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Comparative oral and intravenous pharmacokinetics of phlorizin in rats having type 2 diabetes and in normal rats based on phase II metabolism†

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Phlorizin (PHZ), a type of dihydrochalcone widely found in *Rosaceae* such as apples, is the first compound discovered as a sodium-glucose cotransporter (SGLT) inhibitor. It has been confirmed to improve the symptoms of diabetes and diabetic complications effectively. Like other flavonoids, the bioavailability challenge of PHZ is the wide phase I and II metabolism in the digestive tract. In this study, we investigated the pharmacokinetics and contribution of phase II metabolism after the oral and intravenous administrations of PHZ in rats having type 2 diabetes (T2D) and in normal rats. The phase II metabolism characteristics of PHZ were investigated by treating plasma samples with β-glucuronidase/sulfatase. The contribution ratio of phase II metabolism of PHZ ranged from 41.9% to 69.0% after intravenous injection with three doses of PHZ in normal rats. Compared with the observations for normal rats, AUC_{0-t} and C_{max} of PHZ significantly increased and $T_{1/2}$ of PHZ significantly decreased in T2D rats. PHZ was converted into phloretin (PHT) through an enzyme-catalyzed hydrolysis reaction, and PHT was further transformed into conjugates with glycose after both oral and intravenous administrations. Moreover, it was found that the bioavailability of PHZ was about 5% in T2D rats, which was significantly higher than that in normal rats (0%). In conclusion, compared with the observations for normal rats, the pharmacokinetic characteristics of PHZ significantly changed in T2D rats through oral and intravenous administrations. The bioavailability of PHZ significantly increased in T2D rats. Besides, the phase II metabolites of PHT were the major existing forms in blood after oral and intravenous administrations. Our results indicated that the phase II metabolism characteristics of PHZ should be considered when PHZ is applied for the treatment of diabetes as a drug or functional food.

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1. Introduction

Phlorizin (PHZ, also referred to as phloridzin), a dihydrochalcone, is a glucoside of phloretin (PHT) and belongs to the family of bicyclic flavonoids. This compound is widely distributed in some daily consumed edible fruits, such as apples, and their vegetative tissues (such as leaves and bark) and seeds. It was isolated and identified for the first time from apples by a French scientist and discovered to be the first sodium-glucose-linked transporter (SGLT) inhibitor in the world.[1] Recent research has shown that the long-term use of PHZ can reduce blood glucose level and lipid metabolism in diabetic mice.[2–4] In a previous research, we found that PHZ can also enhance insulin sensitivity by changing the structure of gut microbiota in obese mice with T2D (*db*/*db*).[5] The hypoglycemic mechanism of PHZ was deduced to inhibit the sugar transport by SGLT in both the kidney and small intestine. Hence, it decreased glucose absorption and reabsorption in the small intestine and kidney, respectively. These results suggested that PHZ holds great potential for the treatment of diabetes. However, previous studies suggested that the bioavailability of PHZ after oral administration is very poor *in vivo*.[6] Crespy and colleagues reported that the phase II metabolites were over 95%,[7] and the concentration of PHZ in blood was quite low in 4 hours after the oral administration of

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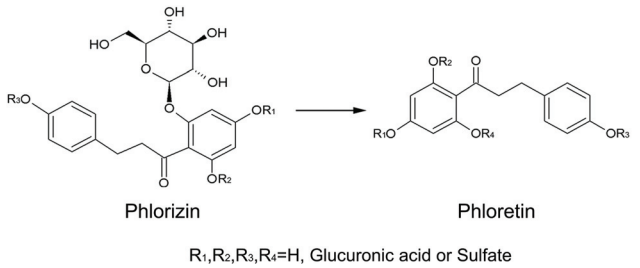

Fig. 1 The possible phase II conjugations of PHZ and PHT in plasma through phase II metabolic pathway.

PHZ (Fig. 1), which restricts its clinical therapeutic value.[8] Unfortunately, the pharmacokinetic study of this compound was not comprehensively and systematically investigated by Crespy and colleagues. Hence, it is difficult to evaluate the metabolic features based on a complete time–concentration curve and pharmacokinetic parameters. Therefore, it is necessary to comprehensively investigate the pharmacokinetics of PHZ *in vivo*. PHZ is always hydrolyzed into phloretin (PHT) by two enzymes, namely, lactase-phlorizin hydrolase (LPH) and β-glucosidase, which are widely distributed in the intestinal tract.[9] It was reported that PHT, phloretic acid, and phloroglucinol were detected in rat's urine after the gavage of PHZ for a period of time.[10]

Oral administration is adopted in most of the pharmacological and pharmacodynamic studies on PHZ. Compared with oral administration, intravenous injection can avoid numerous problems, including poor aqueous solubility of the drug, first-pass metabolism of the drug in the liver and acidic gastric environment, and the presence of digestive enzymes causing degradation of the drug.[11] It is thus necessary to investigate the pharmacokinetics and bioavailability of PHZ in oral and intravenous administrations.[12]

The incidence of diabetes is increasing year by year all over the world with the improvement of modern diet. Over 400 million people suffer from diabetes, and more than 90% of them are identified to have T2D,[13] which raises a great threat to the public health.[14–17] Researchers have shown that the metabolism of the drug under diabetic condition is quite different from that in normal condition,[18] where distribution and biotransformation are altered.[19] Reports have demonstrated that the pharmacokinetics of some novel drugs for diabetes mellitus, such as Canagliflozin, can be different in patients having diabetes compared with that in normal subjects,[20] where AUC, C_{max}, and $T_{1/2}$ in patients with diabetes are substantially altered.[21]

Drugs are often used for patients in a disease state; thus, it is considered that a drug metabolism study should be evaluated under the pathological state. In this study, we first combined a high-calorie diet and low dosage of streptozotocin (STZ) to induce T2D rats, which was followed by investigation of the pharmacokinetics of PHZ in T2D rats and in normal rats. Then, the pharmacokinetic behaviors of PHZ in the diabetic state and in the normal state were compared to determine whether the bioavailability and pharmacokinetic parameters of PHZ are different under these two conditions. In this article, we established a new HPLC method for the simultaneous and rapid quantitative analysis of PHZ and its metabolites in blood samples by treating with and without β-glucuronidase and sulfatase. Additionally, the pharmacokinetic characteristics of PHZ in T2D rats and in normal rats were investigated based on phase II metabolism.

