs. that of the T2DM group; mean plasma concentration-time profiles of BBR (B) and its main metabolite, M1 (C), M2 (D), M3 (E), M4 (F) in T2DM rats after oral administration of BBR or BBR+AST ($n=5-6$); Mean plasma concentration-time profiles of AST (G) and its main metabolite, CAG, (H) in T2DM rats after oral administration of AST or BBR+AST ($n=5-6$).

AUC_(0-t) 1.70-fold ($P < 0.05$), and the CAG AUC_(0-∞) 2.29-fold ($P < 0.05$). The $t_{1/2}$, MRT_(0-t), and MRT_(0-∞) was not statistically significant difference between the AST and BBR+AST groups.

These results indicated that AST had few significant effects on the pharmacokinetic parameters of BBR and the main metabolites in T2DM rats. Similarly, BBR had no significant effect on the pharmacokinetic parameters of AST but BBR significantly increased the exposure of CAG *in vivo*.

3.4 Molecular docking

Molecular docking of BBR and AST with ABC transporters 1 and 2 of the ABCB1/P-gp was performed separately to explain the potential mechanism by which BBR and AST reduced exposure in normal rats.

The ABCB1/P-gp has two nucleotide-binding domains (ABC transporters 1 and 2) and 12 transmembrane

helices [29, 30]. The results of molecular docking showed that BBR and AST bound to ABCB1/P-gp at ABC transporter 1 or 2 with binding energies < -7 kcal/mol, which indicated stable binding. The ligand binding pocket is mainly polar and contains 5 hydrophilic residues, including Thr-1046, Ser-1071, Ser-1072, Ser-1077, and Gln-1081, while residues Tyr-1044 and Tyr-1087 provide hydrophobic contacts. AST bound to Gly-533 of ABC transporter 1 with a binding energy of -8.1 kcal/mol (Figure 5A), while BBR bound to Glu-493 of ABC transporter 1 with a binding energy of -8.3 kcal/mol (Figure 5B). AST also bound to Asp-1200, Gln-1081, and Lys-1076 of ABC transporter 2 with a binding energy of -8.0 kcal/mol (Figure 5C), while BBR bound to Arg-664 of ABC transporter 2 with a binding energy of -7.0 kcal/mol (Figure 5D). The molecular docking results indicated that BBR and AST bound to ABCB1/P-gp tightly, which may lead to the structural changes in ABCB1/P-gp and

Table 7 | Pharmacokinetic parameters of seven components in T2DM rats after administration of BBR, AST, and BBR+AST (n=5-6).

Compound	Group	C _{max} (ng·ml ⁻¹)	T _{max} (h)	t _{1/2} (h)	AUC _(0-t) (h·ng·ml ⁻¹)	AUC _(0-∞) (h·ng·ml ⁻¹)	MRT _(0-t) (h)	MRT _(0-∞) (h)
BBR	BBR	32.27±25.47	2.71±2.90	17.41±8.16	297.01±247.80	376.12±219.74	15.19±8.21	36.71±20.71
	BBR+AST	13.21±13.29	0.56±0.72	15.82±7.24	191.53±233.93	248.69±216.84	17.88±7.33	23.33±11.41
	BBR	0.68±0.12	5.11±9.38	11.83±9.54	10.72±5.84	20.98±23.19	20.07±7.29	26.78±13.69
	BBR+AST	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
M2	BBR	3.10±2.20	12.13±17.84	8.63±2.61	41.45±26.96	42.77±28.04	19.42±4.47	31.43±14.56
	BBR+AST	6.75±13.39	12.67±8.82	22.83±14.49 [#]	80.12±128.65	124.01±136.62	20.61±3.60	23.13±16.60
M3	BBR	4.03±2.18	0.26±0.37	27.00±22.59	36.47±14.58	71.99±32.65	24.47±3.92	53.16±31.66
	BBR+AST	4.27±8.30	14.00±8.29 ^{##}	15.54±12.61	41.00±48.33	110.84±120.48	17.81±3.91 ^{##}	21.21±17.37 [#]
M4	BBR	0.68±0.36	5.68±9.21	19.41±11.64	8.97±4.19	11.32±5.21	16.09±5.65	31.39±12.54
	BBR+AST	0.85±1.05	1.22±2.34	17.74±5.93	11.35±16.45	14.30±18.19	18.38±6.88	36.16±13.94
AST	AST	156.17±62.36	3.60±1.67	14.33±6.11	2923.84±977.75	3517.81±867.43	17.98±7.14	42.70±20.28
	BBR+AST	261.54±411.67	9.33±7.76	18.60±9.76	3895.91±3676.89	5771.03±4188.40	17.34±5.57	29.44±15.49
CAG	AST	197.50±143.17	3.55±2.58	18.06±12.52	3416.75±1479.31	5194.69±1534.69	17.84±6.19	38.85±16.55
	BBR+AST	751.40±353.97 ^{##}	0.88±0.56 [#]	26.25±15.40	5797.91±1522.43 [#]	11917.97±6552.90 [#]	16.31±3.68	33.40±18.02

