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Pharmacokinetic Profile of Total Quercetin after Single Oral Dose of Tartary Buckwheat Extracts in Rats

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ABSTRACT: As an important edible and medicinal material, tartary buckwheat (*Fagopyrum tataricum* Gaertn.) is commonly used as a kind of food or drug in eastern Asian countries. To investigate the pharmacokinetic profile of total quercetin after a single oral dose of tartary buckwheat extract in rats, a sensitive, simple, and accurate HPLC−UV method was developed to determine total quercertin following plasma enzyme hydrolysis with glucuronidase/sulfatase. Eighteen male Wistar rats were randomly assigned into three groups, which were given three different doses of tartary buckwheat extract. The pharmacokinetic results showed that the area under the plasma concentration−time curve (AUC) of total quercetin after single oral doses of tartary buckwheat extract presented a linear relationship. The peak plasma concentrations (C_{max}) of total quercetin afte plasma enzyme hydrolysis with glucuronidase/sulfatase were 0.55 ± 0.26, 1.10 ± 0.53, and 2.05 ± 0.26 μg/mL; the peak times (T_{max}) were 2.33 ± 1.94, 2.75 ± 3.67, and 2.50 ± 1.82 h; the areas under the curves (AUC_{0-36h}) were 5.29 ± 1.35, 10.02 ± 4.43, and 22.51 ± 3.05 $\mu g \cdot mL^{-1} \cdot h^{-1}$ for three doses of tartary buckwheat extract (60, 120, and 240 mg/kg). The present study has provided a basic pharmacokinetic profile of total quercetin after a single oral dose of tartary buckwheat extract in rats.

KEYWORDS: Tartary buckwheat extract, total quercetin, phase II metabolites, β-glucuronidase, sulfatase

INTRODUCTION

For a long time, tartary buckwheat (*Fagopyrum tataricum* Gaertn.) has been widely used as daily diet and traditional medicine, especially in some Asian countries, such as China, India, Bhutan, Nepal, etc.[1,2] The major chemical components in this plant have been demonstrated to be flavonoids such as rutin, quercetin, and quercetrin.[1−6] As important functional foods, tartary buckwheat products are also rich in vitamins B1, B2, and B6,[2] various essential proteins,[7] various essential trace elements,[8] crude fiber (dietary fiber), and D-chiro-inositol.[4,9] Tartary buckwheat has multiple biological functions; for example, D-chiro-inositol can lower the blood glucose level in KK-Ay mice,[9] and recent studies have also revealed the potential beneficial effects of quercetin and its glycosides (quercetrin, rutin, etc.) on human health, such as antioxidant activity,[5,6,10] antivascular fragility,[11] antiatherosclerosis,[12] lowering blood pressure,[13] and enhancing vascular permeability.[14]

Our previous experiments indicated that the main chemical components in tartary buckwheat ethanol extract were quercetin and its related glycosides,[15] which were the primary bioactive constituents in tartary buckwheat.[7] However, so far, there are no reports on the pharmacokinetics profile of flavonoids in tartary buckwheat products after a single oral dose to rats, so the aim of this study was to investigate the pharmacokinetics profile of the flavonoids in tartary buckwheat products.

To our knowledge, due to the ubiquitous distribution of quercetin and its glycosides in the plant kingdom, numerous reports were available on both the pharmacokinetic profiling and the mechanism understanding of these types of compounds during the past few decades, especially quercetin. Numerous studies had provided the evidence that after oral administration of glycosides of quercetin (rutin or quercetrin) to rats, pigs, and humans, the glycoside forms of quercetin were always hydrolyzed to their aglycons in the small or large intestine by the glycosidase activity of intestinal bacteria[13,16−20] and then metabolized to their glucuronides and sulfate conjugates (phase II metabolites) during the process of absorption and/or circulation (as shown in Figure 1.).[14,17,18,21−23] It was reported that the methylated metabolites of quercetin (isorhamnetin and tamarixetin) were also present in conjugated forms in the plasma.[13,24,25] Furthermore, isorhamnetin had been identified as a phase II metabolite of quercetin in some mammalian species.[13,17,26−29] The O-methylation of quercetin (isorhamnetin) and another O-methylated metabolite of quercetin (tamarixetin) were catalyzed by catechol-O-methyltransferase.[30] It had to be mentioned above that in vitro the phase II metabolites of quercetin, including O-methylated products of quercetin (isorhamnetin and tamarixetin), were the main exhibited forms in the plasma after a single oral application of quercetin and its glycosides. Subsequently, multimonitoring of various structurally related flavonoids was necessary while the pharmacokinetic profiles of quercetin and its glycosides were investigated.

