Absorption and biotransformation of four compounds in the Guizhi decoction in the gastrointestinal tracts of rats

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Abstract

OBJECTIVE: To study the absorption and biotransformation of liquiritin, cinnamic acid, paeoniflorin, and glycyrrhizic acid in the Guizhi decoction (GZD) in the gastrointestinal tracts of rats.

METHODS: A simple and reliable high-performance liquid chromatography method was established and validated for the analysis of the four components of GZD simultaneously in the gastrointestinal tracts of rats. Rats were randomly divided into in situ gastrointestinal loop model, in vitro anaerobic culture model, and blank control groups. All rats were fasted for 12 h and anesthetized using 20% urethane. Subsequently, the abdominal cavity of each rat was opened, and the stomach, duodenum, jejunum, ileum, cecum, and colon were ligated. For the in situ gastrointestinal loop model group, 2.5 mL of GZD (1.0 g crude drug/mL, 37 °C) were injected into the gastrointestinal tract. The abdominal incision was covered with warm, wet cotton, and animals were maintained at 25 °C. Then, we collected the gastrointestinal tract content after 1.5 h. For the in vitro anaerobic culture model group, the gastrointestinal tract contents of rats were collected and then cultured in 2.5 mL of GZD in an anaerobic environment at 25 °C for 24 h. For the blank control group, rats received the same volume of a normal saline solution instead of GZD. High performance liquid chromatography was used to detect the liquiritin, cinnamic acid, paeoniflorin, and glycyrrhizic acid concentrations in each group and calculate the absorption and biotransformation rates of each ingredient.

RESULTS: Cinnamic acid (low polarity) was more easily absorbed by each gastrointestinal part than the higher-polarity glycosides. However, the absorption rate in the cecum was higher than that in other parts. The four compounds, cinnamic acid, liquiritin, paeoniflorin, and glycyrrhizic acid, were transformed completely within 24 h in the cecum and colon, whereas they were hardly transformed in the stomach, excluding glycyrrhizic acid. In addition, all ingredients had higher biotransformation rates in the distal small intestine than that in the proximal small intestine.

CONCLUSION: Although a portion of the glycosides in GZD was directly absorbed as the prototype forms in the gastrointestinal tract, they were primarily metabolized and transformed into their corresponding metabolites by intestinal flora near the distal small intestine before their absorption.
INTRODUCTION

In the practice of Traditional Chinese Medicine (TCM), medicines are usually administered as oral dosage forms, such as decoctions, pulvis, pills, and tinctures, making oral administration the major route for drug delivery of TCM prescriptions.\textsuperscript{1-3} Gastrointestinal absorption and biotransformation, which involve the transport of drugs across gastrointestinal epithelial cells into the blood circulation, are important components of pharmacokinetics studies, and they also important prerequisites for establishing the effectiveness and bioavailability of oral drugs. Knowledge of the dynamic absorption processes of active components in the digestive tract is critical for understanding the function, mechanisms, curative effects, and bioavailability of TCM.

The Guizhi decoction (GZD) is a TCM prescription recorded in On Harm Caused by Cold.\textsuperscript{4} According to pharmacodynamics research, GZD has the function of bidirectional regulation for testing body temperature, blood pressure, peristalsis, and immune function.\textsuperscript{5} Modern pharmacological research and clinical experience revealed that paeoniflorin, liquiritin, cinnamic acid, cinnamaldehyde, and glycyrrhizic acid are the major active constituents of GZD.\textsuperscript{6,7} Phenylpropanoids, including cinnamic acid and cinnamaldehyde, are the major components of Guizhi (Ramusulus Cinnamomi). Pharmacokinetic analyses illustrated that a portion of cinnamic acid was completely metabolized to cinnamaldehyde.\textsuperscript{8} The monoterpeneoid paeoniflorin is one of the major active ingredients in Baishao (Radix Paeoniae Alba), and it has been widely used as an anti-nociceptive and anti-inflammatory agent in China\textsuperscript{9} and other countries.\textsuperscript{10,11} However, the bioavailability of paeoniflorin results is low because of its poor absorption. Liquiritin and glycyrrhizic acid are flavonoids and triterpenoid saponins, respectively. They are the most abundant components Gancao (Radix Glycyrrhiza), and have anti-tumor and anti-thrombotic biological activities.\textsuperscript{12,13} In previous studies, a high-performance liquid chromatography (HPLC) method with multi-wavelength ultraviolet detection was developed to detect the components of GZD simultaneously.\textsuperscript{14} Based on this HPLC method, we determined the contents of the active ingredients of GZD. In the current study, in vivo gastrointestinal absorption and in vitro biotransformation experiments were used to compare the absorption and metabolism of four compounds in GZD quantitatively, thereby providing an important material basis for further pharmacodynamics and pharmacokinetics research in vivo.