[#]P<0.05, ^{##}P<0.01, ^{###}P<0.001 vs. the BBR or AST group.

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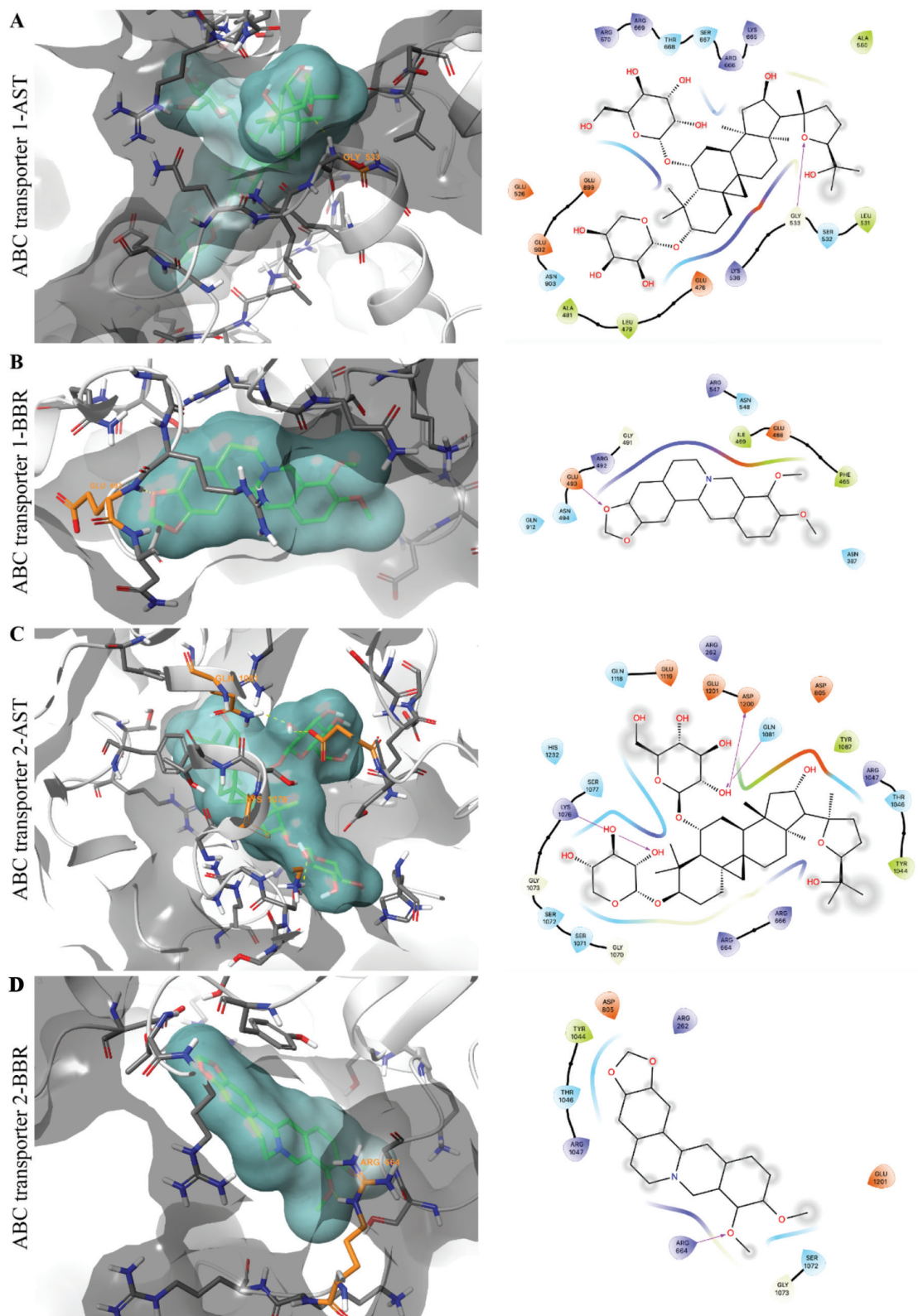


