Absorption characteristics of the total alkaloids from Mahonia bealei in an in situ single-pass intestinal perfusion assay

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Available online 20 July 2014

[ABSTRACT]
AIM: To investigate the absorption characteristics of the total alkaloids from Mahoniae Caulis (TAMC) through the administration of monoterpene absorption enhancers or protein inhibitors.

METHOD: The absorption behavior was investigated in an in situ single-pass intestinal perfusion (SPIP) assay in rats.

RESULTS: The intestinal absorption of TAMC was much more than that of a single compound or a mixture of compounds (jatrorrhizine, palmatine, and berberine). Promotion of absorption by the bicyclic monoterpenoids (borneol or camphor) was higher than by the monocyclic monoterpenes (menthol or menthone), and promotion by compounds with a hydroxyl group (borneol or menthol) was higher than those with a carbonyl group (camphor or menthone). The apparent permeability coefficient (Papp) of TAMC was increased to 1.8-fold by verapamil, while it was reduced to one half by thiamine. The absorption rate constant (Ka) and Papp of TAMC were unchanged by probenecid and pantoprazole.

CONCLUSION: The intestinal absorption characteristics of TAMC might be passive transport, and the intestinum tenue was the best absorptive site. In addition, TAMC might be likely a substrate of P-glycoprotein (P-gp) and organic cation transporters (OCT), rather than multidrug resistance protein (MRP) and breast cancer resistance protein (BCRP). Compared with a single compound and a mixture of compounds, TAMC was able to be absorbed in the blood circulation effectively.

[KEY WORDS] Mahoniae Caulis; Total alkaloids; In situ single-pass intestinal perfusion; Absorption enhancers; Protein inhibitors

[CLC Number] R969.1

Introduction
Mahoniae Caulis, as an important traditional Chinese medicine (TCM), has been officially listed in the Chinese Pharmacopoeia (2010 Edition) as Mahonia bealei (Fortune) Carrière and Mahonia fortunei (Lindl.) Fedde (Berberidaceae)
no literature for the simultaneous determination of berberine, palmatine, and jatrorrhizine in perfusates. Therefore, little information is available about the absorption parameters of TAMC.

Accordingly, the present study was carried out to investigate the intestinal absorptive characteristics of TAMC in the presence of absorption enhancers and protein inhibitors separately in an in situ SPIP, determining jatrorrhizine, palmatine, and berberine simultaneously in the perfusate by high-performance liquid chromatography (HPLC). The absorption-enhancing agents were the monoterpenoids borneol, camphor, menthol, and menthone (Fig. 2). In addition, verapamil [7], probenecid [8], pantoprazole [9], and thiamine [10] were employed as protein inhibitors of P-gp, MRP, BCRP, and OCTN, respectively. This research provided predictive information regarding a compatibility mechanism of TAMC for the first time.

Materials and Methods

Animals

Male SD rats (200 ± 20 g) were obtained from the Experimental Animal Center of Suzhou University, Suzhou, China. The animals were housed under standard conditions of light and dark cycles with food and water ad libitum in China Pharmaceutical University (Nanjing, China). All experiments were approved by the Animal Ethics Committee of China Pharmaceutical University, and every effort was made to minimize stress to the animals.

Apparatus

The HPLC analysis was carried out using an Agilent 1100 series HPLC instrument (USA) with a Hedera ODS-2 column (250 mm × 4.6 mm, 5 μm) and a HL-2 peristaltic pump (Shanghai HuXi Analysis Instrument Factory Co, Shanghai, China).

Chemicals and reagents

The reference standards, such as berberine (purity ≥ 98.0%), palmatine (purity ≥ 98.0%), and jatrorrhizine (purity ≥ 98.0%) were all purchased from the Jiangsu Institute for Food and Drug Control (Nanjing, China). Methanol and acetonitrile were of chromatographic grade (Merck Company Inc, Germany). Borneol, camphor, menthol, and menthone were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Verapamil, probenecid, pantoprazole, and thiamine were bought from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

Krebs–Ringer (K–R) buffer solution

The K-R buffer solution consisted of 7.8 g NaCl, 0.35 g KCl, 1.37 g NaHCO3, 0.02 g MgCl2, 0.32 g NaH2PO4, 0.37 g CaCl2, and 1.40 g glucose dissolved in 1 000 mL of distilled water.

Preparation of TAMC

Mahoniae Caulis, collected from Yunnan Province (China), were dried in an oven at 60 °C and ground before reflux extraction with 70% ethanol for 4 h. It was identified by the author and a herbarium sample was deposited in the State Key Laboratory of Natural Products and Functions, China Pharmaceutical University. The total content of jatrorrhizine, palmatine, and berberine in the extract was determined to be 61.32% (28.07% of jatrorrhizine, 17.48% of palmatine, and 15.77% of berberine) by HPLC after purifying by macroporous resin.

Preparation of drug solutions

The TAMC was dissolved in the K-R buffer solution to prepare different concentrations of test solutions during the in situ SPIP. Borneol, camphor, menthone, and menthol were dissolved separately in the minimum amount of ethanol to obtain a solution and diluted with K-R buffer solution containing TAMC, and the amount of ethanol was 0.5% in the final perfusion solutions. Verapamil, probenecid, pantoprazole, and thiamine were directly dissolved in the solution of TAMC.

