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Research paper

Difference in absorption of the two structurally similar flavonoid glycosides, hyperoside and isoquercitrin, in rats

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Abstract

The present study was to investigate oral absorption of the two similar flavonoid glycosides, isoquercitrin (IQ, quercetin-3-*O*-glucoside) and hyperoside (HP, quercetin-3-*O*-galactoside) in rats. Two groups of male SD rats received an oral dose of either IQ (4.5 mg/kg) or HP (6.0 mg/kg). Blood samples were collected via jugular vein at time intervals after drug administration and the plasma concentrations of the studied compounds were analyzed by HPLC. The stability of IQ and HP in the GI tract was also measured by incubation with various GI contents from rats. The results showed that unchanged IQ was barely detectable whereas the glucuronidated quercetin (the aglycone of IQ) was found to be the major form in plasma after oral administration of IQ. In contrast, HP could not be detected in plasma neither as unchanged form nor its aglycone or conjugated aglycone form. Additional in vitro stability studies demonstrated that HP is more stable than IQ in the GI tract. This suggests that IQ could be hydrolyzed easier than HP to its aglycone in GI tract before being absorbed. In conclusion, IQ, as a flavonoid glucoside, could be rapidly absorbed and transformed into glucuronidated quercetin and such absorption might be related to the hydrolysis of the type of sugar moieties attached to its aglycone molecule.

Keywords: Isoquercitrin; Hyperoside; Flavonoids; Absorption

1. Introduction

Flavonoids are a large and complex group of polyphenolic compounds widely distributed throughout the plant kingdom. They are common dietary components of fruits, vegetables and beverages, and are usually present in plant tissue as the form of glycosides [1,2]. Flavonoids exhibit a multitude of biological activities, such as anti-bacteria, anti-inflammation, anti-allergy and anti-oxidation [3,4]. Epidemiological studies have shown that the dietary intake of flavonoids is inversely associated with the incidence of coronary heart disease and cancer [5,6]. Due to their abundance in dietary products and their potential beneficial pharmacological and nutritional effects, the flavonoids are of considerable interest for drug as well as health food supplement.

Even though a lot of investigations have been conducted for the absorption and bioavailability of flavonoid glycosides, the results are quite contradictory. Early studies hypothesized that flavonoid glycosides would not enter the systemic circulation, neither as the natural glycosides nor as the aglycone hydrolysis products. It was believed that cleavage at the central heterocyclic ring by intestinal bacteria would occur effectively generating phenolic acid fission products [7,8]. At the later stage of flavonoid study, it was generally believed that the flavonoid glycosides have to be hydrolyzed to its aglycone before being absorbed [9–11]. However, recent studies reported that some flavonoids glycosides can be detected in human or rat plasma as its intact form [12,13], indicating that flavonoid glycosides may be able to be absorbed before being hydrolyzed.

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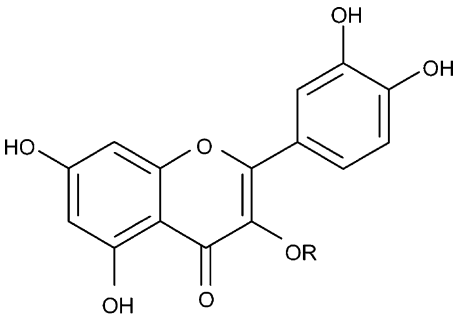

Hyperoside R = Galactose
Isoquercitrin R = Glucose

Fig. 1. Chemical structures of hyperoside and isoquercitrin.

In most of the above studies, the plasma samples were all subjected to the hydrolysis and the concentrations of the aglycone in the plasma were examined. However, both flavonoid glycoside and the glucuronidated conjugate of the aglycone could be hydrolyzed by the β-glucuronidase [8,14], which was generally used for the hydrolysis treatment. Thus, the study could not confirm whether the detected aglycone is from flavonoid glycoside absorbed as its intact form or from the flavonoid glucuronide formed during the absorption of its aglycone after the hydrolysis of flavonoid glycoside in GI tract.