2. Materials and methods

2.1 Drugs and reagents

PHZ and PHT (purity >98%) were supplied by Zhongren Biotechnology, Inc., (Hunan, China). Baicalein (internal standard, IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). β-Glucuronidase (type H-3, from *Helix pomatia*; 1 unit will liberate 1.0 μg of phenolphthalein from phenolphthalein glucuronide at 37 °C and pH 5.0 after incubation for 30 min) and sulfatase (type H-1, from *H. pomatia*; 1 unit will hydrolyze 1.0 μmol of *p*-nitrocatechol substrate at pH 5.0 at 37 °C after incubation for 30 min) were purchased from Sigma-Aldrich (St Louis, MO). STZ was purchased from Boaigang Trade co, Ltd (Beijing, China). Formic acid (HPLC grade) was purchased from Kelong Chemical Reagent factory (Chengdu, China). Methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO). Water (HPLC-grade) was prepared in an ultrapure water system (UPT-11, Chengdu, China). All other reagents used in this study were of analytical grade.

2.2 Animals

Twenty-four SPF male Wistar rats (4 weeks, 200 ± 10 g) were purchased from Dashuo Laboratory Animal Technology Co. (Chengdu, China). Six rats were randomly selected and housed in a temperature-controlled (25 °C) room with a dark period from 20:00 to 08:00. The rats were given a controlled diet for 7 days, which was relatively flavonoid-free and contained 10% mineral, 18% crude protein, 4% crude fat, and 5% crude fibre.

Other 24 SPF male Wistar rats (4 weeks, 200 ± 10 g) were given high-calorie food (12.1% crude protein, 21.4% crude fat, and 20% carbohydrate) for 1 month to induce insulin resistance and then, 45 mg kg^{-1} dose of STZ was administered to each rat by intraperitoneal injection. Six rats were randomly selected and housed in a temperature-controlled (25 °C) room with a dark period from 20:00 to 8:00 and with access to food from 8:00 to 20:00. Three days after the injection of STZ, indexes related to the T2D model in rats were detected for the validation of success in the STZ-induced T2D rats, including food intake, water intake, fasting insulin (FINS), fasting blood glucose (FBG), insulin resistance index (HOMA-IR) and other data.

2.3 Animal experiments

The 24 male Wistar rats were randomly divided into 4 groups for intravenous pharmacokinetics research, including 18 normal

rats and 6 STZ-induced T2D rats. Animals were fasted for 12 h but had free access to water before the experiment. The rats were anesthetized by an intraperitoneal injection of urethane (750 mg kg^{-1}) and then fixed on the operating table. Three different dosages of PHZ (5, 10, and 20 mg kg^{-1}) were intravenously given to the normal groups, and 10 mg kg^{-1} of PHZ was intravenously given to the T2D rats. The rats were given 0.1 mL PHZ solution (PHZ was dissolved in 0.9% sodium chloride solution at different concentrations). Approximately 0.5 mL of venous blood was collected *via* the jugular vein at 0, 1, 3, 5, 10, 15, 30, 45, 60, 90, 120, 180, and 240 min after administration.

The other 24 male Wistar rats were randomly divided into 4 groups and used for the oral pharmacokinetics study. Three groups including 18 STZ-induced T2D rats and one group including 6 normal rats were fasted for 12 h and had free access to water before the experiment. Three different doses of PHZ (50, 100, and 200 mg kg^{-1}) were given to the T2D groups by gavage, and 100 mg kg^{-1} dose of PHZ was given to normal rats by gavage. Approximately 0.5 mL of blood was collected *via* the caudal vein at 0, 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after administration.

Blood samples were collected in 1.5 mL tubes containing heparin sodium for anticoagulation. The samples were centrifuged (3000*g*, 10 min) after collection. To avoid PHZ degradation in the high pH environment, 20 μL 0.5 M acetic acid (containing 2 mg mL^{-1} of vitamin) was added to 100 μL blood sample to harvest the supernatant and prevent the loss of PHZ. All the samples were stored at −20 °C until analysis.

The animal experiments in this study were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Chengdu University.

2.4 HPLC assay

A Waters chromatographic system (Waters, Milford, MA) was utilized consisting of a 2695 separations Module and 2996 photodiode array detector. The tested analytical column was a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Dikma, Beijing, China). The column was maintained at 25 °C. A mixture of solvent A (0.4% formic acid in water) and solvent B (acetonitrile) was used as the mobile phase at a flow rate of 0.8 mL min^{-1} under gradient elution programs as follows: 0–6 min, 80% A; 6–18 min, linear gradient from 80% to 30% A; 18–20 min, 30% A; 20–23 min, linear gradient from 30% to 80% A; 23–25 min, 80% A. The injection volume was 10 μL and the injector needle was washed in 50% methanol. The optimal detection UV wavelength was 285 nm.

2.5 Plasma sample preparation

An aliquot of 100 μL of frozen plasma sample was thawed to room temperature, and these samples were treated for 30 min at 37 °C in the presence of 1 unit of β-glucuronidase and 1 unit of sulfatase. Subsequently, the treated solutions were spiked with 20 μL of baicalein working solution (IS, 12.5 μg mL^{-1}). After being briefly shaken, each mixture was vortex-mixed with 1 mL of ethyl acetate for 2 min at 2000 rpm (Vortex Genius 3, IKA, Germany). The tube was centrifuged at 4 °C for 10 min at 12 000*g* (EBA21 table centrifuge, Hettich, Germany). The upper organic phase was transferred to another 1.5 mL polypropylene tube and evaporated to dryness under a stream of nitrogen at 40 °C (N-EVAP 11155, Organomation). The residue was re-dissolved in 100 μL methanol and thoroughly vortex-mixed for 2 min at 2000 rpm. Then, the solution was centrifuged at 12 000*g* for 5 min and finally, an aliquot (10 μL) of the solution was injected into the chromatographic system for determination.[22]

2.6 Standard solution and quality control (QC)

Stock solutions of PHZ (1 mg mL^{-1}), PHT (2 mg mL^{-1}), and IS (100 μg mL^{-1}) were prepared in methanol and stored in −20 °C. Stock solutions were further mixed and diluted with methanol to yield standard working solutions at concentrations of 0.5, 2, 5, 20, 50, 100, 200, 400, 500 μg mL^{-1} for PHZ and 0.32, 0.8, 3.2, 316, 80, 160, 320, 480, 640 μg mL^{-1} for PHT. These working solutions were used to make the calibration standard and QC samples. Calibration standards in rat plasma were prepared at the final plasma concentrations of 0.1, 0.4, 1, 4, 10, 20, 40, 80, 100 μg mL^{-1} for PHZ and 0.064, 0.16, 0.64, 3.2, 16, 32, 64, 96, 128 μg mL^{-1} for PHT. The QC samples were prepared separately in the same way for low, middle, and high concentrations: 0.4, 10, and 80 μg mL^{-1} for PHZ and 0.16, 16, and 96 μg mL^{-1} for PHT. All stock solutions, working solutions, and QC samples were immediately stored at −20 °C and brought to room temperature before using them.

