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ORIGINAL ARTICLE

Assessment of intestinal absorption of total flavones of *Hippophae rhamnoides L.* in rat using in situ absorption models

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Abstract

Purpose: The objective of this study was to investigate the absorption behavior of total flavones of *Hippophae rhamnoides L.* (TFH) (the sum of isorhamnetin and quercetin as the index component) in the rat intestine using in situ circulation method. *Methods*: The accumulated TFH absorption and related absorption parameters were calculated. Furthermore, the influences of Cremophor ELP and the P-glycoprotein inhibitor, verapamil, on the intestinal absorption of TFH were studied using the in situ circulation model. *Results and Discussion*: The results showed that the absorption of TFH increased linearly with its concentration, indicating that a passive diffusion process was dominated. There were no significant differences in the absorption of TFH in three small intestine segments of duodenum, jejunum, and ileum and at different concentrations of Cremophor ELP ranging from 0.25% to 1% ($P > 0.05$). With the presence of P-gp inhibitor, verapamil, in the circulation fluid, the accumulated absorption of TFH did not increase significantly ($P > 0.05$). Further studies on the solubility and permeability enhancement of TFH should be investigated to develop new TFH products with high bioavailability.

Key words: *Absorption behavior; Cremophor ELP; in situ circulation; P-glycoprotein inhibitor; total flavones of* Hippophae rhamnoides L.

Introduction

Total flavones of *Hippophae rhamnoides L.* (TFH) are extracted from a Chinese herbal medicine, Sea buckthorn[1]. It is reported that, in the high-pressure liquid chromatography (HPLC) chromatograms of TFH, 12 compounds have been identified, such as quercetin 3-*O*-glucoside, isorhamnetin 3-*O*-rutinoside, quercetin, kaempferol, isorhamnetin, and so on[2]. With their major constituents including quercetin, isorhamnetin, and kaempferol[3,4], TFH have been demonstrated with most of the bioactive properties of Sea buckthorn. Animal and human studies suggested that sea buckthorn flavonoids might have antioxidant, anti-ulcerogenic, and hepato-protective actions, which also can scavenge free radicals, lower blood viscosity, lower blood pressure, enhance cardiac function, and suppress platelet aggregation[5–7]. Despite these claimed biological activities, TFH have not been tested rigorously for their absorption in appropriate models. They are expected to have poor bioavailability because most of the flavonoids have poor bioavailabilities, which were the results of poor intrinsic permeability and efflux by apical efflux transporters such as P-glycoprotein[8–12]. In addition, TFH are poorly water soluble, which might be one of the reasons for their low and erratic oral bioavailability[13,14].

Following oral administration, dissolution of the drug molecule in the gastrointestinal (GI) milieu is a prerequisite for the absorption process. Actually our research group has prepared solid dispersions of TFH, and the dissolution enhancement of TFH was obtained by solvent method[15]. But preliminary intestinal absorption studies showed that there was no significant difference in accumulated amount of TFH between TFH group and TFH solid dispersions group. It was indicated that intestinal

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absorption characteristics of drugs are very important for oral drug delivery system. According to the Biopharmaceutical Classification System (BCS)[16] poorly water soluble compounds are classified as either class 2 or class 4 compounds. For class 2 compounds, which have high intestinal permeability properties, absorption level is dictated by the dissolution properties of the molecule in the GI fluids. BCS class 4 compounds, which are characterized by both low solubility and poor intestinal wall permeability, are generally poor drug candidates (unless the dose is very low)[17,18]. Nevertheless, information on the absorption and bioavailability of TFH is still very limited and which class TFH belongs to is not ascertained yet. And TFH preparations are marketed mainly as tablets and capsules in China. So, for these oral drug delivery formulations, a more detailed mechanistic investigation including the transport mechanisms and the transport rate is essential. Also it was warranted for developing new TFH dosage forms with high bioavailability.

There are several methods extensively used to investigate the intestinal absorption behavior of drug compounds in the form of in situ circulation method, intestinal loops, isolated mucosa, everted sacs, and Caco-2 cell lines[19–24]. The related absorption information and the factors influencing drug absorption can be obtained by using these methods properly. Among them, the in situ circulation method is carried out in anesthetized animals with the aid of the in situ rat gut perfusion technique, which normally provides very realistic absorption rate. Moreover, the in situ circulation method is considered to be the choice for absorption mechanism studies because the intestinal manipulation is minimal, the aqueous diffusion layer remains unchanged, and normal blood supply is maintained, leading to perfect sink conditions throughout the test. Furthermore, this methodology is found to be simple and highly accurate for predicting intestinal absorption in humans[25,26].