Therefore, in the present study, we plan to investigate the oral absorption of the two flavonoid glycosides, isoquercitrin (IQ, quercetin-3-*O*-glucoside) and hyperoside (HP, quercetin-3-*O*-galactoside) (Fig. 1), by measuring their intact forms as well as their glucuronidated metabolite in plasma. The doses of IQ and HP were chosen as 4.5 and 6.0 mg/kg, respectively, which ratio was based on their contents in hawthorn phenolic extract [15] and our previous pharmacokinetic study on hawthorn extract [16]. The purposes of the current studies are to: (1) find out the detailed absorption processes of the studied natural flavonoids; (2) investigate the influence of the sugar moieties on the absorption of flavonoids.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade isoquercitrin (IQ, quercetin-3-*O*-glucoside) and hyperoside (HP, quercetin-3-*O*-galatoside) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Naringin used as internal standard for HPLC assay was obtained from Signa (St. Louis, MO, USA). HPLC-grade Acetonitrile was obtained from Labscan (Labscan Asia Co. Ltd, Thailand). All other chemicals and solvents used were of analytical grade and were obtained from Sigma or BDH Laboratory Supplies (Poole, Dorset, England). Distilled and deionized water was used throughout the study.

2.2. Animals

Male Sprague–Dawley rats aged 7 weeks (200–220 g) were supplied by and bred at the Laboratory Animal Service Center at the Chinese University of Hong Kong. The rats were housed in an air-conditioned room (temperature, 23 ± 2 °C; relative humidity, 55 ± 5%) and kept on a light/dark cycle of 12/12 h. They had free access to standard rodent diet (Prolab RHM 3000; PMI Nutrition International, Inc., Brentwood, MO, USA) and water before the experiment.

On the day before the experiment, a light surgery for the rat was performed. A polyethylene catheter (0.40-mm ID, 0.80-mm OD, Portex Limited, Hythe, Kent, England) was cannulated into the right jugular vein under light anaesthesia. After surgery, the rat was placed individually in metabolic cages. The animal was then allowed to recover for 24 h and fasted overnight prior to each experiment.

2.3. Drug administration and blood sampling

The dosing solutions of IQ and HP were freshly prepared by dissolving in saline containing 2% DMSO before experiment. Two groups of male Sprague–Dawley rats (200–220 g) received an oral dose of IQ (4.5 mg/kg, $n = 9$) and HP (6.0 mg/kg, $n = 4$), respectively, by gastric gavages. In addition, a higher dose of HP (30 mg/kg, $n = 2$) was also given to rats to further confirm the findings. Blood samples (0.2 ml) were withdrawn via the catheter before and at 2, 5, 10, 15, 20, 30, 40 and 50 min post-dosing and collected into heparinized centrifuge tubes. At the last time point, the collection volume of blood was doubled in order to improve the detection sensitivity of compounds for HPLC analysis. After each blood sampling, 0.2 ml of heparinized normal saline solution (20 IU/ml) was immediately injected back into the catheter to prevent coagulation. The collected blood samples were immediately centrifuged at 4000 rpm for 5 min. The plasma was then separated and stored at −80 °C for analysis. The urine and faeces samples were collected over 12 and 24 h, respectively, post-dose. All the faeces collected were homogenized in 100 ml of water. The mixture was sonicated for 30 min and centrifuged at 6000 rpm for 10 min. The supernatant was collected and stored at −80 °C until analysis.

2.4. In vitro stability of IQ and HP

The stability of IQ and HP in the rat plasma and the gastrointestinal (GI) tract was evaluated by incubation in plasma, buffer solutions with pH 1.2 (similar to the pH in stomach) and 6.8 (similar to the pH in small intestine and colon), and following various GI content solutions (stomach, duodenum, jejunum, ileum), respectively.

2.4.1. In plasma

Fresh rat plasma obtained from rats was spiked with a standard solution of either IQ or HP at a final concentration

of 20 μg/ml. After vortexing, the mixture was incubated in a 37 °C water bath with continuous shaking. The samples (0.1 ml) were taken at time intervals up to 4 h and analyzed by HPLC/UV described as below. The experiments were performed in triplicate.

2.4.2. In gastrointestinal (GI) tract

A standard solution containing either IQ or HP (100 μg/ml in 2% of DMSO aqueous solution) was spiked into the following solutions, leading to a final concentration of 20 μg/ml for each compound. (1) pH 1.2 buffer containing 0.2 g sodium chloride and 0.7 ml hydrochloric acid in 100 ml water. (2) pH 6.8 containing 0.68 g potassium dihydrogen phosphate and 7.7 ml of 0.2 N sodium hydroxide in 100 ml water. (3) Various GI content solutions, prepared from the stomach, duodenum, jejunum, ileum and colon of the rat (see preparation described below). After vortex-mixing, the solutions were incubated in a 37 °C water bath and samples were removed at appropriate intervals (60, 120 and 240 min for the buffer solutions, and 5, 30, 60, 120 and 240 min for the GI solutions) for HPLC analysis. Each test was performed in triplicate.