In this study, based on available reports, an appropriate assay employing sulfatase and β-glucuronidase hydrolysis for determining aglycons of quercetin, isorhamnetin, and tamarixetin was to be developed and applied to investigate the pharmacokinetics profile of the flavonoids in tartary buckwheat extract. However, in

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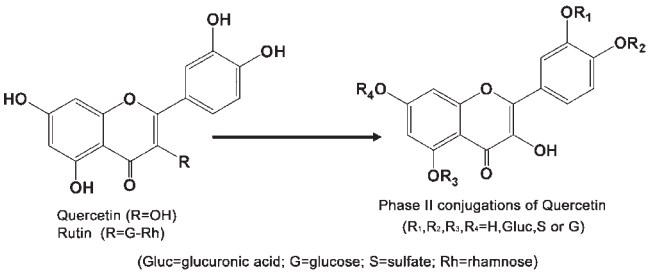

Figure 1. Phase II conjugations of quercetin and rutin in plasma are possible in phase II metabolic pathway.

our pre-experiment, when a single high oral dose (240 mg/kg) of tartary buckwheat extract was given to rats (equivalent to both 46.82 mg/kg rutin and 11.57 mg/kg quercetin), the maximum concentration of isorhamnetin was below the lower limit of quantification of our method, and neither rutin nor tamarixetin was detected in plasma.

Therefore, an appropriate analytical method employing sulfatase and β-glucuronidase hydrolysis for determining total aglycon of quercetin was developed and applied to investigate the pharmacokinetics profile of the flavonoids in tartary buckwheat extract.

MATERIALS AND METHODS

Materials. Quercetin, rutin, and baicalein (internal standard) were obtained from 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 at 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). Ethyl acetate (chemical pure) was purchased from ARK Chemicals (Chengdu, China) and redistilled before extraction. Methanol and acetonitrile (HPLC grade) were purchased from Dikma Technologies (Beijing, China). Formic acid (HPLC-grade) was purchased from the Chengdu Reagent Co. (Chengdu, China). Water (HPLC-grade) was prepared in an ultrapure water system (UPA, Chongqing, China). All other reagents used in the study were of analytical grade.

Tartary Buckwheat Extracts. Tartary buckwheat extract was obtained from Maoxian Qiang Medicine Institute (Sichuan, China) as a powder extracted with 60% ethanol from buckwheat seeds. The contents of rutin and quercetin in tartary buckwheat powder extract were 0.1951 and 0.0482 g/g, respectively (the contents of rutin and quercetin were determined by HPLC−UV assay[15]). The content of total flavonoids (expressed as rutin equivalents, the amount of total flavonoids was determined with the colorimetric assay[31]) in tartary buckwheat powder extract was 0.2645 g/g.

Animals. Eighteen male Wistar rats (about 200 g) were purchased from Laboratory Animals Center of Sichuan University, P.R. China. Every six rats were housed in one cage in a temperature-controlled room, with a dark period from 22:00 to 08:00 and access to food from 8:00 to 16:00. The rats were given a controlled diet for at least 7 days, which was relatively flavonoid-free and contained 70% wheat starch, 15% casein, 10% egg white, and 5% mineral mixture.

Animal Experiments. Eighteen male Wistar rats (about 200.0 g) were randomly divided into three groups. Six male Wistar rats in each group were investigated in each experiment. The rats were fasted for 12 h but given free access to water before experiment. The rats were given free access to water throughout the process of experiment, and after 12 h, the rats were given access to food from 8:00 to 16:00.