Therefore, to develop a theoretical basis for understanding the oral absorption of TFH by the main index component, isorhamnetin and quercetin, characterization of their intestinal transport mechanism and intestinal absorption is imperative. In this work, a rat in situ intestinal perfusion technique was adopted to investigate and characterize the intestinal absorption of TFH with the index component, isorhamnetin and quercetin, in different intestinal segments, at different concentrations and P-gp-mediated intestinal transport.

Materials and methods

Materials

TFH powder was obtained from Sichuan Medco Pharmaceutical (Sichuan, China). Isorhamnetin\quercetin and verapamil were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cremophor ELP was received as gift samples from BASF, Ludwigshafen, Germany. Methanol of HPLC grade was purchased from Hanbang Chemical Co. (Jiangyin, Jiangsu, China). Water was ultrafiltered through a Millipore filtration system (Milli-Q®). Ethanol and other chemicals used were of AR grade.

Analytical methods

Samples were assayed for TFH content (the sum content of isorhamnetin and quercetin) by HPLC. The HPLC system consisted of a pump (Agilent 1200 Series; Agilent Technologies Co. Ltd., Santa Clara, CA, USA) and a 250 mm long C_{18} column (Kromasil 100-5C_{18}; EKA Chemicals AB, Bohus, Sweden). The mobile phase was composed of methanol, water, and phosphoric acid at a ratio of 50:49.8:0.2. Flow rate was 1 mL/min. The oven temperature was 35°C. The sample was filtered with a 0.22-μm membrane filter and then detected at wavelength of 371 nm. The injection volume was 20 μL. The relative retention time of isorhamnetin and quercetin was about 20.3 and 10.5 minutes (Figure 1), respectively. A linear relationship between the isorhamnetin and quercetin concentration in the range of 1.225–19.6 and 0.5125–8.2 μg/mL, respectively, and the peak area was found. The calibrations were $C = 0.0107A + 0.1227$ ($R^2 = 0.9991$) and $C = 0.0116A + 0.4485$ ($R^2 = 0.9981$), respectively.

Preparation of perfusates (TFH in Krebs–Ringer fluid)

TFH is a hydrophobic natural product and insoluble in water but easily soluble in ethanol. Some proper solvents and surfactant can be applied to intestinal absorption experiment for their solubilization[27,28]. Thus, TFH should be dissolved in ethanol first and then added into Krebs–Ringer (K–R) fluid with a small amount of Cremophor ELP, which was a solubilizer for TFH. The percent of TFH/ethanol/Cremophor ELP in K–R fluid was 0.05–0.15%, 2%, 0.25–1%, respectively.

Stability of TFH in Krebs–Ringer fluid

Preliminary investigations of the process for TFH K–R fluid revealed that pH highly influenced the stability of TFH in K–R fluid. Considering the pH condition in GI, two pH values (6.8 and 7.4) were chosen for further studies. TFH K–R fluid was prepared as described above and pH was adjusted to 6.8 and 7.4, respectively. They were allowed to stand at 37°C for 5 hours. At predetermined intervals (0, 1, 2, 3, 4, and 5 hours), 1 mL samples were withdrawn from each vessel, filtered with a 0.22-μm membrane filter. The TFH content (the sum content of isorhamnetin and quercetin) in each sample was determined by HPLC as described above, and the