2.7 Specificity and selectivity

The specificity of the method was evaluated by analyzing six separate rat plasma samples to determine the potential interferences at the LC peak region for each analyte and IS.

2.8 Calibration curve

The linearity was studied and detected in rat plasma at nine different concentrations of PHZ (0.1, 0.4, 1, 4, 10, 20, 40, 80, 100 μg mL^{-1}) and PHT (0.064, 0.16, 0.64, 3.2, 16, 32, 64, 96, 128 μg mL^{-1}). The calibration curves of PHZ and PHT were fitted by least-squares linear regression using 1/concentration ($1/c^2$) as a weighting factor of the peak-area ratio of PHZ and PHT to baicalein (internal standard) *versus* the injected concentration of PHZ and PHT in each calibration sample. The acceptable calibration curve must have a correlation coefficient (r^2) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value for the lower limit of quantification (LLOQ), and deviation was set at ±20%.

2.9 Lower limit of quantification (LLOQ) and limit of detection (LOD)

The lower limit of quantification (LLOQ) and limit of detection (LOD) for PHZ and PHT were determined by diluting the calibration samples. LLOQ was defined as the lowest plasma concentration of PHZ and PHT quantified with a coefficient of

variation of less than 20%. The LOD was considered as the concentration of PHZ and PHT that provides a signal corresponding to 3 times the HPLC background signal.

2.10 Precision and accuracy

Six replicates containing PHZ and PHT for three different QC samples at low, medium, and high concentration levels in plasma were analyzed to estimate the intra-assay precision and accuracy. The inter-assay precision and accuracy were determined by repeating the analysis of the three concentration levels of QC samples on three consecutive days. The criteria for acceptability of the data included accuracy within ±15% standard deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, which should not be over ±20% of SD.

2.11 Stability

The stability of the analytes in rat plasma was assessed by analyzing four replicates (n = 4) of QC samples at the low, medium, and high concentrations under a variety of storage and process conditions. The spiked plasma samples were analyzed after storage at room temperature for 24 h at −20 °C for 30 days and after three freeze–thaw cycles from −20 °C to room temperature.

2.12 Recovery

The extraction recoveries of the analytes were calculated by comparing the peak areas of the analytes spiked before extraction to those of analytes spiked after extraction. At the three QC concentration levels mentioned above, six replicates were tested.

2.13 Bioavailability

The absolute bioavailabilities of PHZ in T2D rats and normal rats were calculated using the following equation: F = ((AUC(ov) × D(iv))/((AUC(iv) × D(ov)) × 100%; here, F is the absolute bioavailability of PHZ *via* oral administration, AUC (ov) and AUC(iv) are the areas under the curve of oral administration pharmacokinetics and intravenous pharmacokinetics, and D(ov) and D(iv) are the doses of oral administration and intravenous injection, respectively.

2.14 Data analysis

Pharmacokinetic calculations were optimized by compartmental and non-compartmental models with the software DAS 2.0.1 (Drug and Statistics, DAS version 2.0.1, Anhui, China). The areas under concentration–time curves (AUC) were calculated according to the trapezoidal method. The following non-compartmental pharmacokinetic parameters were derived *via* standard methods: AUC from time zero to infinity ($AUC_{0-\infty}$) and from time zero to the time of the last measurable concentration (AUC_{0-t}), peak concentration (C_{max}), peak time (T_{max}), and terminal $T_{1/2}$. Pharmacokinetic parameters were also determined by fitting the pharmacokinetic compartmental models to the plasma concentration–time profiles for each animal after administration.

3. Results

3.1 T2D rat model validation

The T2D rat model was validated by measurement of food intake, water intake, body weight, fasting blood glucose, fasting insulin, and insulin homeostasis. The experimental results (ESI Table 1†) showed that the amounts of water, food intake, and body weight significantly increased. In T2D rats, it was found that fasting blood glucose levels, fasting insulin, and calculated HOMA-IR increased up to 23.6 ± 7.6 mmol L^{-1}, 27.2 ± 2.2 mU L^{-1}, and 29.8 ± 5.4, respectively, which were significantly higher than those of normal rats ($p < 0.01$). Based on these data, we concluded that this batch of rats was considered as T2D rats and was suitable for the indicated experiments.

3.2 Methodology validation

Specificity was evaluated by comparing the retention times of two analytes and IS on chromatograms. Representative chromatograms of blank, spiked, and rat plasma samples were obtained after vein administration of PHZ (Fig. 2). No significant interfering peaks were observed around the retention times of each analyte. The mean equation (curve coefficients ± SD) of the calibration curves (n = 6) was obtained from six single batches in routine unknown plasma sample analysis. It was shown that the slope of the calibration curves was stable for PHZ within the concentration range of 0.1–100 μg mL^{-1} and for PHT within the concentration range of 0.064–128 μg mL^{-1}. The regression coefficients (r^2) of the calibration curves remained excellent; both were higher than 0.99. This result indicated that the established HPLC method was suitable for the analysis of PHZ and PHT in plasma from low to high concentrations. The lower limit of quantification (LLOQ) was achieved as the lowest point on the standard curve: 0.4 μg mL^{-1} for PHZ with RSD of 10.36% and 0.128 μg mL^{-1} for PHT with RSD of 1.87% (n = 5). The limits of detection (LOD) of the analytes in plasma were determined to be 0.0300 μg mL^{-1} for PHZ and 0.0256 μg mL^{-1} for PHT.

The inter-assay and intra-assay accuracy and precision data are shown in ESI Table 2.† These quality control samples (n = 5) representing the low, medium, and high concentrations contained 0.4, 10, and 80 μg mL^{-1} of PHZ and 0.16, 16, and 96 μg mL^{-1} of PHT, respectively. The precision (RSD) and the biases in each case were less than 10%. These results showed that the present method has good precision and accuracy. The extraction recoveries of the analytes and internal standard are shown in ESI Table 3.† The mean extraction recoveries of PHZ and PHT (n = 3) from the spiked plasma were satisfactory at low, medium, and high concentrations; the extraction recoveries were as follows: 82.71 ± 2.19%, 85.44 ± 0.94%, and 83.94 ± 0.08% for PHZ and 81.02 ± 1.80%, 89.60 ± 1.23%, and 84.15 ± 1.18% for PHT. The mean extraction recovery of internal standard (n = 3) from the spiked plasma was 75.55 ± 3.76%.