Figure 5 | Molecular docking of AST and BBR with ABCB1/P-gp.

Molecular docking of AST and BBR with ABC transporters 1 and 2 of ABCB1/P-gp was performed separately. AST and BBR were colored green and the surrounding residues in the binding pockets and the backbone of the ABC transporters 1 and 2 were depicted as other colors in the cartoon.

further affected the efflux process of BBR and AST from intestinal cells. The molecular docking results explained the reason why BBR and AST reduced the internal exposure and the main metabolites in normal rats.

3.5 Difference in gut microbiota between normal and T2DM rats

The differences in gut microbiota between normal and T2DM rats were compared by 16S rRNA sequencing. A Venn diagram shows the number of ASVs in the two groups. Mutual ASVs (1191 and 581) were detected in the normal and T2DM groups, respectively. Among the ASVs, 201 were shared by the two groups (Figure 6A). The Shannon and ACE indices of α -diversity were used to evaluate the diversity and richness of gut microbiota. Lower microbial community diversity was noted in the T2DM group compared to the normal group ($P < 0.01$; Figure 6B). β -diversity was used to measure the difference in colony composition among individuals in the two groups. Principal coordinates analysis (PCoA) based on Binary-Jaccard distance metrics was used to assess structural and compositional differences in gut microbiota between the two groups. The T2DM group was significantly distinct from the normal group (Figure 6C). The specific gut microbiota was identified by the linear discriminant analysis (LDA) effect size (LEfSe) analysis to determine the difference in gut microbiota between the two groups (Figure 6D). Fifty-four different microbial genera were identified, of which 17 were highly abundant in the T2DM group (Figure 6E). Of the 17 highly abundant genera, *Bacteroides* and *Enterococcus* produce β -glucosidase [31–33], while *Corynebacterium* and *Enterobacter* produce β -xylosidase [34, 35]. These two enzymes can hydrolyze AST to produce CAG.

4. DISCUSSION

In conclusion, we established a UPLC-QqQ-MS/MS analysis method for the simultaneous determination of BBR, AST, and the main metabolites, and studied the drug-drug interactions between BBR and AST in normal and T2DM rats. BBR and AST mutually inhibited absorption in normal rats. The situation was different in T2DM rats. Specifically, AST had few significant effects on the pharmacokinetic parameters of BBR and the main metabolites in T2DM rats. Similarly, BBR had no significant effect on the pharmacokinetic parameters of AST but BBR will significantly increase the exposure of CAG *in vivo*. There are still some issues worth discussing.