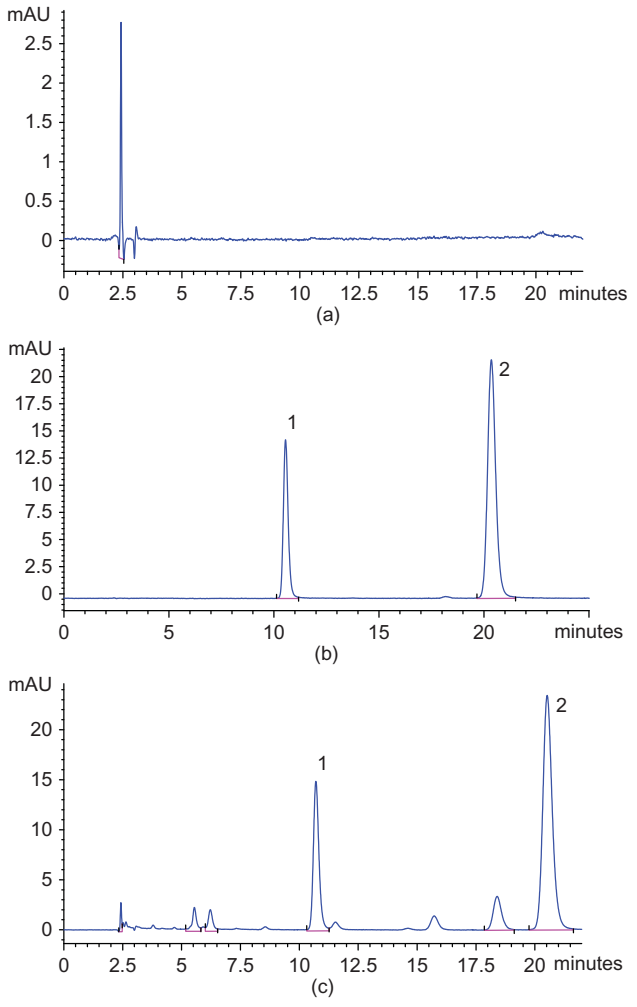

Figure 1. Chromatographs of (a) blank K–R fluid (b) reference substances (c) sample solution: (1) quercetin; (2) isorhamnetin.

stability was evaluated from the data. All experiments were carried out in triplicate.

Physical adsorption of TFH

The 10 cm long segment of each intestine region was excised and everted. The segment was tied in both ends and immersed in TFH K–R fluid of known concentrations (100 μg/mL) for 2 hours at 37°C. The intestine segment was then removed and the drug concentration was detected (C_t) and compared with the original concentration in the solution (C_0). The percentage of drug that remained in the solution was calculated from $(C_t/C_0) \times 100\%$. All experiments were carried out in triplicate.

In situ uptake experiment

Male Sprague–Dawley (SD) rats, weighing from 250 to 300 g, were supplied by Shanghai SLAC Laboratory Animal Co., Ltd. [Certificate no.: SCXK (Shanghai, China) 2008-0016]. The experiments were carried out after approval by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine. The rats were fasted for 12 hours before the experiment but were allowed free access to water. The in situ circulation method was used in these experiments. SD rats were anesthetized with 25% urethane solution (1 mg/kg). A midline abdominal incision was made and the small intestine was exposed. The bile duct was ligated to avoid bile secretion into the perfusate. The whole small intestine as one segment (from duodenum to ileum) was rinsed with normal saline at 37°C for 20 minutes until the washing appeared clear. After that, the glass tubings connected to silicone tubing were cannulated into both ends of the small intestine and secured with suture thread. Then, the small intestine was replaced in the abdomen and the cannulas were connected to a peristaltic pump. The perfusate (100 mL) was firstly perfused with a pump at 5 mL/min through the small intestine. When the perfusate appeared at the distal end of the segment, the timer was reset and the flow rate was decreased to 2.5 mL/min. The perfusate was recollected into a reservoir, which was maintained at a temperature of 37 ± 0.5°C throughout the course of an experiment. Each perfusion experiment lasted for 125 minutes, and 0.5 mL perfusion was quantitatively taken at 25-minute intervals. Then the equal volume of fresh blank K–R solution was added into the recirculation fluid. The samples were filtered through 0.22-μm membrane filters. The first 20% of the filtrate was discarded and then the total concentration of the TFH in the subsequent filtrate was analyzed by HPLC. After perfusion, the whole small intestine was excised and the area of the segment was measured.

Stability of TFH in the intestine circulation fluid

The whole small intestine was isolated and cannulated for circulation. The K–R fluid containing 0.5% of Cremophor ELP and 2% ethanol (100 mL) was recirculated through the whole intestine for 2 hours at 37°C to get the blank circulation fluid. Solutions (the pH adjusted to 6.8 and 7.4) containing TFH at the concentration of 100 μg/mL were prepared by the blank circulation fluid and allowed to stand at 37°C for 3 hours. The TFH concentrations before and after the incubation were determined by HPLC as described above, and the stability was evaluated from the data. All experiments were carried out in triplicate.