2.4.3. Preparation of GI content solutions

Five rats were sacrificed after an overnight fast. The whole gastrointestinal tract of each rat was removed immediately. The contents of the stomach, small intestine and colon were collected and suspended with five volumes of ice-cooled simulated gastric fluids (for stomach) or intestinal fluid (for intestine and colon), according to the method described previously [17]. For the small intestine, the duodenum, jejunum and ileum were separated and tested accordingly [18]. The first 20-cm segment from the pylorus was designated as the duodenum. The next 20-cm segment between the pylorus and the caecume was designated as the jejunum. The distal 20-cm segment of the intestine proximal to the caecume was designated as the ileum. All suspensions were mixed extensively by vortexing and then centrifuged at 6000 rpm for 5 min. The supernatants were kept on ice for subsequent experiments as described above.

2.5. HPLC/UV analysis

The drug concentrations of IQ, HP as well as the glucuronidated quercetin in plasma, urine, faeces or incubation solutions for stability study were determined by using an HPLC method previously developed by us with modifications [15]. For urine and faeces samples, an aliquot of urine (0.5 ml) or faeces supernatants (3 ml) was spiked with 50 μl of 20% ascorbic acid and extracted three times with 3 ml of ethyl acetate saturated with water. The combined ethyl acetate solution was concentrated to dryness and then reconstituted with 0.5 ml of the mobile phase. After centrifugation at 13,000 rpm for 10 min, the supernatant (100 μl) was injected into HPLC for analysis. The detection limits for both HP and IQ were 30, 30 and 15 ng/ml in plasma, urine and faeces supernatant, respectively. The recovery of both HP and IQ were higher than 95% in plasma and in urine, and higher than 75% in faeces, respectively. The intra-day ($n=5$) and inter-day ($n=4$) accuracy/precision of both HP and IQ were better than 10/3% and −10/6% in plasma.

2.6. LC/MS/MS identification

LC/MS/MS were used to identify IQ and HP in rat plasma following their oral administration. It was performed