MATERIALS AND METHODS

Chemicals and GZD preparation

Guizhi (Ramusulus Cinnamomi) 9 g, Shanyao (Rhizoma Dioscoreae Opposita) 9 g, Shengjiang (Rhizoma Zingiberis Recens) 9 g, Dazao (Fructus jujubae) 9 g, and Gancao (Radix Glycyrrhiza) 6 g were purchased from Beijing Tongrentang Pharmacy (Beijing, China) and authenticated by Beijing University of Chinese Medicine Professor Liu Changsheng (Beijing, China), with the results being in line with the standards of Pharmacopoeia of the People’s Republic of China (Edition 2015).\textsuperscript{15} The aforementioned crude drugs were prepared into a water decoction (1.0 g crude drug/mL) at concentrations of 0.51 mg/mL for liquiritin, 2.63 mg/mL for paeoniflorin, 0.17 mg/mL for cinnamic acid, and 0.95 mg/mL for glycyrrhizic acid. Cinnamic acid (110 786-201 604), paeoniflorin (110 736-201 741), liquiritin (111 610-201 106), and glycyrrhetinic acid (110 723-201 514) were purchased from National Institutes for Food and Drug Control (Beijing, China). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Hampton, NH, USA). All other chemicals involved in this research were of analytical grade. Tyrode’s solution (pH 7.2-7.4) consisted of the following components: NaCl 8.0 g/L, KCl 0.2 g/L, CaCl\textsubscript{2} 0.2 g/L, NaH\textsubscript{2}PO\textsubscript{4} 1.0 g/L, NaH\textsubscript{2}PO\textsubscript{4} 1.0 g/L, MgCl\textsubscript{2} 0.2 g/L, and glucose 1.0 g/L.

Animals

Male Sprague-Dawley rats [(300 ± 20) g] were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). They were housed under sterile conditions and 12-h/12-h light/dark cycles. The room temperature and relative humidity were maintained at (23 ± 3) °C and 50% ± 10%, respectively. The principles of laboratory animal care and all protocols were in accordance with the relevant national legislation and local guidelines, and all procedures were approved by the Animal Care and Use Committee of the Institute of Basic Theory for Chinese Medicine of China Academy of Chinese Medical Sciences (Date: August 6, 2014; No. 201408-16).

Instrumental configuration

An Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) was coupled with a quaternary pump, autosampler, and diode array detector. Water was prepared using a Mill-Q ultrapure water system (Millipore Corp., Billerica, MA, USA). ZH-Super constant temperature water bath (Beijing zhonghui tiancheng technology Co., Ltd., Beijing, China), and tabletop high-speed refrigerated centrifuge (Sigma-Aldrich, Schnelldorf, Germany) were also used.
Separation was performed using a Diamond C18 column (4.6 × 250 mm, 5 µm) with a column temperature of 35 °C. A gradient elution with water containing 0.1% phosphoric acid (a) and acetonitrile (b) was regarded as the mobile phase using the following sequence: A: B 85%: 15%, 0-31 min; and 63%: 37%, 32-54 min. The flow rate was set at 1.0 mL/min, and the injection volume was 10 µL. The elution was detected using a diode array detector for paeoniflorin, glycyrrhizic acid, liquiritin, and cinnamic acid at 250 nm. The chromatographic area of the four components was substituted into the standard curve to calculate each concentration.
**Statistical analysis**
The content of each component of GZD was considered the initial concentration in gastrointestinal absorption experiments, whereas it was considered the final concentration in the samples. The absorption rate was calculated by dividing the concentration difference between the initial and terminal doses by the initial dose. The transformation rate of each component in the *in vitro* experiment was calculated using the same method applied for the absorption rate. Data were expressed as the mean ± standard deviation (±σ) and analyzed using Statistical Product and Service Solutions 20.0 (STRONG-VINDA, Beijing, China) One-way analysis of variance and least significant difference-<i>t</i> were conducted to test the differences between groups, and <i>P</i> < 0.05 denoted statistical significance.