Site dependency of drug absorption

The perfusion experiment was carried out as described above. The four intestinal sections were isolated and cannulated (all were 10 cm long): the duodenum, the

jejunum, ileum, and colon, which were used to investigate the site dependency of TFH absorption. TFH solutions at the TFH concentration of 100 μg/mL were recirculated through each section of the intestine and the drug that remained in the perfusate at 2 hours was determined. The amount absorbed in each section of the intestine was compared from the data.

Effect of TFH concentration on drug absorption

Solutions containing different concentration of TFH (50/100/150 μg/mL) were prepared and were perfused through the intestine section for 125 minutes as described above. The volume of perfusate was 100 mL and the concentration of TFH (the sum content of isorhamnetin and quercetin) was determined by HPLC. The accumulated drug absorption and related absorption parameters were calculated and compared.

Effect of Cremophor ELP concentration on drug absorption

To evaluate the effect of the surfactant concentration in the circulation fluid on drug absorption, TFH solutions containing different concentrations of Cremophor ELP were recirculated through the whole small intestine segment for 125 minutes. TFH solutions (100 μg/mL) were prepared in normal saline; Cremophor ELP was then added to the solution to make a final concentration between 0.25% and 1%. After circulation, the accumulated drug absorption was calculated and compared.

Effect of P-gp inhibitor on TFH absorption

The P-gp efflux inhibitor verapamil (final concentration 10/50/100 μM) was added to the TFH (100 μg/mL) containing circulation fluid and then the fluid was circulated for 2 hours using the above-mentioned method. TFH concentration in the perfusate after circulation was determined and the amount absorbed was calculated. The difference of the drug absorbed with the presence of P-gp inhibitor was compared with that without verapamil.

Data analysis

The drugs' absorption rate constant (K_a) in the intestine was calculated as follows:

$$\ln X = \ln X_0 - K_a t \tag{1}$$

where t is the sampling time and X_0 and X are the drug's initial and remaining quantity in the perfusate, respectively. Regressing the logarithm of the drug remaining in the intestinal perfusate and sampling time, the regression coefficient is K_a.

The drugs' absorption half-life ($t_{1/2}$) in the intestine was calculated as follows:

$$t_{1/2}\,(\mathrm{h}) = \frac{0.693}{K_a} \tag{2}$$

The absorptive fraction in unit time (P%) was calculated as follows:

$$P\%\,(\mathrm{h}^{-1}) = \frac{(C_0V_0 - C_tV_t)}{C_0V_0}t \times 100\% \tag{3}$$

where C_0 and C_t are the initial and final drug concentrations in perfusate, respectively; V_0 and V_t are the initial and final volumes of perfusate; and t is the time of circulation of perfusate.

The apparent parameter of permeability (cm/s) was calculated as follows:

$$P_{app} = \frac{K_a}{A} \times 3600 \tag{4}$$

where K_a is the drugs' absorption rate constant and A is the area of intestine segment.

Statistical analysis

All data were shown as mean ± SD. The statistical difference between treatment groups was evaluated using analysis of variance (ANOVA) and the identification of significances was carried out with Student's t-test; $P < 0.05$ was considered to be statistically different.

Results and discussion

Stability of TFH in Krebs–Ringer fluid

After standing in 37°C water bath for 3 hours, the remaining amount of TFH in K–R fluid with pH 6.8 and 7.4 was 99.54 ± 1.10% and 90.14 ± 0.98% ($\bar{x} \pm \mathrm{SD}$, $n = 3$), respectively. But the remaining amount of TFH decreased to 90.66 ± 1.23% and 81.67% 5 hours later. The drug was found to be stable in K–R fluid (pH 6.8) at least for 3 hours but unstable in K–R fluid (pH 7.4) from above data. It is indicated that the pH of TFH K–R fluid probably should be adjusted to 6.8 in the following experiments.

Physical adsorption of TFH

The physical absorption and stability tests were performed to make sure that the drug disappearance from the luminal content was only because of genuine absorption. After incubation with the excised intestinal section at 37°C, the percentage of drug that remained for duodenum, jejunum, ileum, and colon was 98.32 ± 0.44%,

98.68 ± 0.31%, 98.98 ± 0.32%, and 99.59 ± 0.45%, respectively. These results indicated that the physical absorption of TFH to the lumen is negligible.