TCM has a long history in the treatment of T2DM in which drug-drug interactions have an important role. However, the composition of TCM is complex and the complexity becomes even greater after the compatibility of TCM. How to study the drug-drug interactions in TCM has always been a challenge. Studying the drug-drug interactions between the main active components of TCM can provide a research foundation and experimental reference for comprehensively elucidating the

drug-drug interactions in TCM. *Coptis chinensis* Franch. and *Astragalus membranaceus* (Fisch.) Bunge are commonly used in TCM for the treatment of T2DM. The current study focused on BBR, the main active component of *Coptis chinensis* Franch., and AST, the main active component of *Astragalus membranaceus* (Fisch.) Bunge and the drug-drug interactions of two compounds were studied in normal and T2DM rats, which provide the experimental basis for elucidating the synergistic mechanism of *Coptis chinensis* Franch. and *Astragalus membranaceus* (Fisch.) Bunge and provide a data reference for guiding the clinical application of TCM.

BBR is the main active component of *Coptis chinensis* Franch. For many years BBR has been used in the treatment of intestinal infectious diseases with remarkable curative effects. In recent years BBR has been reported to have good effects in treating metabolic cardiovascular diseases, such as T2DM, hyperlipidemia, and obesity [23, 25, 26, 36–39]. However, the oral bioavailability of BBR is very poor. Introducing other compounds to improve the oral bioavailability of BBR may further improve the therapeutic effect. After the combined use of BBR and AST, it was found that AST could not significantly promote the oral bioavailability of BBR. Because BBR can exert therapeutic effects on metabolic cardiovascular diseases by regulating the gut microbiota [24, 40, 41], whether the introduction of AST has an impact on the gut microbiota and whether the impact of BBR on the gut microbiota will further affect the metabolism of AST *in vivo* are scientific questions that warrant further exploration. We are conducting corollary studies and hope to present this part of the study to readers in the future.

An interesting phenomenon was discovered in the current study. The drug-drug interactions between BBR and AST showed a significant difference in normal and T2DM rats. BBR and AST mutually reduce internal exposure in normal rats for the following reasons: BBR or AST may increase the activities of efflux transporters, which led to a decrease in the $AUC_{(0-t)}/AUC_{(0-\infty)}$ of BBR and AST in rats; BBR and AST may competitively bind to drug transporters, leading to a decrease in the $AUC_{(0-t)}/AUC_{(0-\infty)}$ of BBR and AST in rats; and BBR or AST may induce an increase in liver drug-metabolizing enzyme activity, leading to an increase in their metabolism. We have preliminarily confirmed through molecular docking that BBR and AST are potential substrates of P-gp, which may be one of the reasons why BBR and AST mutually reduce the internal exposure *in vivo*. AST had no significant effect on the internal exposure of BBR and its metabolites in T2DM rats but BBR can significantly increase the $AUC_{(0-t)}/AUC_{(0-\infty)}$ of AST and its metabolite, CAG, *in vivo*. The reasons for these findings are worth considering and exploring further. A long-term high-fat diet could significantly affect the composition of the gut microbiota in T2DM rats. The difference in gut microbiota was involved in the drug-drug interactions between BBR and AST and had a major role. The gut microbiota that produced β -glucosidase and β -xylosidase were