**Gastrointestinal absorption experiments**

In Situ Stomach Absorption: after fasting for 12 h, the rats were anesthetized *via* an intraperitoneal injection of 20% urethane (1.0 g/kg). The abdominal cavity of each animal was opened, and the stomach was exposed. Both the cardia and pylorus were ligated after injecting 2.5 mL of GZD (37 °C). Then, the stomach was returned to the abdominal cavity, which was covered with warm, wet cotton, and each animal was maintained at an ambient temperature of approximately 25 °C. After 1.5 h, the stomach was removed again, and the liquid medicine inside it was added to a graduated cylinder with 50 mL. The internal surface of the stomach was flushed with K-R buffer repeatedly. Then, the liquid medicine and buffer solution were mixed together and diluted with K-R buffer to a volume of 50 mL. For the blank control group, GZD was replaced with the same volume of a normal saline solution. Intestinal Loop Absorption: after fasting for 12 h, the rats were anesthetized *via* an intraperitoneal injection of 20% urethane (1.0 g/kg). The abdominal cavity of each rat was opened along the mediolateral line. The two ends of the duodenum, jejunum, ileum, cecum, and colon were ligated and injected with 2.5 mL of GZD (37 °C), after which they were returned to the abdominal cavity. The incision was covered with warm, wet cotton, and the animals were maintained at an ambient temperature of approximately 25 °C. After 1.5 h, the intestinal tract of each animal was removed again, and the liquid medicine from each tract was exported to a graduated cylinder with 50 mL. The internal surface of the stomach was flushed several times with a small amount of K-R buffer. Then, the liquid medicine and buffer solution were mixed together and diluted with K-R buffer to a volume of 50 mL.

**In vitro biotransformation experiments**

After fasting for 12 h, the rats were sacrificed, and the abdominal cavity of each animal was opened immediately. The gastrointestinal tract (stomach, duodenum, jejunum, ileum, and colon) was quickly ligated from both two ends. Then, the content in these tissues were collected separately. After adding 2.5 mL of GZD (37 °C) to the content of each component of the gastrointestinal tract, the tissues were cultured in an anaerobic environment at 37 °C. After 24 h, the mixture was removed, and a 3-fold excess of a methanol solution was added to stop the reaction.

**Sample preparation**

A tabletop high-speed refrigerated centrifuge was used to centrifuge the samples at 11180 × g for 20 min (4 °C). Then, the supernatant was filtered using a 0.45 µm filter membrane. Next, 10 µL of filtrate were injected into the HPLC system for analysis.

**RESULTS**

**Method validation**

We previously established an HPLC method for detecting several components of GZD simultaneously. The experimental results illustrated that this method has favorable precision, repeatability, and recovery rates for determining the contents of the samples accurately. Figure 1 presents the typical chromatograms of a mixed standard substance containing liquiritin (212.5 ng/mL), cinnamic acid (187.5 ng/mL), paoniflorin (417.6 ng/mL), and glycyrrhizic acid (217.5 ng/mL), as well as various gastrointestinal sections in the blank rat content sample and a content sample after injecting GZD into the gastrointestinal tract at different sites. In this experiment, all chromatograms were detected at a wavelength of 250 nm, and the results revealed that no endogenous components interfered with component detection.

**Absorption rates**

HPLC was used to determine the concentrations of the four examined components of GZD in the gastrointestinal tracts of rats, and their absorption rates were calculated, as shown in Table 1. The order of absorption rates of liquiritin in the tissues were cecum > jejunum > colon, duodenum, and stomach. The compound was digested and absorbed completely in the cecum. That for paoniflorin was cecum and ileum > colon > stomach and duodenum > jejunum, whereas that for glycyrrhizic acid was cecum > stomach > jejunum, ileum, and colon. Glycyrrhizic acid was hardly absorbed in the duodenum. However, cinnamic acid was completely digested and absorbed in the intestine. These results illustrated that the absorption of cinnamic acid, which has low polarity, was better throughout the gastrointestinal tract, whereas the high-polarity glycosides liquiritin, paoniflorin, and glycyrrhizic acid were better absorbed in the cecum (Figure 2).