In the stability study (results are shown in ESI Table 4†), the change in the initial concentrations was used to evaluate the variation of PHZ and PHT concentrations; the change for all was less than 15% at the low, medium, and high concentration

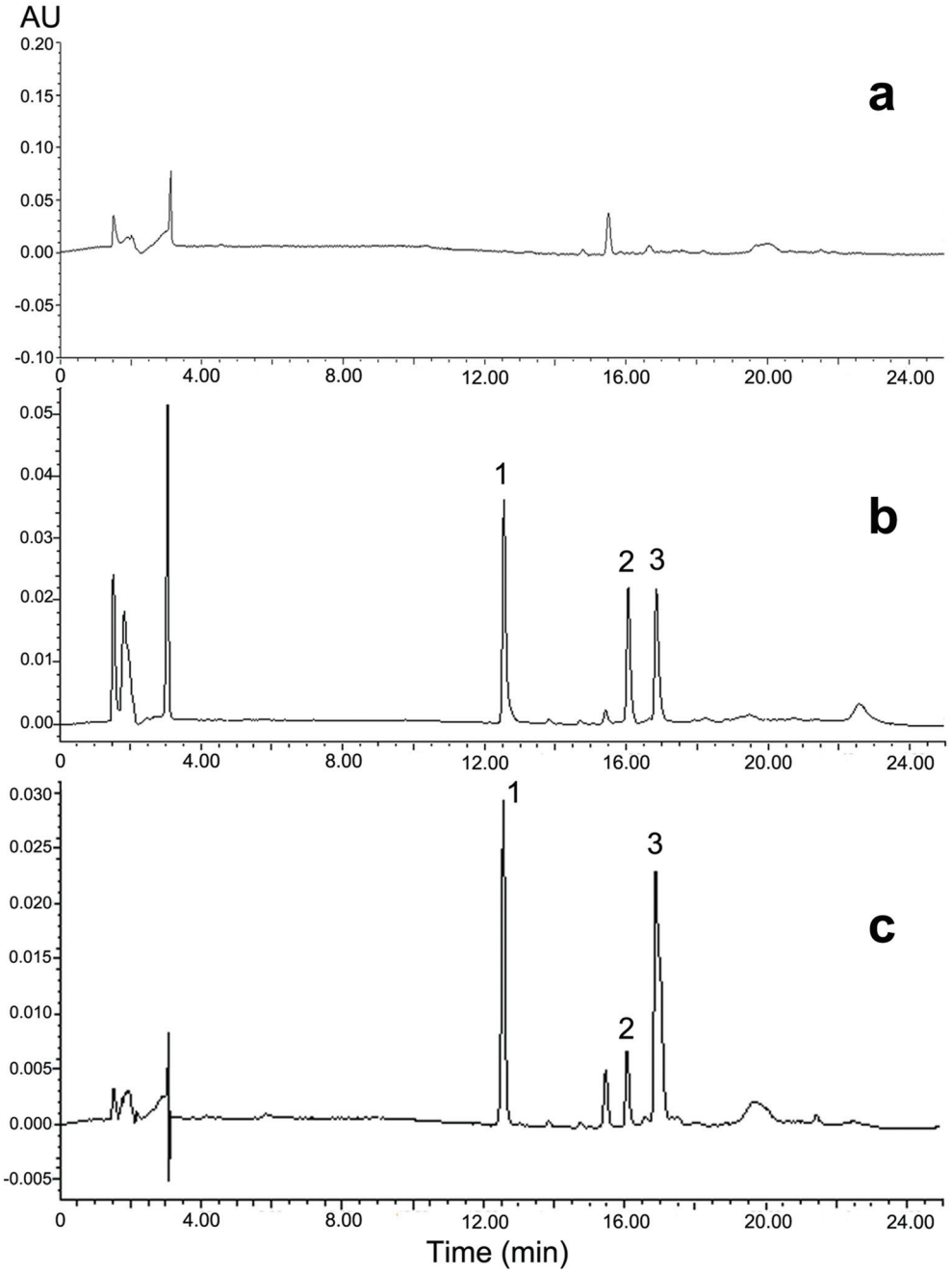

Fig. 2 Typical chromatograms of (a) blank Wistar rat plasma, (b) blank plasma spiked with PHZ (4 μg mL^{-1}), PHT (1.6 μg mL^{-1}), and IS (2.5 μg mL^{-1}), and (c) Wistar rat plasma obtained from an experimental rat 30 min after an intravenous injection dose of PHZ (10 mg kg^{-1}) (peak 1, PHZ; peak 2, PHT; peak 3, IS, baicalein).

levels. The results indicated that the analytes were stable in plasma either kept at room temperature for 24 h or stored at −20 °C for at least 1 month. PHZ and PHT were also stable after three freeze–thaw cycles from −20 °C to room temperature.

3.3 Pharmacokinetic results

Study on the intravenous pharmacokinetics of PHZ indicated that PHT was only detected after enzyme hydrolysis through comparing the chromatograms of blood samples with or without enzyme treatment (β-glucuronidase and sulfatase) (Fig. 3). The concentrations of the parent PHZ, total phlorizin including the parent and conjugations of PHZ (TPHZ), and total phloretin including the parent and conjugations of PHT (TPHT) in normal rats' plasma after intravenous injection of PHZ are shown in Fig. 4. It was shown that the pharmacokinetics of PHZ, TPHZ, and TPHT after the intravenous injection of PHZ were non-linear pharmacokinetics and exhibited slight

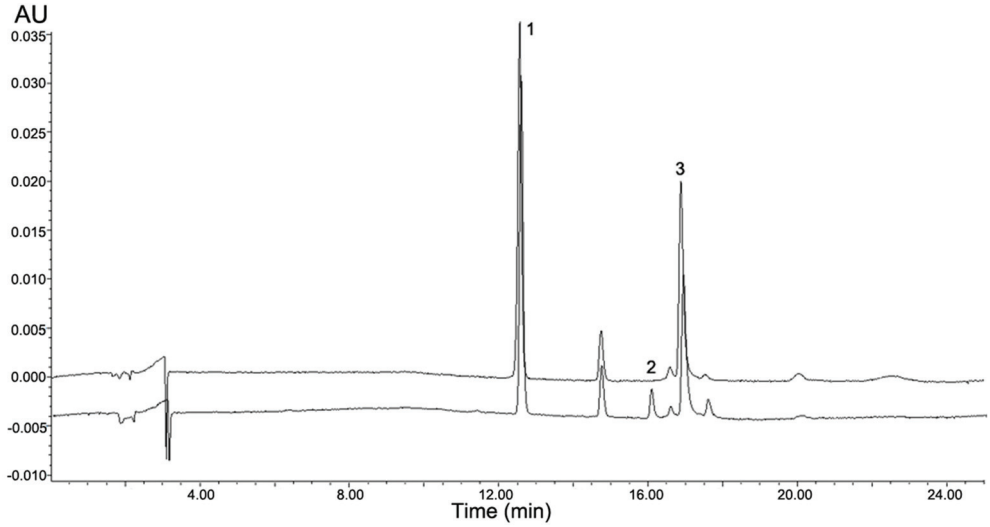

Fig. 3 Chromatograms of plasma sample after intravenous injection; the under line is the chromatogram of plasma through enzymatic hydrolysis (peak 1, PHZ; peak 2, PHT; peak 3, IS).