Three different doses of tartary buckwheat extract (60, 120, and 240 mg/kg; equivalent11.71 mg/kg rutin and 2.89 mg/kg quercetin, 23.41 mg/kg rutin and 5.78 mg/kg quercetin, and 46.82 mg/kg rutin and 11.57 mg/kg quercetin, respectively) were investigated. On the day of the experiments, the male Wistar rats were given approximately 4 mL of tartary buckwheat extract solution via oral administration. (tartary buckwheat powder extract was dissolved in sodium chloride solution at different concentrations).

Blood samples (approximately 0.3 mL of venous blood) were collected via tail at 0.25, 0.50, 1.00, 1.50, 2.00, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 18.00, 24.00, and 36.00 h. Blood samples were collected in tubes containing lithium−heparin anticoagulant and centrifuged (2500*g*, 3 min) within 3 min after collection (EBA21 table centrifuge). Then, due to the instability of flavonoids at high pH,[24] a volume of 100 μL of harvested plasma was acidified with 10 μL of 0.5 M acetic acid, which contained 2 mg of vitamin C/mL, to prevent loss of flavonoids. All samples were stored at −20 °C until assay.

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 Sichuan University.

Chromatographic Conditions. Liquid chromatography analysis was performed on a Waters chromatographic system (Waters, Milford, MA) consisting of a 515 pump and a 2487 dual λ absorbance detector. The tested analytical column was Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm; Dikma, Beijing, China). The column temperature was maintained at 30 °C. The mobile phase was acetonitrile−0.2% formic acid in water (38:62 v/v) at a flow rate of 1 mL/min. The injection volume was 20 μL and the injector needle was washed in methanol. The optimal detection UV wavelength was 370 nm.

Hydrolysis of Plasma Sample with β-Glucuronidase and Sulfatase. A volume of 100 μL of sample plasma, control plasma for calibration, and QC samples was removed from the freezer, thawed at room temperature, and then were made ready for extraction in 2.5 mL polypropylene tubes. After the pH was adjusted to 5.0 with 0.5 M acetic acid (containing 2 mg of vitamin C/mL), 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 (internal standard, 40 μg/mL). After being briefly shaken, each mixture was vortex-mixed with 1.5 mL of ethyl acetate (redistilled) for 2 min at 2000 rpm (Vortex Genius 3, IKA, Germany). The tube was centrifuged for 5 min at 8000*g* (EBA21 table centrifuge, Hettich, Germany). The upper organic phase was transferred to another 2.0 mL polypropylene tube and evaporated to dryness under a stream of nitrogen at 37 °C (N-EVAP 11155, Organomation). The residue was reconstituted in 100 μL of methanol, then the solution was centrifuged at 8000*g* for 5 min, and finally an aliquot (20 μL) of the solution was injected into the chromatographic system for determination.