**Transformation rates**

The transformation rates of liquiritin, cinnamic acid,
Figure 2 Absorption of the four compounds in the rat stomach (stom.), duodenum (duod.), jejunum (jeju.), ileum (ileu.), cecum (cecu.), and colon (colo.)
A: liquiritin; B: cinnamic acid; C: paeoniflorin; D: glycyrrhizic acid.

Table 2 Transformation rates of the four compounds (% ± s)

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Stomach</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon</th>
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<tbody>
<tr>
<td>Liquiritin</td>
<td>10</td>
<td>4.625±0.207</td>
<td>8.537±6.348</td>
<td>6.448±1.005</td>
<td>99428±0.514</td>
<td>98937±0.822</td>
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<tr>
<td>Cinnamic acid</td>
<td>10</td>
<td>1.535±0.217</td>
<td>33.427±5.025</td>
<td>20.004±2.305</td>
<td>99.889±0.002</td>
<td>99.921±0.018</td>
<td>99.934±0.036</td>
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<tr>
<td>Paeoniflorin</td>
<td>10</td>
<td>3.428±0.924</td>
<td>2.365±0.424</td>
<td>2.485±0.992</td>
<td>9.928±1.308</td>
<td>99.788±0.022</td>
<td>99.922±0.001</td>
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<tr>
<td>Glycyrrhizic acid</td>
<td>10</td>
<td>40.427±1.417</td>
<td>0.433±0.105</td>
<td>4.358±1.138</td>
<td>2.823±0.501</td>
<td>96.71±3.13</td>
<td>97.915±0.983</td>
</tr>
</tbody>
</table>

Notes: *P < 0.05, versus the rate in the stomach; †P < 0.05, versus the rate in the duodenum; ‡P < 0.05, versus the rate in the jejunum; ‡‡P < 0.05, versus the rate in the ileum.

paeaniflorin, and glycyrrhizic acid in GZD were obtained via anaerobic culturing for 24 h in vitro, and the rates are presented in Table 2. The results indicated that all compounds were completely transformed in the cecum and colon within 24 h. Liquiritin, cinnamic acid, and paeoniflorin were hardly metabolized in the stomach. The transformation rates of the four compounds were lower in the proximal small intestine than in the distal small intestine (Figure 3).

**DISCUSSION**

In this study, the intestinal loop method was adopted to explore the absorption of GZD in different parts of the gastrointestinal tract in rats. In the early stage of the experiment, we found that liquiritin, paeoniflorin, and glycyrrhizic acid were hardly excreted in their original forms in urine and feces. Small amounts of liquiritin, paeoniflorin, and glycyrrhizic acid were absorbed into blood in their original forms after the oral administration of GZD, but the compounds were largely metabolized by intestinal flora before absorption. The experimental results demonstrated cinnamic acid was mostly absorbed as a prototype form that could be detected in portal vein blood, which might be related to its low polarity and the proximal location in the gastrointestinal tract. However, the results of in vitro tests revealed that cinnamic acid was not significantly transformed after reaching the distal small intestine. It has been reported that cinnamic acid, which has higher bioavailability, was absorbed quickly into blood and then excreted in urine mainly as hippuric acid after the intragastric administration of Guizhi (Ramulus Cinnamomi). According to previous studies, the absorption rates of paeoniflorin and glycyrrhizic acid in the intestinal tract were extremely low, which resulted in lower...
bioavailability. In fact, pharmacological activity was produced by their metabolites rather than their prototype forms. Paeonimetabolone I was the most abundant metabolite of paeoniflorin when cultured with bacteria in human excrement. At present, a variety of paeoniflorin-related metabolites can be detected in rat blood, and studies have examined the in vivo pharmacokinetics of the metabolites in urine, such as paeonimetabolin I, which have important pharmacodynamic effects in the body.

As a major component of Gancao (Radix Glycyrrhiza), glycyrrhizic acid can be mainly metabolized to glycyrrhetinic acid by intestinal flora, whereas a small proportion can be metabolized to 18β-glycyrrhetinic acid and subsequently hydrolyzed to 3-epi-18β-glycyrrhetinic acid as the intermediate products. However, its final metabolite is still glycyrrhetinic acid, which has strong pharmacological activity, and its content in vivo increases with increasing metabolic time.

In conclusion, our findings suggest that although a small portion of the glycosides in GZD was directly absorbed as their prototype forms in the gastrointestinal tract, they were primarily metabolized and transformed into the corresponding products by intestinal flora near the distal small intestine before their absorption into blood.

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