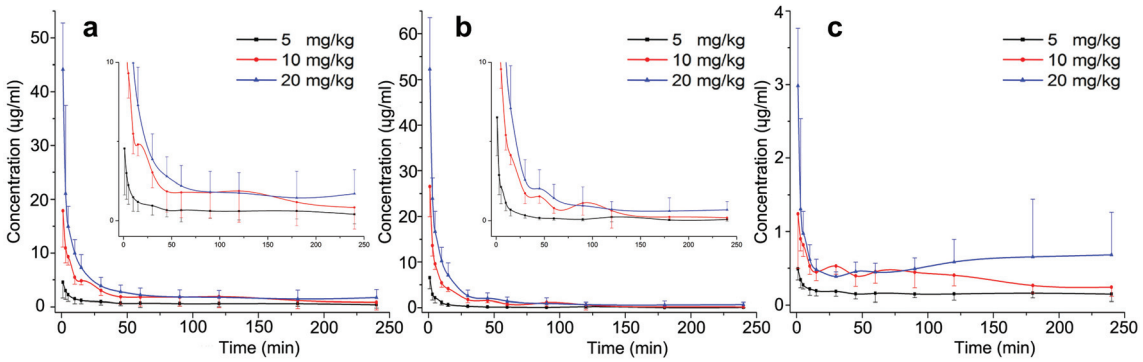

Fig. 4 The mean (±SD, n = 6) plasma concentration–time profile of PHZ after enzymatic hydrolysis (a), parent PHZ (b), and PHT after enzymatic hydrolysis (c) in rats after intravenous injection of three different doses of PHZ (5, 10, and 20 mg kg^{-1}).

bimodal phenomenon. The pharmacokinetic profile features of PHZ were similar to those of TPHZ, but the parameters were different. The estimated pharmacokinetic parameters of PHZ, TPHZ, and TPHT are listed in Table 1. The AUC_{0-t} values of PHZ before enzyme treatment were 66.47 ± 39.17, 333.17 ± 91.24, and 516.47 ± 249.35 mg L^{-1} min^{-1}, and the peak plasma concentrations of PHZ were 6.48 ± 2.42, 24.94 ± 6.62, and 45.09 ± 11.21 μg mL^{-1}. After enzyme hydrolysis, the AUC_{0-t} values of TPHZ were 174.32 ± 128.96, 525.3 ± 132.5, and 739.43 ± 355.01 mg L^{-1} min^{-1}, and the peak plasma concentrations were 4.59 ± 2.92, 25.46 ± 6.77, and 44.24 ± 11.8 μg mL^{-1}. The time curve of TPHT was associated with the three concentrations. The AUC_{0-t} values of TPHT through enzyme treatment were 38.86 ± 16.32, 108.06 ± 19.9, and 149.3 ± 82.79 mg L^{-1} min^{-1}, and the peak plasma concentrations were 0.48 ± 0.15, 1.86 ± 0.55, and 2.97 ± 0.98 μg mL^{-1}. This indicated that the systemic exposure (AUC) of PHZ changed with enzyme hydrolysis after the intravenous injection of PHZ.

In Fig. 5, the histogram is drawn depending on the area under the curve of the parent PHZ, phase II conjugations of phlorizin (CPZ), and the conjugated form of phloretin (CPT); the histogram shows that PHZ accounts for the maximum percentage, CPZ takes the second place and CPT accounts for the minimum area of all. This proved that the prototype of PHZ was still the major form in blood after intravenous injection; the metabolism contribution ratios of PHZ were 30.9%, 52.6%, and 58.1% at the three dose groups. CPZ in the metabolites also exhibited a significant contribution ratio, which accounted for about 50.7%, 30.3%, and 25.1% at the three dose groups. CPT showed contribution of 18.3%, 17.1%, and 16.8%, which indicated that CPT was produced steadily and might not change with the increase in dosage. In brief, the total phase II metabolism contributions were 69.0%, 47.4%, and 41.9% at the three dose groups.

After comparing the pharmacokinetics of T2D rats to that of normal rats (Fig. 6), we found that the AUC_{0-t} values of PHZ

Table 1 Pharmacokinetic parameters of PHZ and its metabolites after three different doses of intravenous injections in normal rats and single dose of intravenous injection in T2D rats (n = 6)

		TPHZ				PHZ			TPHT			
		NR			T2D	NR						T2D
Parameters		L	M	H	M	L	M	H	L	M	H	M
$AUC_{(0-t)}$	mg L^{-1} min^{-1}	174.3 ± 128.9	525.3 ± 132.5	739.4 ± 355.0	703.9 ± 496.6	66.4 ± 39.1	333.1 ± 91.2	516.4 ± 249.3	38.8 ± 16.3	108.0 ± 19.9	149.3 ± 82.7	183.6 ± 140.9
$AUC_{(0-\infty)}$	mg L^{-1} min^{-1}	450.9 ± 645.5	777.7 ± 334.1	1197.5 ± 888.7	1338.2 ± 1916.6	68.6 ± 40.2	342.2 ± 94.0	579.8 ± 344.3	106.1 ± 48.6	147.0 ± 46.3	294.6 ± 210.7	200 ± 164.7
$t_{1/2}$	min	192.2 ± 207.3	209.1 ± 148.8	171.0 ± 45.3	155.9 ± 199.6	74.5 ± 27.2	62.3 ± 21.4	75.0 ± 47.2	353.6 ± 347.3	113.7 ± 65.4	121.4 ± 72.9	57.9 ± 27.5
C_{max}	mg L^{-1}	4.5 ± 2.9	25.4 ± 7.6	44.2 ± 11.8	39.5 ± 12*	6.4 ± 2.4	24.9 ± 6.6	45.0 ± 11.2	0.48 ± 0.15	1.86 ± 0.55	2.97 ± 0.98	7.4 ± 4.6*
$MRT_{(0-\infty)}$	min	80.6 ± 23.1	151.5 ± 99.9	217.1 ± 116.4	184.1 ± 280	67.3 ± 44.9	52.8 ± 20.9	67.2 ± 46.2	505.4 ± 490.2	180.9 ± 87.8	226.6 ± 104.8	91.2 ± 54.4*

NR: normal rats; T2D: type 2 diabetes; TPHZ, total phlorizin, phlorizin after enzymatic hydrolysis; PHZ, parent phlorizin or original phlorizin; TPHT, total phloretin, phloretin after enzymatic hydrolysis. L: 5 mg kg^{-1}; M: 10 mg kg^{-1}; H: 20 mg kg^{-1}. Compared with NR, differences were assessed by One-way ANOVA and denoted as follows: *$P < 0.05$, **$P < 0.01$.