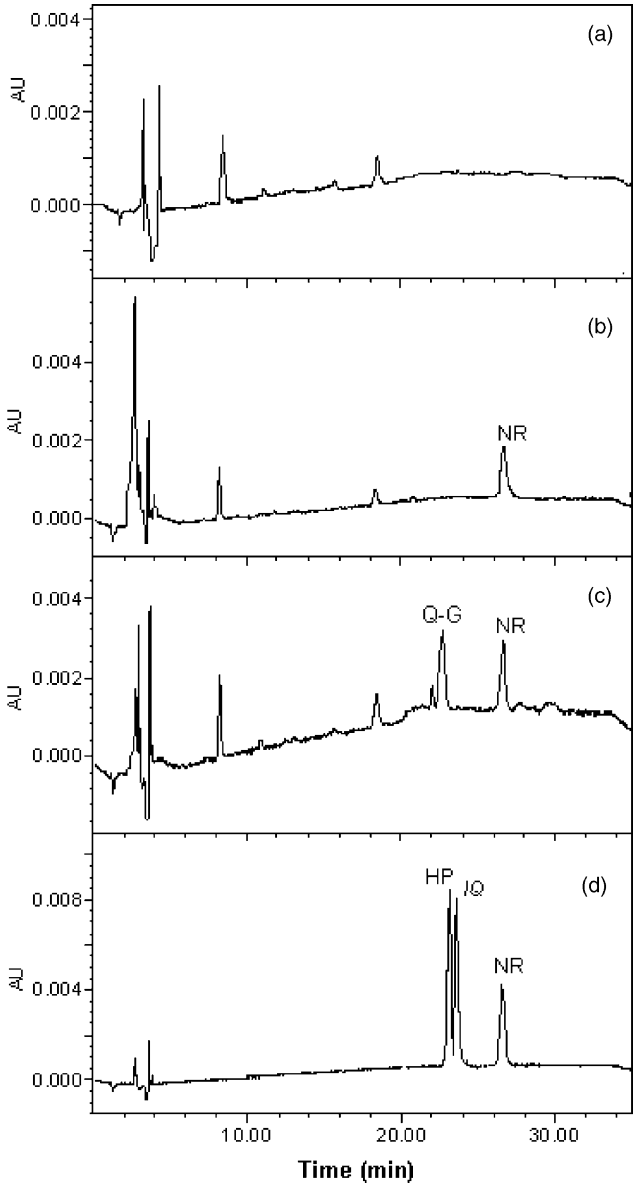

Fig. 2. HPLC/UV (360 nm) chromatograms of plasma samples of rats before (a) and after oral administration of hyperoside (HP, b) or isoquercitrin (IQ, c) at 10 min post-dosing, as well as the standards, HP, IQ and naringin (NR, used as internal standard) (d).

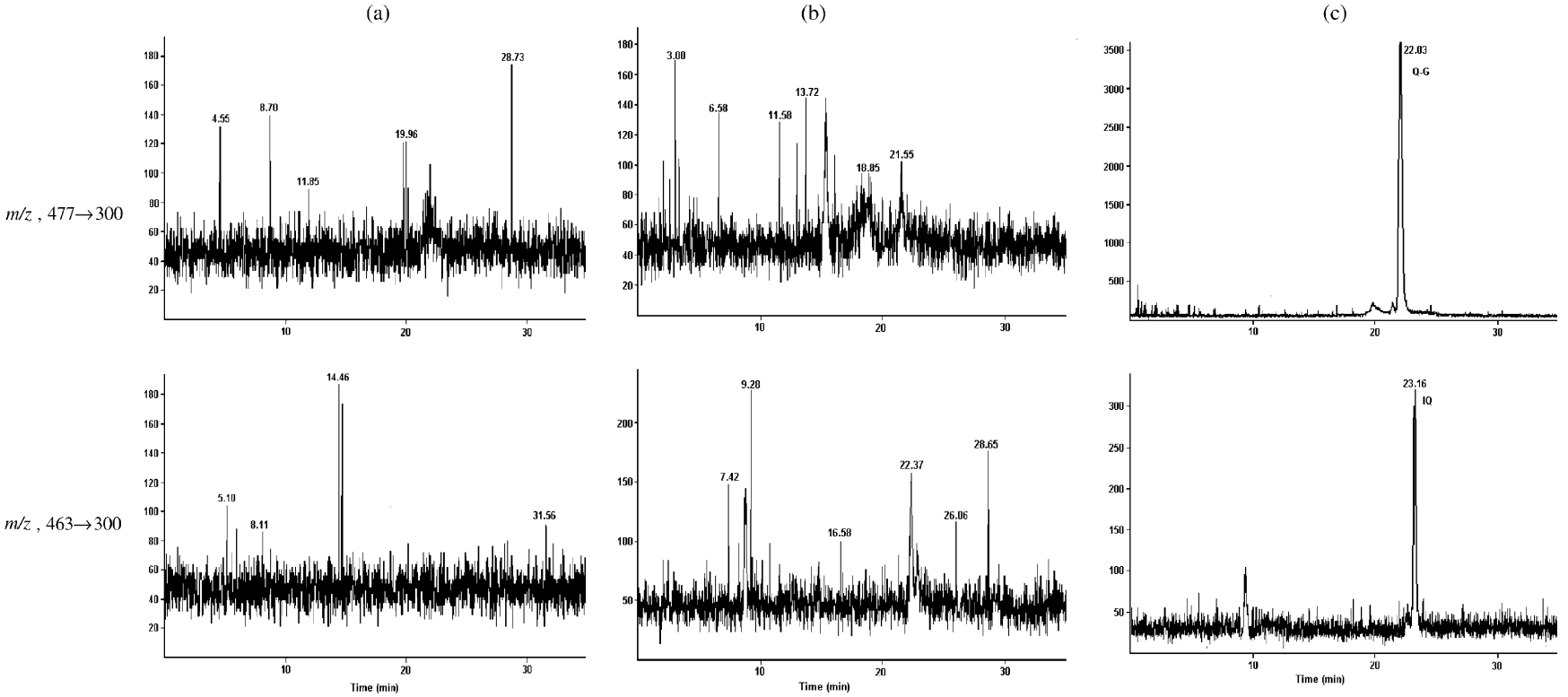

Fig. 3. LC/MS/MS analysis of hyperoside (HP, *m/z* 463→300), isoquercitrin (IQ, *m/z*, 463→300) and quercitin glucuronide (*m/z* 477→300) in plasma samples before (a) and after oral administration of HP (6.0 mg/kg, (b) and IQ (4.5 mg/kg, (c) to rat at 10 min post-dosing.