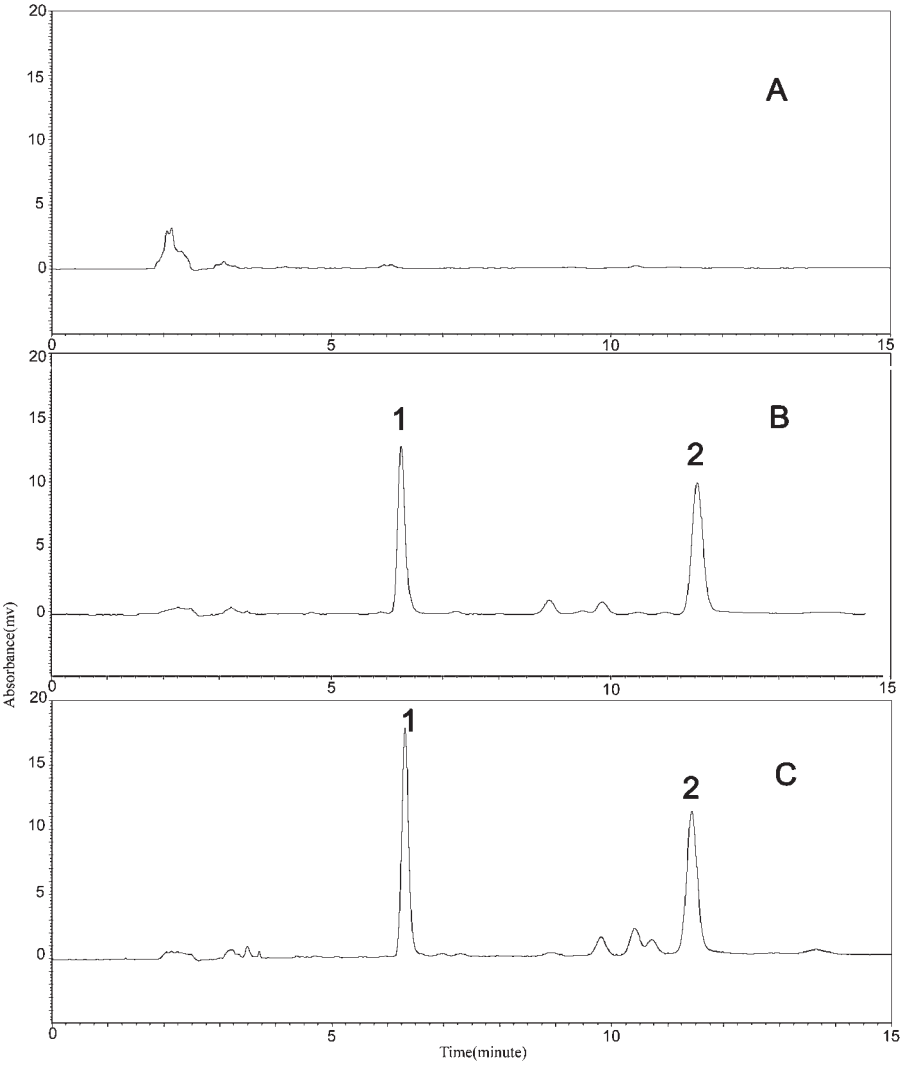

Figure 2. Typical chromatograms of (A) blank Wistar rat plasma, (B) blank Wistar rat plasma spiked with quercetin (0.97 μg/mL) and internal standard (baicalein, 8.00 μg/mL), and (C) Wistar rat plasma obtained from an experimental rat 1.5 h after an oral dose of tartary buckwheat extract (120 mg/kg, equivalent to 23.41 mg/kg rutin and 5.78 mg/kg quercetin). Peak 1, quercetin; peak 2, internal standard (baicalein).

Method Validation. The specificity/selectivity of the method was investigated by screening six separate blank rat plasma samples and looking for endogenous peaks that accounted for more than 20% of the peak area of quercetin or the internal standard in the low concentration standard.[25]The calibration curves of quercetin were fitted by least-squares linear regression using 1/concentration ($1/c^2$) as weighting factor of the peak-area ratio of quercetin to baicalein (internal standard) versus the injected concentration of quercetin in each calibration samples. Calibration curves of quercetin were quantified in three individual runs, whose coefficient of determination values (r^2) were required to be no less than 0.99.

The lower limit of quantification (LLOQ) and limit of detection (LOD) of quercetin were determined by diluting the calibration samples. The LLOQ was defined as the lowest serum concentration of quercetin quantified with a coefficient of variation of less than 20%. The LOD was considered as the concentration of quercetin that provides a signal corresponding to 3 times the HPLC background signal.

For intraday accuracy and precision, a single batch of samples was analyzed, and for interbatch inaccuracy and imprecision, three batches of samples were analyzed on three separate days within 1 week. Each batch contained a calibration curve.

The extraction recoveries of quercetin from plasma were determined at four concentrations (0.06, 0.24, 0.97, and 3.88 μg/mL; n = 3), by comparing the areas of extracted samples with nonextracted samples (pure methanol solutions of quercetin and internal standard mixture were evaporated to dryness under a stream of nitrogen at 37 °C following the sample preparation operation). The extraction recovery of the internal standard baicalein was determined at the concentration of 8 μg/mL (n = 12).