Fig. 5 Metabolic contribution ratio of PHZ and metabolites *in vivo* after intravenous injection of PHZ.

and PHT in T2D rats after intravenous injection of PHZ were significantly higher than those in normal rats by 33.99% and 70.01%, respectively. The $T_{1/2}$ values decreased by about 25% for PHZ and 50% for PHT. MRT of PHZ increased, while that of PHT decreased. The pharmacokinetic parameters are listed in Table 1.

In the oral pharmacokinetic study, the blood samples were all treated with β-glucuronidase and sulfatase after the rats were gavaged with PHZ. We found that there was a trace of PHZ or a concentration lower than the LOD in the normal rats' blood. Nevertheless, PHZ could be continuously and obviously detected in the T2D rats. Both in the normal and T2D rats, the contents of PHT in blood were much higher than the contents of PHZ.

Three dosages of PHZ were administered to T2D rats for the pharmacokinetic study. The results showed that the time–concentration curves of PHZ and PHT correlated with the dosage (Fig. 7). The results also showed that a complete pharmacokinetic profile for PHZ was observed for T2D rats; the AUC_{0-t} values of TPHZ were 5.23 ± 3.17, 6.78 ± 2.93, and 5.11 ± 4.07 mg L^{-1} h^{-1}. The AUC_{0-t} values of TPHT were 15.88 ± 4.26, 31.64 ± 18.92, and 68.36 ± 23.87 mg L^{-1} h^{-1}. The corresponding pharmacokinetic parameters are shown in Table 2. With the dosage of 100 mg kg^{-1} of PHZ, the AUC_{0-t} values of TPHZ and TPHT in T2D rats after oral administration were 6.78 ± 2.93 mg L^{-1} h^{-1} and 31.64 ± 18.92 mg L^{-1} h^{-1}, respectively, while that in normal rats was absent and it was 15.28 ± 3.73 mg L^{-1} h^{-1}, which indicated that the systemic exposure of TPHZ and TPHT significantly increased after the oral administration of PHZ in T2D rats. This conclusion was identical to that of the intravenous pharmacokinetic study. The pharmacokinetic parameters are compared in Table 2. After comparing the pharmacokinetic parameters for T2D rats with that for normal rats, we inferred that AUC_{0-t}, C_{max}, T_{max}, and MRT of TPHT in the T2D rats significantly increased, and $T_{1/2}$ decreased by about 42%. Multiple peaks were also observed in the concentration–time curves, which suggested enterohepatic recirculation or intestinal secretion mediated by membrane

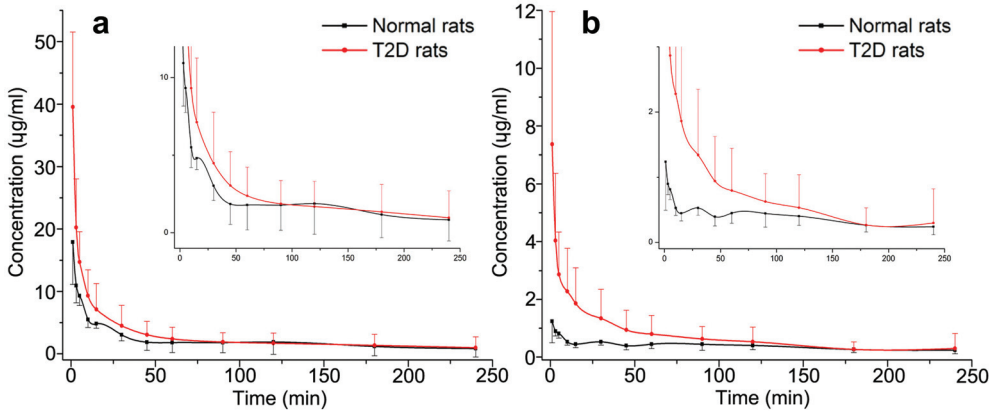

Fig. 6 The plasma concentration–time (mean ± SD, n = 6) profile of PHZ (a), PHT (b) in normal and T2D rats after intravenous injection of PHZ (10 mg kg^{-1}).

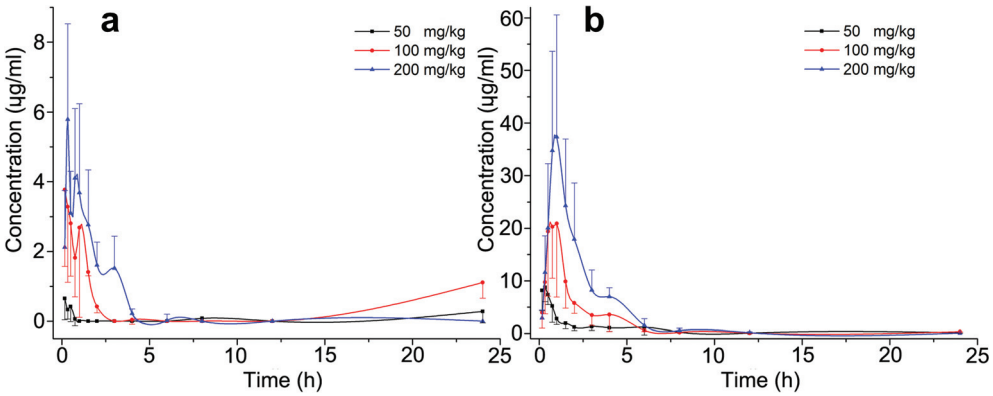

Fig. 7 The plasma concentration–time (mean ± SD, n = 6) profile of PHZ (a), PHT (b) in STZ-induced T2D rats after oral administration of three different doses of PHZ (50, 100, and 200 mg kg^{-1}). Plasma sample hydrolyzed by β-glucuronidase and sulfatase.