Stability of TFH in the intestine circulation fluid

Recovery of TFH from the blank circulation fluid was conducted by adding a known concentration of TFH in the medium and kept at 37°C for 3 hours. The results showed that the remaining amount of TFH in two kinds of circulation fluid (pH 6.8 and 7.4) were 100.26 ± 1.78% and 88.41 ± 0.63% ($\bar{x} \pm \mathrm{SD}$, $n = 3$), respectively. It meant that the circulation fluid (pH = 6.8) did not disturb the analysis of TFH and TFH was stable in the circulation fluid (pH 6.8) during the experimental period but not stable in the circulation fluid (pH 7.4). In other words, the pH could highly influence the stability of TFH in the intestine circulation fluid, so the pH of intestine circulation fluid should be rigidly controlled.

Site dependency of drug absorption

Figure 2 displayed the site dependency of TFH absorption. It is shown in Figure 2 that there is no significant difference in the accumulated drug absorption among duodenum, jejunum, and ileum ($P > 0.05$). The drug absorption from these three sections was much higher than that from the colon ($P < 0.01$). The results illustrated that TFH was mainly absorbed from the upper part of intestine rather than the colon and the absorption of TFH had not the specific site in the upper three intestine segments. Therefore, the whole small intestine segment was selected for the evaluation of the effect of drug concentration, Cremophor ELP concentration, and P-gp inhibitor on the absorption of TFH.

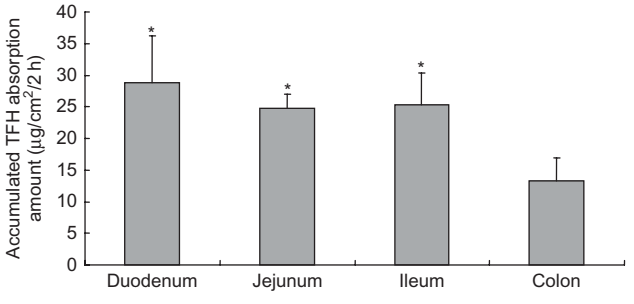

Figure 2. Site dependency of TFH absorption at intestinal segments: duodenum, jejunum, ileum, and colon. The SD of each mean value is also presented ($n = 6$ in each group). *$P < 0.01$ versus colon group. There were no significant differences in the accumulated TFH absorption amount among duodenum, jejunum, and ileum segments, but these three segments had significant differences to colon according to the one-way ANOVA.

Effect of TFH concentration on drug absorption

To examine the effect of concentration on intestinal permeability, perfusates at the concentrations of 50, 100, and 150 μg/mL were dispended and the absorptive profiles were investigated. A summary of these data is given in Table 1. The drugs' absorption rate constant (K_a) at 100 and 150 μg/mL had no statistical difference ($P > 0.05$), but had significantly statistical difference with 50 μg/mL group ($P < 0.01$). As for the P% of these three groups, the result was the same. In other words, the absorptive profiles of TFH might be independent of the concentration at high concentration (100–150 μg/mL) but were dependent of the concentration at low concentration (<100 μg/mL). The absorptive profile with these three concentrations is shown in Figure 3. It is obviously seen from Figure 3 that the absorption processes fit first-order processes.

The P_{app} values of TFH in the rat intestinal perfusion at the concentration of 50, 100, and 150 μg/mL were $(0.7497 \pm 0.1262)\times10^{-6}$, $(0.4863 \pm 0.1874)\times10^{-6}$, and $(0.3714 \pm 0.1132)\times10^{-6}$ cm/s, respectively. It is reported that compounds with an average P_{app} in rats of approximately $<0.03 \times 10^{-4}$ cm/s are poorly absorbed, whereas those with P_{app} of about $>0.2 \times 10^{-4}$ cm/s are completely absorbed[25]. On the basis of these permeability data, TFH might be classified as a low permeability class drug according to the BCS. It was reported that apple pectin could significantly enhance the intestinal absorption of quercetin, which might be attributed to alteration of the absorptive capacity of the small intestine[29]. And some quercetin prodrugs, quercetin-glutamic acid conjugate, or easter-based precursors of quercetin showed remarkable increases in water solubility and cell permeability compared with quercetin[30,31]. So permeability enhancement of TFH also needs to be considered during the design of TFH dosage forms.