In the stability study, the variation of quercetin concentrations was expressed in mean percentages of change of the initial concentrations. Stability studies of quercetin included (a) long-term stability of plasma samples kept frozen at −20 °C for 8 weeks, (b) stability at room temperature for 8 h, (c) stability after three freeze/thaw cycles, and (d) stability of the extracted sample at room temperature.

Data Analysis. Pharmacokinetic calculations were optimized by the compartmental and noncompartmental 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

Table 1. Precision and Accuracy of Quercetin Assay

	intraday (n = 5)		interday (n = 5 × 3)	
nominal conc. (μg/mL)	accuracy (bias, %)	precision (RSD, %)	accuracy (bias, %)	precision (RSD, %)
0.03	3.08 ± 1.49	1.44	3.50 ± 2.39	2.31
0.24	−3.04 ± 4.61	4.76	−3.22 ± 5.41	5.59
0.97	6.56 ± 6.28	6.72	−7.34 ± 5.43	5.43
3.88	4.44 ± 7.78	7.45	3.11 ± 8.52	8.26

Table 2. Extraction Recovery of Quercetin and Internal Standard[a]

nominal conc. (μg/mL)	area (mv·min, methanol)	area (mv·min, plasma)	recovery (%)
quercetin (3.38)	693 998 ± 5866	530 020 ± 4002	77.49 ± 0.89
quercetin (0.97)	166 086 ± 3917	125 556 ± 1066	75.63 ± 1.69
quercetin (0.24)	36 954 ± 288	29 724 ± 354	80.44 ± 1.52
quercetin (0.03)	10 682 ± 74	9008 ± 517	84.32 ± 4.65
internal standard (8.00)	174 064 ± 1446	131 497 ± 2291	75.55 ± 1.42

[a] Quercetin, n = 3; internal standard, n = 3 × 4.

calculated according to the trapezoidal method. The following noncompartmental 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 pharmacokinetic compartmental models to the plasma concentration−time profiles for each animal after oral dosing.

RESULTS

Method Validation Results. Under the validated HPLC system, typical chromatograms of blank rat plasma sample, blank sample spiked with quercetin (0.97 μg/mL) and internal standard (baicalein, 8.00 μg/mL), and sample obtained 1.5 h after a single oral dose of tartary buckwheat extract (120 mg/kg) from a rat are shown in Figure 2. No interference was presented in the chromatographic separation, and each target peak had good resolution.

The mean equation (curve coefficients ± SD) of the calibration curves (n = 6) was obtained from six single batches in routine unknown plasma sample analysis. The slope of the calibration curves appeared stable for quercetin within the concentration range 0.03−7.76 μg/mL. The regression coefficient (r^2) of the calibration curves remained excellent, always higher than 0.99. This result showed the usefulness of the present HPLC method in assays of quercetin from low to high plasma levels.

The lower limit of quantitation (LLOQ) was achieved as the lowest point on the standard curve, 0.03 μg/mL for quercetin with RSD of 8.49% (n = 5). The limit of detection (LOD) of the quercetin in plasma was determined to be 0.01 μg/mL.

The inter- and intra-assay accuracy and precision data are shown in Table 1.

These quality control samples (n = 5), representing very low, low, medium, and high concentrations, contained 0.03, 0.24, 0.97, and 3.38 μg/mL quercetin. The precision (RSD) and the biases in each case were less than 10%. These results showed that the present method had good precision and accuracy.

The extraction recoveries of quercetin and internal standard are shown in Table 2. The mean extraction recoveries of quercetin (n = 3) from spiked plasma were satisfactory at very low, low, medium, and high concentrations, which varied from 75.63% ± 1.69% to 84.32% ± 4.65%. The mean extraction recovery of internal standard (n = 12) from spiked plasma was 75.55% ± 1.42%.

In the stability study, the variation of quercetin concentrations was expressed in mean percentages of change of the initial concentrations, which were all less than 15% at very low, low, medium, and high concentration levels. The results indicated that quercetin was stable in plasma kept at room temperature for 12 h or for at least 2 months when stored at −20 °C. The quercetin was also stable after three freeze−thaw cycles and in the extracted sample kept at room temperature for 12 h.