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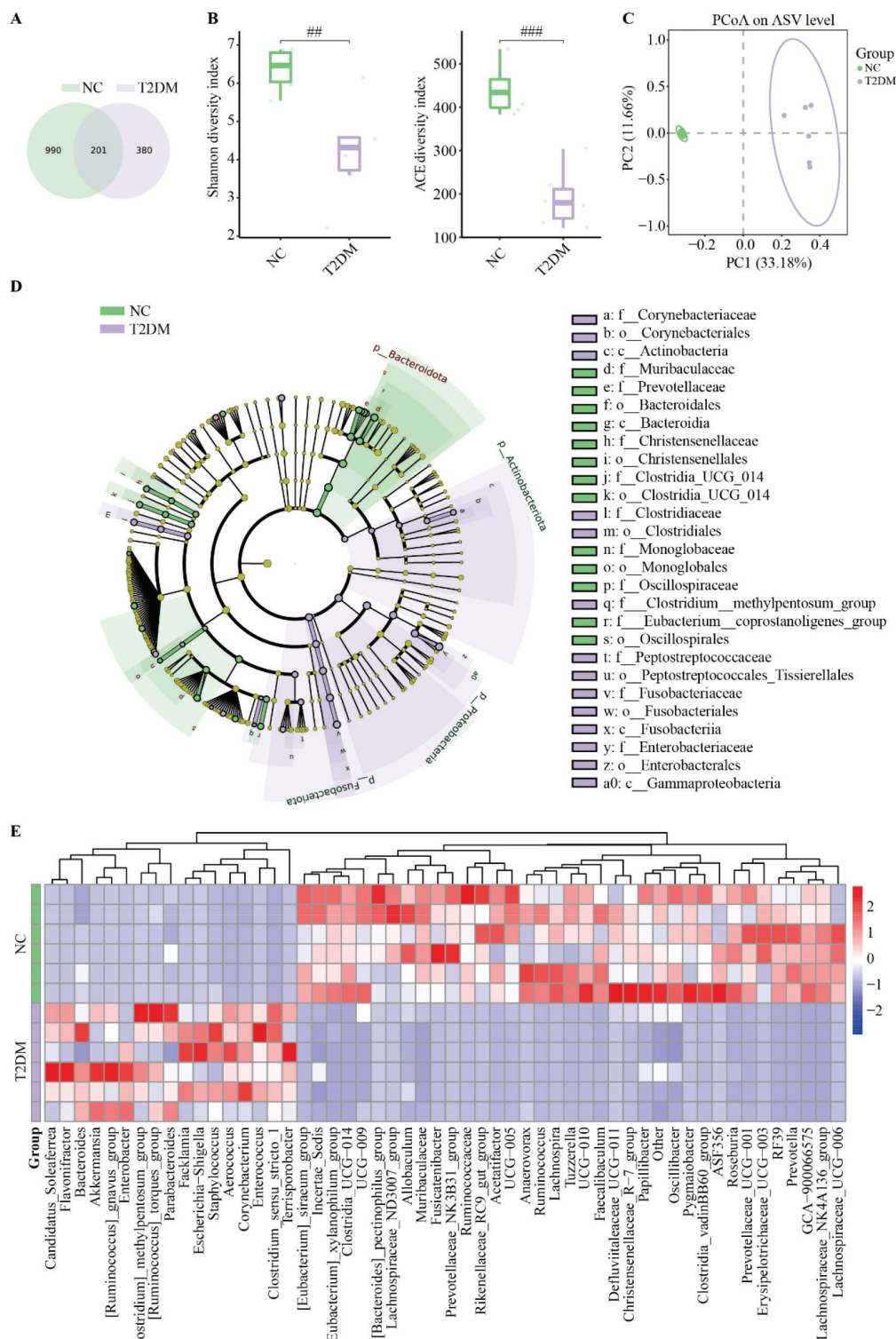


Figure 6 | 16S rRNA analysis of the gut microbiota in normal and T2DM rats.

(A) Venn diagram of the gut microbiota in the two groups. (B) Shannon and ACE indices used to represent the α -diversity of fecal gut microbiota are shown (## $P < 0.01$, ### $P < 0.001$). (C) PCoA plot based on Binary-Jaccard distance metrics of β -diversity for the two groups ($P < 0.001$). (D) Taxonomy cladogram obtained using LEfSe analysis. (E) The heat map of differential abundance bacteria at the genus level. The displayed data came from 6 rats in each group.

highly abundant in the T2DM rats based on 16S rRNA sequencing. β -glucosidase and β -xylosidase hydrolyze AST into CAG, so the drug-drug interactions between AST and BBR in T2DM rats were blocked and the level of CAG exposure was increased. Of course, further experimental verifications are needed to determine the specific reason so that we can better understand the scientific connotation of TCM compatibility.