Table 1. Absorption parameters of TFH in different concentrations ($n = 6$, $\bar{x} \pm \mathrm{SD}$).

C (μg/mL)	0	50	100	150
K_a (h^{-1})	Not found	0.2946 ± 0.0587	0.1683 ± 0.0393*	0.1289 ± 0.0298*
P%(h^{-1})	Not found	22.33 ± 3.63	13.86 ± 2.23*	11.38 ± 2.04*
P_{app} ($\times10^{-6}$, cm/s)	Not found	0.7497 ± 0.1262	0.4863 ± 0.1874*	0.3714 ± 0.1132*

*$P < 0.01$ versus 50 μg/mL group. The statistical evaluation of the results was performed using SPSS 13.0.

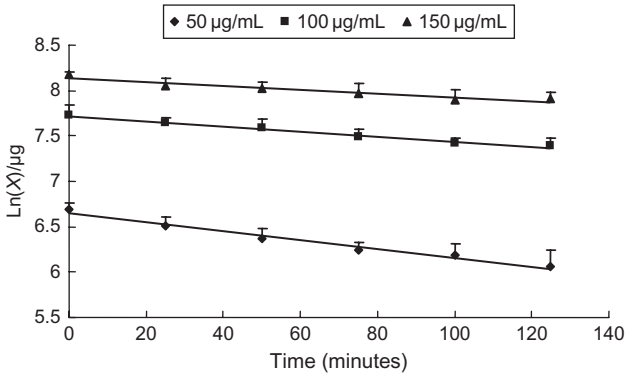

Figure 3. The absorptive rate of TFH of the rat small intestine in situ after with perfusing 125 minutes with different concentrations. Each datum represents the mean ± SD of six rats.

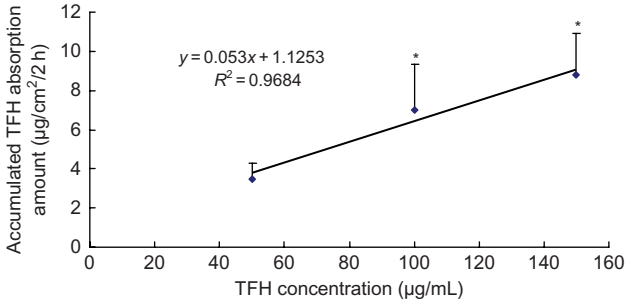

Figure 4. Accumulated TFH absorption amount at the concentrations of 50, 100, and 150 μg/mL. The SD of each mean value is also presented ($n = 6$ in each group). There were good linear relationship between the accumulated TFH absorption amount and the TFH concentration tested. *$P < 0.01$ versus 50 μg/mL group.

In this case, the accumulated drug absorption within 125 minutes increased with TFH concentrations. A linear relationship was observed between the accumulated drug absorbed and TFH concentration in the range of the concentration tested (Figure 4), which conformed to the former reports for isorhamnetin and quercetin[32,33]. The results illustrated that the transport of TFH through the whole small intestine segment at the concentration ranges from 50 to 150 μg/mL might be a passive diffusion process. In addition, the results also indicated that there is no significant difference in the accumulated drug absorption between 100 μg/mL group and 150 μg/mL group ($P > 0.05$). The accumulated drug absorption in 50 μg/mL group is highly significantly different with 100 μg/mL group and 150 μg/mL group ($P < 0.01$), which meant that the absorption mechanism might be different between high and low TFH concentration. Therefore, active transport could not be excluded at these three concentrations.

Intestinal absorption is a complex process where not all underlying mechanisms are fully understood, though the in situ technique provides the advantages of experimental control (e.g., compound concentration, pH, and osmolality), ability to study regional differences, and an intact intestinal blood supply and innervations of the animal[34]. It is likely that no single experimental method will be ideal to study absorption process and that maximum information will often require corroborative evidence from more than one method[35]. Therefore, further studies on the everted gut sac, the human Caco-2 cells (the human colon carcinoma cells), and in vivo animal experiments are in progress to verify the present results.

Effect of Cremophor ELP concentration on drug absorption

The accumulated drug absorption of TFH solution containing 0.25%, 0.5%, and 1% Cremophor ELP were 6.978 ± 2.862, 6.304 ± 1.523, and 6.787 ± 1.600 μg/cm^2/2h as shown in Figure 5, respectively. There was no statistical significance among these three groups ($P > 0.05$), indicating that the Cremophor ELP concentration ranging from 0.25% to 1% had no influence on TFH absorption.