Table 2 Pharmacokinetic parameters of PHZ and PHT in T2D rats after three different doses of oral administrations of PHZ and single dose of PHZ (100 mg kg^{-1}) in normal rats (n = 6)

		TPHZ				TPHT			
		T2D			NR	T2D			NR
Parameters		L	M	H	M	L	M	H	M
$AUC_{(0-t)}$	mg L^{-1} h^{-1}	5.23 ± 3.17	6.78 ± 2.93**	5.11 ± 4.07	—	15.88 ± 4.26	31.64 ± 18.92	68.36 ± 23.87	15.28 ± 3.73
$AUC_{(0-\infty)}$	mg L^{-1} h^{-1}	9.98 ± 5.10	11.23 ± 10.28**	5.49 ± 3.85	—	16.25 ± 4.19	40.87 ± 14.77**	68.4 ± 23.88	15.34 ± 3.74
$T_{1/2}$	h	9.45 ± 7.02	2.88 ± 2.36**	3.29 ± 1.86	—	4.17 ± 2.78	1.56 ± 0.92	1.33 ± 0.55	2.72 ± 2.48
T_{max}	H	8.17 ± 12.27	0.42 ± 0.33**	0.64 ± 0.24	—	0.38 ± 0.22	0.79 ± 0.19	0.88 ± 0.14	0.70 ± 0.21
C_{max}	mg L^{-1}	0.79 ± 0.52	5.09 ± 2.13**	3.23 ± 3.14	—	10.45 ± 2.45	24.63 ± 14.16*	36.86 ± 16.16	8.84 ± 2.18
$MRT_{(0-\infty)}$	h	27.48 ± 8.89	5.11 ± 8.58**	5.05 ± 5.82	—	5.40 ± 0.94	2.74 ± 1.79	2.24 ± 0.63	2.52 ± 0.77

NR: normal rats; T2D: type 2 diabetes; TPHZ (total phlorizin): phlorizin after enzymatic hydrolysis; TPHT (total phloretin): phloretin after enzymatic hydrolysis. L: 50 mg kg^{-1}; M: 100 mg kg^{-1}; H: 200 mg kg^{-1}. Compared with NR, differences were assessed by One-way ANOVA and denoted as follows: *P < 0.05, **P < 0.01.

transporters, and this result was consistent with the flavonoid pharmacokinetic profiles.[23]

Since AUC_{0-t} of PHZ was absent in normal rats after oral administration, it was considered that the absolute bioavailability of PHZ in normal rats was 0. The AUC_{0-t} value of TPHZ in T2D rats after intravenous injection of 10 mg kg^{-1} PHZ was 703.9 ± 496.6 mg L^{-1} min^{-1}, and the AUC_{0-t} values of TPHZ in T2D rats were 313.8 ± 190.2, 406.8 ± 175.8, and 306.6 ± 244.2 mg L^{-1} min^{-1} after gavages of 50, 100, and 200 mg kg^{-1} PHZ, respectively; the calculated absolute bio-

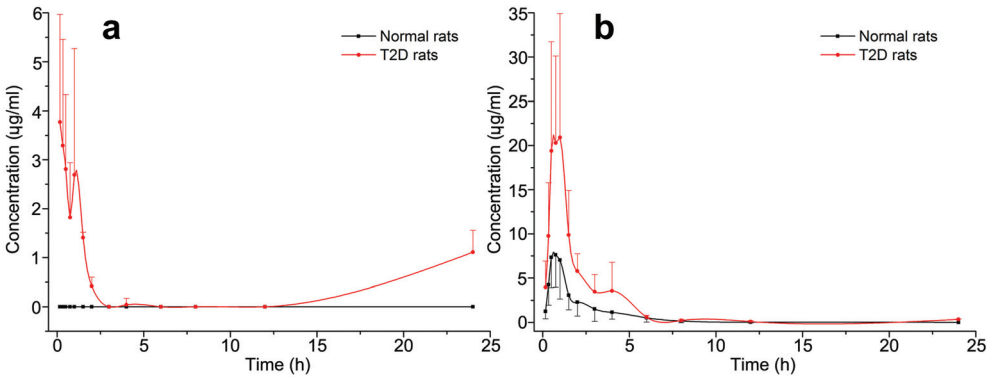

Fig. 8 The plasma concentration–time (mean ± SD, n = 6) profile of PHZ (a), PHT (b) in normal and STZ-induced T2D rats after oral administration of PHZ (100 mg kg^{-1}). Plasma sample was hydrolyzed by β-glucuronidase and sulfatase.

availability values were 8.91%, 5.78%, and 2.18%, respectively. The results indicated that the bioavailability of PHZ significantly increased in the state of T2D (Fig. 8), but the bioavailability was still quite poor.

4. Discussion

The intestinal tract contains abundant LPH and β-glucosidase,[24,25] which can mediate the occurrence of deglycosylation. One report demonstrated that PHZ changed into PHT by LPH and β-glucosidase in the intestinal tract, and a part of PHZ is further involved in an oxidation–reduction reaction, in which it is metabolized into phlorizin acid and phloroglucin.[10] PHZ rapidly and continuously enters the process of phase II metabolism once it is absorbed. Similar to other glycosides of flavonoids,[25] PHZ is always absorbed as its aglycone PHT. PHT is always restored to the glucuronidation/sulfation metabolites in the intestine and liver *in vivo*. Then, the conjugated metabolites are excreted to the duodenum from bile, which are easily hydrolyzed to the free aglycone and then reabsorbed and recirculated in the process of enterohepatic circulation. Drastic phase I and II metabolisms of this compound result in poor bioavailability, which has been proven to be the limitation for the clinical application of PHZ. In our present study, PHZ was administered intravenously to avoid the extreme pH environment and related enzyme action in the gastrointestinal tract. The results showed that the metabolic rate of PHZ significantly decreased compared with that in oral administration, whereas that of original PHZ greatly increased *in vivo*. Nevertheless, it was found that a portion of PHZ occurred in phase I and II metabolites and a number of PHT and conjugated metabolites were produced. A multiple peak phenomenon was also observed in the intravenous pharmacokinetic curve, which suggested that enterohepatic circulation was involved in the metabolizing of PHZ.

Increasing the dosage for achieving better therapeutic effect is one of the most common solutions for drugs with poor bioavailability. However, a high dosage of drugs will inevitably increase the metabolic burden of liver and affect the renal excretion function. Intravenous injection is easier and more efficient than oral administration to achieve the minimum effective dose. Therefore, it is considered that intravenous administration can greatly reduce the dosage of PHZ and avoid harmful side effects caused by excessive medication. In 1933, Chasis H intravenously injected PHZ into a human, and no significant side effects were observed in his study.[26] In our present study, although rats could survive after intravenous injection, it was suggested that further study should be performed to ensure the safety of intravenous injection for PHZ.

The structures of the tissues and organs change under disease conditions, and the changed structures result in a change in the function of the tissues and organs. Based on these reasons, it is considered that a drug's metabolism will be altered in the pathological state.[27] Medicines are only used when people are ill; thus, the pharmacokinetics of the pathological state is more representative in the actual situation. Research shows that diabetes,[28,29] liver injury,[30] inflammation,[31] cerebral ischemia,[32] and other diseases alter the drug's metabolism.[33–35]

Our results showed that the pharmacokinetic parameters of PHZ and PHT in STZ-induced T2D rats were remarkably different from those in normal rats both in oral and intravenous administrations. In particular, the AUC values of PHZ and PHT in T2D rats both in the oral and intravenous administrations of PHZ were significantly larger than those in normal rats. The higher AUC and C_{max} values indicated that the utilization of PHZ in STZ-induced T2D rats increased and systemic clearance decreased. In brief, the bioavailability of PHZ in T2D rats significantly increased than that in normal rats.