Pharmacokinetics Results. In animal experiments, after treatment with β-glucuronidase and sulfatase, the conjugated form of quercetin (glucuronides and sulfates) was quantified, including the metabolites of quercetin and its related glycosides, in rat plasma samples. After a single oral administration of three different doses of tartary buckwheat extract (60, 120, or 240 mg/kg; n = 6) to rats, the concentration−time curves of total quercetin were measured and are shown in Figure 3. Multiple peaks were observed in individual profiles, which suggested enterohepatic recirculation or intestinal secretion mediated by membrane transporters, consistent with the pharmacokinetic profiles of flavonoids.[13,32] It was shown that the concentration−time curves of total quercetin over the tested doses were similar. The C_{max} values of total quercetin were 0.55 ± 0.26, 1.10 ± 0.53, and 2.05 ± 0.26 μg/mL and were achieved at T_{max} of 2.33 ± 1.94, 2.75 ± 3.67, and 2.50 ± 1.82 h, respectively, after three different single oral doses of tartary buckwheat extract to rats. After T_{max}, concentrations of total quercetin declined in a biexponential manner, with a terminal phase beginning at 12−36 h after dosing. Mean terminal $t_{1/2\beta}$ values were 6.64 ± 3.19, 5.58 ± 1.56, and 7.85 ± 3.26 h for the three doses of tartary buckwheat extract, respectively. The other main pharmacokinetic parameters are presented in Table 3.

The results from Table 3 suggested that the pharmacokinetics profile of total quercetin (AUC_{0-t}, $AUC_{0-\infty}$, and C_{max}) after three different single oral doses of tartary buckwheat extract in rats had a linear relationship over the dose tested. The regression coefficients (r^2) of the calibration curves of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were 0.9959, 0.9954, and 0.9988, respectively.

DISCUSSION

In this study, based on available reports, we aimed to develop an appropriate analytical method employing sulfatase and

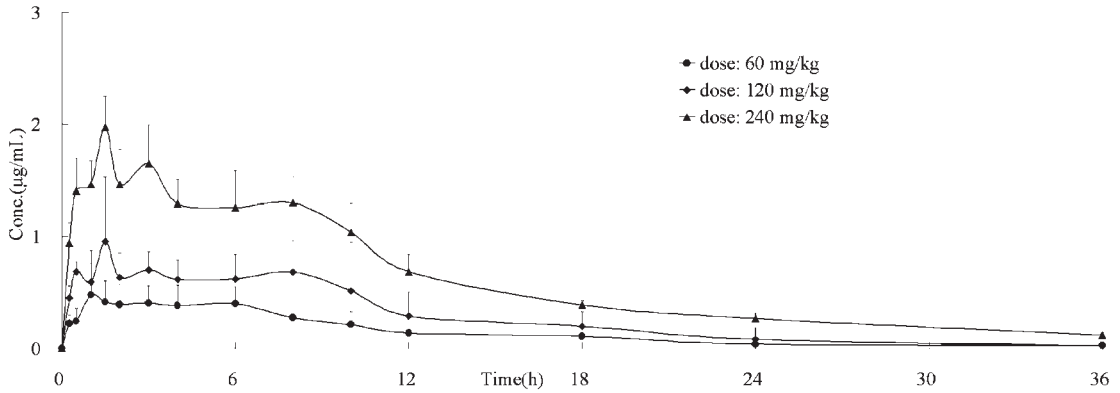

Figure 3. Mean plasma concentration−time profiles of total quercetin after three different single oral doses of tartary buckwheat extract (60, 120, and 240 mg/kg) in rats (n = 6).