Effect of P-gp inhibitor on TFH absorption

The accumulated amount of TFH absorption in the absence and presence of P-gp inhibitor (verapamil) with 10/50/100 μM concentration (as shown in Figure 6) was 6.978 ± 2.863, 4.621 ± 0.896, 5.080 ± 0.976, and 5.710 ± 1.772 μg/cm^2/2h, respectively. Comparing across the control group (in the absence of verapamil) and three verapamil groups, the accumulated amount of TFH in control group was higher than other groups but did

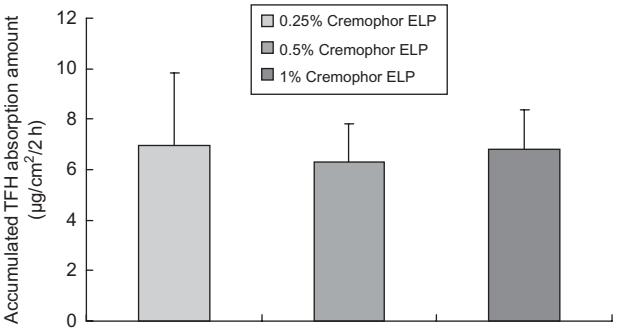

Figure 5. Accumulated TFH absorption at the Cremophor ELP concentrations of 0.25%, 0.5%, and 1%. The SD of each mean value is also presented ($n = 6$ in each group). There were no significant differences in the accumulated TFH absorption amount among the three groups according to the one-way ANOVA.

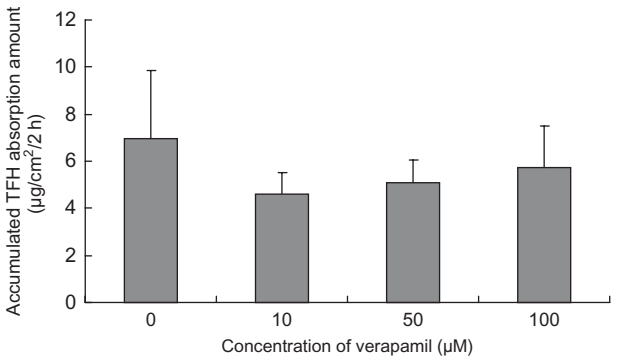

Figure 6. Accumulated TFH absorption at the verapamil concentrations of 10, 50, and 100 μM. The SD of each mean value is also presented (n = 6 in each group). There were no significant differences in the accumulated TFH absorption amount among the four groups according to the one-way ANOVA.

not reach statistical significance (P > 0.05). In these three verapamil groups, the accumulated amount of TFH was not apparently influenced with the increasing of verapamil (P > 0.05). The results indicated that the absorption of TFH at 100 μg/mL was not influenced by the addition of verapamil. Passive diffusion could be the main manner of intestinal absorption. However, active transport could not be excluded below 100 μg/mL as mentioned before. Therefore, further experiments will be carried out to examine whether active transport exist.

Furthermore, it was proven that isorhamnetin and quercetin could enhance the oral absorption of each other in rats. P-gp might play an important role in the absorptive pharmacokinetics interaction between the two components[36]. But this kind of enhancement could not be exhibited significantly in our research. The biotransformation of quercetin and isorhamnetin in rats[37] might result in counteraction of their absorption enhancement. The inter-action among active components and other attendants, TFH dose arrangement chosen in experiments, and inhibitor type also might be the possible reasons. So it is necessary to further explore the effect of P-gp on flavones absorption in herb extracts in the near future.

Conclusion

In conclusion, TFH had some absorption in duodenum, jejunum, and ileum when the perfusates were prepared with ethanol and proper surfactant. The existence of a preferential absorption zone in the small intestine for TFH can be discarded. The concentration of Cremophor ELP below 1% did not affect the accumulated TFH absorption significantly (P > 0.05). With the presence of P-gp inhibitor, verapamil, in the circulation fluid, the accumulated absorption of TFH did not increase significantly (P > 0.05). Passive membrane diffusion dominates the absorptive transport behavior of TFH. Further studies on other absorption models are in progress and the correlations are being established with data generated from different models.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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