Recent reports showed that lipopolysaccharide (LPS) can increase the intestinal tract permeability,[36] thus resulting in increased absorption of medicine and increased drug concentration in blood.[37] Research shows that the level of LPS in T2D or high-fat-diet-induced mice is two- or three-fold higher than that of normal ones.[38,39] P-glycoprotein (P-gp) can limit the absorption of hydrophobic drugs,[40] which reduces the bioavailability of some drugs.[41,42] Studies have confirmed that the expression level of P-gp decreases in the state of diabetes.[42,43] Based on the above points, we concluded that the

intestinal tract permeability increased in T2D rats by the up-regulation of LPS and the down-regulation of P-gp, which resulted in increased systemic exposure (AUC) of PHZ. Moreover, other complications of diabetes such as hepatic lesions, atherosclerosis, and renal diseases may also affect the pharmacokinetics of PHZ. In this study, the half-times of PHZ and PHT increased in T2D rats, which indicated a higher elimination rate than that in normal rats. The results showed that phase II metabolism became more intense in T2D rats; combining the facts that phase II metabolism is the main way to eliminate the drugs and the phase II conjugate form is the main form of the drug excreted,[43] we concluded that the increase in phase II metabolism activity led to increased systemic clearance of PHZ in T2D rats.

Saeko Masumoto *et al.* reported that 2.81 ± 0.85 μmol L^{-1} of PHZ and 87.8 ± 13.5 μmol L^{-1} of PHT were detected in the blood of STZ-induced diabetic rats after feeding with food containing 0.5% PHZ.[2] However, Crespy's study indicated that the PHZ prototype scarcely existed in normal rats.[7] Combined with our experiments, it was deduced that more active phase II metabolism of PHZ and metabolites occurred in T2D rats than in normal rats, which might be due to the increase in UGT activity. Rong Shi's study demonstrated that diabetes mellitus resulted in an increase in the activity of UGT1a9 in *db*/*db* mice.[44] Our results were consistent with Rong Shi's conclusion that the UGT activity should increase in diabetic rats. It has been reported that the activity of β-glucuronidase increases in the diabetic rats' intestinal tract.[45] We speculated that the relative phase II metabolism of PHZ might decrease because of the up-regulation of β-glucuronidase, and the contents of PHZ and PHT can increase in the intestine.

Flavonoids occur in a wide range of phase I and II metabolisms *in vivo*.[46] In general, flavonoid glucuronides are water-soluble and thus, the conjugated compounds are eliminated rapidly *via* urine. Therefore, the phase II conjugates of flavonoids were considered as the decreased activity biological forms *in vivo*.[47] However, we put forward some different points through our study.

In Crespy's study, PHZ was not detected in normal rats after the oral administration of PHZ. Nevertheless, numerous traces of PHT were found in blood after enzyme hydrolysis by β-glucuronidase and sulfatase. Moreover, conjugated forms of PHT accounted for 95% of TPHT *in vivo* after 4 hours of oral administration.[7] In our present study, the phase II metabolism was universally found both in T2D rats and normal rats, which resulted in the major form of phase II conjugates (PHT, existing in blood *in vivo* after oral and intravenous administrations).

PHZ is well-known as the first discovered SGLT inhibitor in the world with specific and competitive inhibition of both SGLT1 and SGLT2 to increase the excretion of urine sugar and subsequently reduce the blood glucose level.[48–50] A number of recent research studies have shown that PHZ significantly improves the blood glucose level and lipid metabolism in diabetic mice;[3,4] it also regulates intestinal flora and increases insulin sensitivity.[5] However, oral administration was adopted in most of the previous studies. Based on the above experimental results, we wanted to know how PHZ exerted its antidiabetic, antioxidant, and anticancer activities under the conditions of a small prototype concentration of this compound *in vivo*.

In the study of antiviral efficacy against feline herpesvirus type 1 (FHV-1) of famciclovir and its metabolites, Allyson D. Groth *et al.* found that famciclovir has no antiviral effect against FHV-1 at any concentration. However, one of the metabolites of famciclovir, Penciclovir, was found to be potent with antiviral activity against FHV-1, and the IC_{50} value reached 0.86 μg mL^{-1}.[51] For Tramadol, a widely used central analgesic drug, it was found that M1 showed the highest affinity to the human μ-opioid receptor among all the five metabolites (M1, M2, M3, M4, and M5). This indicated that the metabolite M1 is responsible for the μ-opioid-derived analgesic effect.[52] Bellou Sofia also found that 6-methoxyequol, a metabolite of isoflavone, showed strong biological activity in inhibiting angiogenesis and suppressing the growth of tumors.[53] It is deduced that the phase II conjugate forms of PHT were the biologically active forms of PHZ *in vivo* due to the phase II conjugates of PHT being the predominant existing forms after the oral administration of PHZ.

In conclusion, the phase I metabolism remarkably decreased by intravenous administration compared with that of traditional oral administration. However, phase II metabolism of this compound was also inevitably found *in vivo*. Phase II conjugates of PHT were the predominant existing forms *in vivo* after oral administration. Hence, we speculated that the phase II conjugated forms of PHT should be the active forms and they are responsible for its activities; further experiments should be performed to validate our speculation. The pharmacokinetic behaviors of PHZ in normal rats and STZ-induced T2D rats were significantly different; the values of AUC and C_{max} increased with the decrease in $T_{1/2}$ in T2D rats compared with the observations for normal rats. In addition, the bioavailability of PHZ and its metabolites in T2D rats was higher than that in normal rats. The pharmacokinetics of PHZ in diabetes might be used for rational reference standard in later drug development and clinical research of PHZ.

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Abbreviations

PHZ	Phlorizin
TPHZ	Total phlorizin

PHT	Phloretin
TPHT	Total phloretin
SGLT	Sodium-glucose cotransporter
T2D	Type 2 diabetes
LPH	Lactase-phloridzin hydrolase
STZ	Streptozotocin
HOMA-IR	Insulin resistance index
FINS	Fasting insulin
FBG	Fasting blood glucose
IS	Internal standard
LLOQ	Lower limit of quantification
LOD	Limit of detection
RSD	Relative standard deviation
SD	Standard deviation
CPZ	Conjugations of phlorizin
CPT	Conjugated form of phloretin
LPS	Lipopolysaccharide

Conflicts of interest

The authors declare no competing financial interests.

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