Table 3. Pharmacokinetics Data of Total Quercetin after Three Different Single Oral Doses of Tartary Buckwheat Extract in Rats (n = 6)

parameters	low dose (60.00 mg/kg)	medium dose (120.00 mg/kg)	high dose (240.00 mg/kg)
$t_{1/2}$, $t_{1/2\alpha}$ (h)	3.81 ± 2.05	2.64 ± 1.67	4.92 ± 3.08
$t_{1/2\beta}$ (h)	6.64 ± 3.19	5.58 ± 1.56	7.85 ± 3.26
$t_{1/2Ka}$ (h)	1.16 ± 1.08	0.89 ± 0.54	1.12 ± 1.19
CL/F ($L \cdot h^{-1} \cdot kg^{-1}$)	11.51 ± 2.50	14.02 ± 8.02	9.86 ± 1.67
AUC_{0-t} ($\mu g \cdot mL^{-1} \cdot h^{-1}$)	5.29 ± 1.35	10.02 ± 4.43	22.51 ± 3.05
$AUC_{0-\infty}$ ($\mu g \cdot mL^{-1} \cdot h^{-1}$)	5.44 ± 1.28	10.49 ± 5.17	24.03 ± 3.33
MRT_{0-t} (h)	9.47 ± 0.68	8.54 ± 2.02	10.30 ± 0.41
$MRT_{0-\infty}$ (h)	11.18 ± 1.12	9.02 ± 2.13	13.22 ± 1.72

β-glucuronidase hydrolysis to monitor all the phase II conjugations of quercetin including the methylquercetins (isorhamnetin and tamarixetin) after single oral application of tartary buckwheat extract to rats. However, no isorhamnetin or tamarixetin was detected in plasma in our present experiment. Therefore, only the total quercetin in the plasma was determined and applied to investigate the pharmacokinetics profile of the flavonoids in tartary buckwheat extract. The objective of our future study should be to establish an analytical method of high sensitivity, such as to determine the phase II conjugations of methylquercetin by an HPLC−MS method.

Quercetin and its glycosides are presented in many plants. The glycosides are always the most abundant form, and free aglycon is uncommon in nature. However, quercetin and its glycosides (quercetrin, rutin, etc.) do coexist in tartary buckwheat extract. Previous studies reported that it reached peak concentration quickly after a single administration of quercetin independently, the peak time was approximately 0.7 ± 0.2 h; however, the glycosides of quercetin, such as rutin, reached peak concentration slowly after a single administration independently, the peak time was approximately 7.0 ± 2.9 h.[18,19,32] In this study, when a single oral administration of tartary buckwheat extract (the ratio of rutin to quercetin was about 4:1) to rats, the peak plasma concentrations of total quercetin were approximately 0.55 ± 0.26, 1.10 ± 0.53, and 2.05 ± 0.26 μg/mL, and the peak times were 2.33 ± 1.94, 2.75 ± 3.67, and 2.50 ± 1.82 h for the three dose of tartary buckwheat extract (60, 120, and 240 mg/kg) respectively. When both quercetin and its glycosides were present in tartary buckwheat extract, the pharmacokinetic profiles of total quercetin changed significantly compared with those of quercetin or rutin independently. That is to say, when the ratio of rutin and quercetin in the tartary buckwheat extract was different, the pharmacokinetic profiles of total quercetin might be also different. However, this hypothesis needs to be investigated in the future.

There was still a need to discuss how to calculate the bioavailability of flavonoids. Should it be calculated by the plasma concentration of only its prototype, the sum of its relative glycosides, or the sum of its relative pharmacologically active metabolites? Recent reports showed that the phase II conjugations of quercetin including the methylquercetins (isorhamnetin and tamarixetin) possessed biological activities.[18,27,33−35] So it is necessary to take into consideration a group of metabolites instead of merely considering the prototype or the sum of antetype and conjugated type.[25] Nevertheless, the present study has provided a basic pharmacokinetic profile of total quercetin after a single oral dose of tartary buckwheat extract consisting of both quercetin and its glycosides to rats, which could provide guidance for further studies on functional food containing these flavonoids.

Until now, the quality control and evaluation method for functional foods was mainly physical and chemical qualitative or quantitative analysis. There was yet no bioavailability assessment, like the drugs. However, the quality control in vitro still could not completely replace that in vivo. Functional foods play an important role in human health, and thus the bioavailability assessment study of these functional foods is necessary.

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