In addition, the current study showed that the pharmacokinetic behaviors of the seven main compounds in normal and T2DM rats were significantly different. The oral bioavailability of BBR in T2DM rats was significantly decreased in the BBR and BBR+AST groups compared to normal rats. The oral bioavailability of AST in the AST and BBR+AST groups increased significantly, which shows that the pharmacokinetic behavior of BBR and AST components is related to the pathologic state of T2DM. It is worth noting that the plasma concentration-time profiles of five alkaloids all had double peaks. A bimodal phenomenon in the pharmacokinetics of alkaloids, such as BBR has been reported, which may be due to the existence of enterohepatic, stomach-intestine, and intestine-intestine circulation in the body.

5. CONCLUSION

BBR and AST reduced the oral bioavailability of each other. The gut microbiota promoted the hydrolysis of AST to produce CAG, while the drug-drug interactions between AST and BBR was blocked.

CONFLICT OF INTEREST

The authors declare no competing conflict of interest.

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DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

REFERENCES

- [1] Williams R, Karuranga S, Malanda B, Saeedi P, Basit A, Besançon S, et al.: Global and Regional Estimates and Projections of Diabetes-related Health Expenditure: Results from the International Diabetes Federation Diabetes Atlas, 9th Edition. *Diabetes Research and Clinical Practice* 2020, 162:108072.
- [2] Magliano DJ, Boyko EJ: *IDF Diabetes Atlas 10th Edition Scientific Committee, IDF DIABETES ATLAS [Internet], 10th Edition*. Brussels: International Diabetes Federation; 2021. Chapter 3, Global Picture.
- [3] GBD 2021 Diabetes Collaborators: Global, Regional, and National Burden of Diabetes from 1990 to 2021, with Projections of Prevalence to 2050: A Systematic Analysis for the Global Burden of Disease Study 2021. *Lancet* 2023, 402:203–234.
- [4] Weng JP, Bi Y: Epidemiological Status of Chronic Diabetic Complications in China. *Chinese Medical Journal* 2015, 128:3267–3269.
- [5] Riddle MC, Cefalu WT, Evans PH, Gerstein HC, Nauck MA, Oh WK, et al.: Consensus Report: Definition and Interpretation of Remission in Type 2 Diabetes. *Diabetes Care* 2021, 44:2438–2444.
- [6] Riddle MC, Cefalu WT, Evans PH, Gerstein HC, Nauck MA, Oh WK, et al.: Consensus Report: Definition and Interpretation of Remission in Type 2 Diabetes. *Diabetologia* 2021, 64: 2359–2366.
- [7] Chan SP, Colagiuri S: Systematic Review and Meta-analysis of the Efficacy and Hypoglycemic Safety of Gliclazide Versus Other Insulinotropic Agents. *Diabetes Research and Clinical Practice* 2015, 110:75–81.
- [8] Green JB, Bethel MA, Armstrong PW, Buse JB, Engel SS, Garg J, et al.: Effect of Sitagliptin on Cardiovascular Outcomes in Type 2 Diabetes. *The New England Journal of Medicine* 2015, 373:232–242.
- [9] Lalau JD, Kajbaf F, Bennis Y, Hurtel-Lemaire AS, Belpaire F, De Broe ME: Metformin Treatment in Patients With Type 2 Diabetes and Chronic Kidney Disease Stages 3A, 3B, or 4. *Diabetes Care* 2018, 41:547–553.
- [10] Zaccardi F, Htike ZZ, Webb DR, Khunti K, Davies MJ: Benefits and Harms of Once-Weekly Glucagon-like Peptide-1 Receptor Agonist Treatments: A Systematic Review and Network Meta-analysis. *Annals of Internal Medicine* 2016, 164:102–113.
- [11] Nauck MA, Wefers J, Meier JJ: Treatment of Type 2 Diabetes: Challenges, Hopes, and Anticipated Successes. *The Lancet Diabetes & Endocrinology* 2021, 9:525–544.
- [12] Morita Y, Murayama H, Odawara M, Bauer M: Treatment Patterns of Drug-naïve Patients with Type 2 Diabetes Mellitus: A Retrospective Cohort Study Using a Japanese Hospital Database. *Diabetology & Metabolic Syndrome* 2019, 11:90.
- [13] Xu Y, Wang L, He J, Bi Y, Li M, Wang T, et al.: Prevalence and Control of Diabetes in Chinese Adults. *JAMA* 2013, 310:948–959.
- [14] He H, Deng J, Yang M, An L, Ye X, Li X: Jatrophi rhizome from *Rhizoma Coptidis* Exerts an Anti-obesity Effect in db/db Mice. *Journal of Ethnopharmacology* 2022, 298:115529.
- [15] Yang Y, Cao S, Xu W, Zang C, Zhang F, Xie Y, et al.: Dual Modulation of Gut Bacteria and Fungi Manifests the Gut-based Anti-hyperlipidemic Effect of *Coptidis Rhizoma*. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie* 2022, 153:113542.
- [16] China Pharmacopoeia Committee: *Pharmacopoeia of the People's Republic of China, First Division*, 2020 ed. Beijing: China Medical Science and Technology Press; 2020, 1156.