by a PE SCIEX API 2000 tandem mass spectrometer equipped with an electrospray ionization source, two Perkin–Elmer PE-200 series micropumps and autosampler (Perkin–Elmer, Norwalk, CT, USA). The mass spectrometer was set in the negative ion mode with multiple reaction monitoring (MRM). The transition of the parent molecular ions *m*/*z* 463 (M-1) and 477 (M-1) to the product ions *m*/*z* 300 were used for monitoring of the two compounds and their glucuronidated metabolites, respectively. HPLC condition and plasma sample preparations were the same as described in Section 2.5, except for the mobile phase, which was 22% acetonitrile in 0.1% acetic acid. A 20% of the eluent was introduced into mass spectrometer for analysis and the other 80% was split off. Under these conditions, a detection limit of 5 ng/ml was achieved for the two compounds.

3. Results

3.1. Absorption of the intact form of IQ and HP

Representative HPLC/UV chromatograms of the rat plasma samples before and after oral administration of IQ and HP are shown in Fig. 2. The peaks of the studied compounds were identified by comparing their retention time and online UV spectra with those of the authentic samples, and further confirmed by LC/MS/MS method using MRM detection mode (Fig. 3). The results showed that glucuronidated quercetin was detected as the major form in plasma after oral administration of IQ (Fig. 4). For HP, neither its intact form nor its aglycone (quercetin, obtained after enzymatic hydrolysis by β-glucoronidase and sulfatase) [8,14] could be detected in plasma, urine or faeces by HPLC/UV. These results demonstrated that the absorption of orally administered HP and IQ are different.

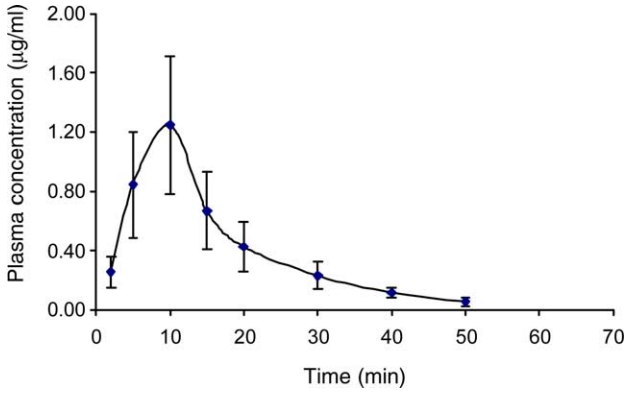

Fig. 4. Plasma concentration versus time profile of quercetin glucoronidate (expressed as isoquercitrin equivalent) in rats after oral administration at a dose of 4.5 mg/kg. Data are expressed as mean ± SD ($n = 9$).

3.2. In vitro stability of IQ and HP in GI tract

The two compounds were both stable in the buffer solutions at pH 1.2 or 6.8 at 37 °C with no significant change during the 4 h of incubation. These results indicate that there should be no chemical degradation occurred in the GI tract for these two compounds.

When incubated with GI contents, IQ and HP were found to vary in stability (Fig. 5). The two compounds were found to be stable in the stomach and relatively unstable in the small intestine and colon contents. After 30 min of incubation, IQ lost 36, 70, 96 and 100% in the duodenum, jejunum, ileum and colon contents, respectively, whereas HP only lost 2, 3, 9 and 14%, respectively, in the corresponding GI content solutions during the same incubation period.

In rat plasma at 37 °C, both IQ and HP were stable with no change during 4 h of incubation, which indicates that there might be no degradation occurred for the two compounds in the systemic circulation system.