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- [17] China Pharmacopoeia Committee: *Pharmacopoeia of the People's Republic of China, First Division, 2020 ed.* Beijing: China Medical Science and Technology Press; 2020, 1548.
- [18] China Pharmacopoeia Committee: *Pharmacopoeia of the People's Republic of China, First Division, 2020 ed.* Beijing: China Medical Science and Technology Press; 2020, 1871–1874.
- [19] Meng FC, Wu ZF, Yin ZQ, Lin LG, Wang R, Zhang QW: Coptidis Rhizoma and Its Main Bioactive Components: Recent Advances in Chemical Investigation, Quality Evaluation and Pharmacological Activity. *Chinese Medicine* 2018, 13:13.
- [20] Wang J, Wang L, Lou GH, Zeng HR, Hu J, Huang QW, et al.: Coptidis Rhizoma: a Comprehensive Review of Its Traditional Uses, Botany, Phytochemistry, Pharmacology and Toxicology. *Pharmaceutical Biology* 2019, 57(1):193–225.
- [21] Fu JY, Zhang Y: Mechanism of Astragali Radix-Coptis Rhizoma Pair in Treating Type 2 Diabetes Mellitus Based on Network Pharmacology. *Zhongguo Zhong Yao Za Zhi* 2021, 46:4808–4815.
- [22] Ma H, He K, Zhu J, Li X, Ye X: The Anti-hyperglycemia Effects of Rhizoma Coptidis Alkaloids: A Systematic Review of Modern Pharmacological Studies of the Traditional Herbal Medicine. *Fitoterapia* 2019, 134:210–220.
- [23] Kong WJ, Vernieri C, Foiani M, Jiang JD: Berberine in the Treatment of Metabolism-related Chronic Diseases: A Drug Cloud (dCloud) Effect to Target Multifactorial Disorders. *Pharmacology & Therapeutics* 2020, 209:107496.
- [24] Wang Y, Shou JW, Li XY, Zhao ZX, Fu J, He CY, et al.: Berberine-induced Bioactive Metabolites of the Gut Microbiota Improve Energy Metabolism. *Metabolism* 2017, 70:72–84.
- [25] Zhang H, Wei J, Xue R, Wu JD, Zhao W, Wang ZZ, et al.: Berberine Lowers Blood Glucose in Type 2 Diabetes Mellitus Patients through Increasing Insulin Receptor Expression. *Metabolism* 2010, 59:285–292.
- [26] Wang C, Yuan Z, Xie J, Lei Y, Li Y, Huang J, et al.: Integrated Metabolomics and Molecular Docking Reveal Berberrubine Inhibits Thrombosis by Regulating the Vitamin K Catalytic Cycle in Mice. *European Journal of Pharmacology* 2023, 938:175436.
- [27] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al.: Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nature Biotechnology* 2019, 37:852–857.
- [28] Nossa CW, Oberdorf WE, Yang L, Aas JA, Paster BJ, Desantis TZ, et al.: Design of 16S rRNA Gene Primers for 454 Pyrosequencing of the Human Foregut Microbiome. *World Journal of Gastroenterology* 2010, 16:4135–4144.
- [29] Mora Lagares L, Minovski N, Caballero Alfonso AY, Benfenati E, Wellens S, Culot M, et al.: Homology Modeling of the Human P-glycoprotein (ABCB1) and Insights into Ligand Binding through Molecular Docking Studies. *International Journal of Molecular Sciences* 2020, 21:4058.