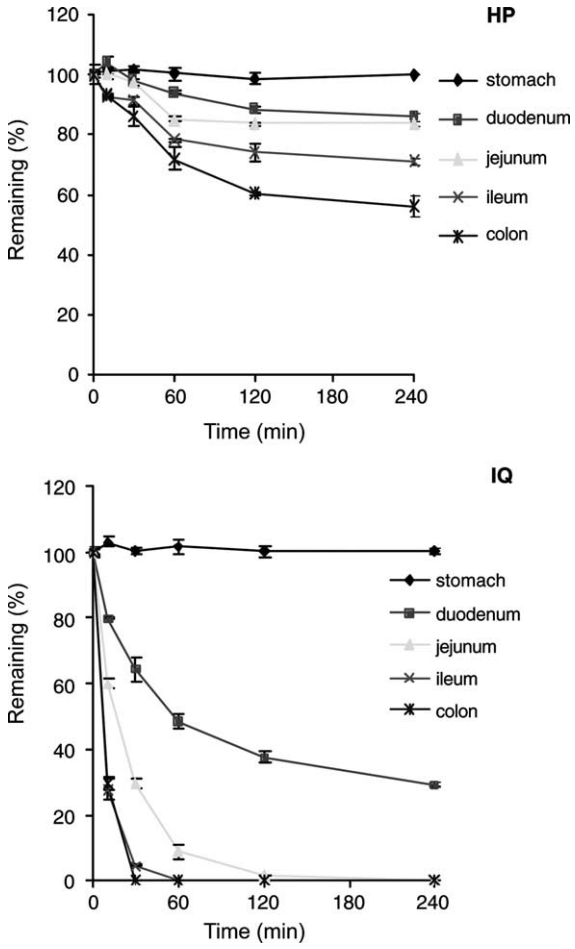

Fig. 5. In vitro stability of hyperoside (HP) and isoquercitrin (IQ) in various gastrointestinal contents including stomach, duodenum, jejunum, ileum and colon. Data are expressed as mean ± SD ($n = 3$).

4. Discussion

IQ and HP have very similar chemical structures (Fig. 1), they are quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside, respectively. However, they showed quite different way of absorption in the present study. IQ, with a glucose sugar moiety, was hydrolyzed first and then entered the systemic circulation rapidly as the quercetin glucuronide form (Figs. 3 and 4).

Using both selective ion monitoring (SIM) and multiple reaction monitoring (MRM) detection models in LC/MS/MS analysis, we were able to detect not only the intact form of IQ (m/z, 463, 463 → 300) with trace amount at retention time of 22.03 min, but also glucuronidated quercetin (m/z, 477, 477 → 300) at 23.16 min as a major detectable peak.

In contrast, HP, with a galactose sugar moiety, was not detected in its intact form by either HPLC/UV or LC/MS/MS methods. In order to clarify whether HP was absorbed into the circulation and was present in plasma as other forms such as conjugated HP, quercetin or conjugated quercetin, the rat plasma samples following oral administration of HP were analyzed for the detection of quercetin after enzymatic hydrolysis treatment. It is believed that the conjugated HP and conjugated quercetin could be changed into quercetin by enzymatic hydrolysis with combined β-glucuronidase and sulphatase. The absence of quercetin in the plasma sample indicated that HP could not be absorbed and presented neither as its conjugated metabolites nor as its aglycone (quercetin). Thus, it is suggested that HP might not be hydrolyzed easily to quercetin in the gut as IQ did or it might degrade completely into phenolic acids by intestinal bacteria in the GI tract [8].

In general, the extent of absorption of a drug is dependent on several factors including its chemical stability from the time of administration to the absorption site, its degradation by intestinal enzymes and/or bacteria, and its mechanism of absorption. To understand the effect of their stabilities in GI tract on the absorption, the chemical and bacterial degradation of IQ and HP were examined. It is found that there is no chemical degradation in the GI tract since the two flavonoid glycosides were stable in both the pH 1.2 and 6.8 buffers. However, their stabilities in various GI content solutions suggest that the degradation of the two compounds in the GI tract were mainly caused by intestinal bacteria, not by chemical instability. Comparing with HP, IQ was even more sensitive to the intestinal bacteria and/or enzymes and unstable in the GI tract. Since IQ was found to be much more absorbable as the quercetin glucuronide form than HP from the current study, it is suggested that the poor bacteria and/or enzymes hydrolysis of HP in the GI tract appears to be an important factor for the poor absorption of HP.

Comparing the differences between IQ and HP in terms of their chemical structure and stability in the GI tract, it is suggested that their difference in enzymatic hydrolysis in gut might be the major cause for their different absorption profiles and this absorption may depend on the type of sugar moiety bonded to their aglycones.

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