- [30] Rosano C, Viale M, Cosimelli B, Severi E, Gangemi R, Ciogli A, et al.: ABCB1 Structural Models, Molecular Docking, and Synthesis of New Oxadiazolothiazin-3-one Inhibitors. *ACS Medicinal Chemistry Letters* 2013, 4:694–698.
- [31] Tao JH, Wang DG, Yang C, Huang JH, Qiu WQ, Zhao X: Biotransformation of Luteoloside by a Newly Isolated Human Intestinal Bacterium Using UHPLC-Q-TOF/MS. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences* 2015, 991:1–8.
- [32] Tao JH, Duan JA, Jiang S, Qian YY, Qian DW: Biotransformation and Metabolic Profile of Buddleoside with Human Intestinal Microflora by Ultrahigh-performance Liquid Chromatography Coupled to Hybrid Linear Ion Trap/Orbitrap Mass Spectrometer. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences* 2016, 1025:7–15.
- [33] Yang J, Qian D, Jiang S, Shang EX, Guo J, Duan JA: Identification of Rutin Deglycosylated Metabolites Produced by Human Intestinal Bacteria Using UPLC-Q-TOF/MS. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences* 2012, 898:95–100.
- [34] Campos E, Negro Alvarez MJ, Sabaris di Lorenzo G, Gonzalez S, Rorig M, Talia P, et al.: Purification and Characterization of a GH43 β -xylosidase from *Enterobacter* sp. Identified and Cloned from Forest Soil Bacteria. *Microbiological Research* 2014, 169:213–220.
- [35] Watanabe A, Hiraga K, Suda M, Yukawa H, Inui M: Functional Characterization of *Corynebacterium Alkanolyticum* β -Xylosidase and Xyloside ABC Transporter in *Corynebacterium Glutamicum*. *Applied and Environmental Microbiology* 2015, 81:4173–4183.
- [36] Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, et al.: Berberine is a Novel Cholesterol-lowering Drug Working through a Unique Mechanism Distinct from Statins. *Nature Medicine* 2004, 10:1344–1351.
- [37] Kong WJ, Wei J, Zuo ZY, Wang YM, Song DQ, You XF, et al.: Combination of Simvastatin with Berberine Improves the Lipid-lowering Efficacy. *Metabolism*, 2008, 57:1029–1037.
- [38] Ren G, Guo JH, Qian YZ, Kong WJ, Jiang JD: Berberine Improves Glucose and Lipid Metabolism in HepG2 Cells Through AMPK α 1 Activation. *Frontiers in Pharmacology* 2020, 11:647.
- [39] Wang C, Wang Y, Ma SR, Zuo ZY, Wu YB, Kong WJ, et al.: Berberine Inhibits Adipocyte Differentiation, Proliferation and Adiposity through Down-regulating Galectin-3. *Scientific Reports* 2019, 9:13415.
- [40] Ma SR, Tong Q, Lin Y, Pan LB, Fu J, Peng R, et al.: Berberine Treats Atherosclerosis via a Vitamine-like Effect Down-regulating Choline-TMA-TMAO Production Pathway in Gut Microbiota. *Signal Transduction and Targeted Therapy* 2022, 7:207.
- [41] Wang Y, Tong Q, Shou JW, Zhao ZX, Li XY, Zhang XF, et al.: Gut Microbiota-Mediated Personalized Treatment of Hyperlipidemia Using Berberine. *Theranostics* 2017, 7:2443–2451.