In situ SPIP of rats

The surgical procedures were carried out using an experimental design adapted from the literature with some modifications [11-12]. Rats were fasted for 12 h and divided into different experimental groups with access to water freely before the perfusion experiment. Under anesthesia, a gentle midline incision was made on the abdomen after placing the animals on a plate to maintain them at 37 °C. In advance, the body temperature of the rats was maintained throughout the experiment by an overhead lamp. The desired intestinal segment of approximately 10 cm was carefully pulled out and small cuts made at the beginning and the end. Then, silicone
tubes were inserted into the two ends and attached to a peristaltic pump. Initially, the intestinal segments were gently flushed with saline (37 °C) until the effluent was clear. The inlet tubing was then perfused with K-R buffer solution for approximately 20 min for balance. Secondly, the drug solution was introduced into the circulation at 0.2 mL·min⁻¹ for 30 min in order to assure steady state conditions. Subsequently, six samples were collected from the outlet of the intestine and weighed using pre-weighed tubes at intervals of 15 min. Then, the samples were weighed and centrifuged (4 500 r·min⁻¹ for 10 min), and the supernatant was filtered through a 0.45 μm Millipore filter and an assay of drug content performed using established HPLC methods. During the experiment, the exposed area was covered with a piece of sterilized gauze to keep it warm and moist by frequent application of warm (37 °C) saline. The intestinum tenue maintained its viability and intact state without disrupting the blood supply throughout the experimental period. Finally, the animals were sacrificed by cervical dislocation before measuring the length of the intestinal segment at the end of the experiment. During the experiment, the drug amount and the total volume of perfusate were changing at all times. Therefore, the gravimetric method was adopted in the study for adjustment.

\[ Ka (s^{-1}) \] and \[ P_{app} (cm \cdot s^{-1}) \] and accumulated amount of TAMC (\[ \sum_k \mu_k \]) were calculated by using the corrected mathematical expression as follows.

\[
Ka = \left(1 - \frac{\rho_{out}V_{out}}{\rho_{in}V_{in}}\right) \frac{V}{\pi r^2 L}
\]

\[
P_{app} = -\ln \left(\frac{\rho_{out}V_{out}}{\rho_{in}V_{in}}\right) \frac{2\pi r L}{a_2}
\]

\[
\sum_{k=1}^{6} a_k = (\rho_{in}V_{in} - \rho_{out}V_{out}) k
\]

Where \( \rho_{out} \) and \( \rho_{in} \) indicated the concentration of TAMC tested in the perfusate at the outlet and the inlet, respectively (μg·mL⁻¹). \( V_{in} \) and \( V_{out} \) were the inlet and outlet volume separately, which were needed to adjust liquid density (mL·min⁻¹) by weighing the contents (using an electronic weighing balance) of a known volume of perfusate (using a micropipette). \( r \) was the radius of the intestinal lumen (cm). \( L \) was the length of the segment perfused (cm). \( v \) was the flow rate of the entering solution (0.2 mL·min⁻¹). \( a_2 \) was the content of absorption at a certain time (μg).

Study for the stability of TAMC

It is essential to ensure that the loss of drug from the perfusates is due to absorption and no other losses (e.g. metabolism, chemical degradation, or adsorption on tubes, and so forth) before carrying out the experiment of SPIP in rats. The tubes and the intestinal wall were not affected by the adsorption of TAMC. In addition, the drug was detected to be stable in the perfusion fluid at different pH and blank intestinal perfusates at 37 °C for 3 h.

Study of the absorption of TAMC in different intestinal segments in situ SPIP in rats

Four intestinal segments of each rat, duodenum (2 cm from proximal end of the pyloric), jejunum (15 cm from the pyloric), ileum (up-bound 10 cm proximal to ileocecal valve) and colon (proximal to ileocecal valve descending), were performed to study the absorption of TAMC to test whether the intestinal absorption of drugs exhibited site-dependent changes. Hence, four intestinal segments for perfusion were simultaneously conducted to determine the best site of TAMC absorption in the intestinal tract at 19.36 μg·mL⁻¹.

Study for the absorption of TAMC at different concentrations in an in situ SPIP in rats

With reference to the human daily dose of TAMC, the dose of the drug was converted from the recommended clinical dose according to the China Pharmacopoeia (2010 Edition). The concentrations of drug solution were settled at 9.68, 19.36, and 48.4 μg·mL⁻¹ in the duodenum segments (~10 cm) in the experiments.

Study for the absorption of TAMC, single compound, and a mixture compounds in an in situ SPIP in rats

Jatrorrhizine, palmatine, berberine, and a mixture of the three monomers were dissolved in K-R buffer solution separately, the mixture of the three monomer content and proportion were brought into correspondence with TAMC. TAMC, at the concentration of 19.36 μg·mL⁻¹, was chosen as the control group to calculate \( Ka \) and \( P_{app} \).

Effects of absorption enhancers on absorption of TAMC in an in situ SPIP in rats

Four absorption enhancers, borneol (50 μg·mL⁻¹), camphor (50 μg·mL⁻¹), menthone (50 μg·mL⁻¹), and menthol (50 μg·mL⁻¹), were researched on the intestinal absorptive characters of TAMC in situ SPIP of rats. The rat duodenum (~10 cm) was used to evaluate the permeability in the presence of these absorption-promoting agents.

Effects of membrane proteins on absorption of TAMC in situ SPIP in rats

Membrane proteins play a major role in affecting drug absorption and oral bioavailability. P-gp, MRP, BCRP, and OCT are the representative proteins in intestinal epithelial cells. Therefore, membrane protein inhibitors were directly added into the perfusate to evaluate their different effects on TAMC in the experiment. K-R buffer solution with verapamil (100 μmol·L⁻¹, P-gp inhibitor), probenecid (100 μmol·L⁻¹, MRP inhibitor), pantoprazole (100 μmol·L⁻¹, BCRP inhibitor), or thiamine (100 μmol·L⁻¹, OCTN inhibitor) was perfused at 0.2 mL·min⁻¹ separately.

Statistical analysis

The statistical analysis was conducted by SPSS version 11.5 software, and all the data were expressed as \( \bar{x} \pm s \). Two-tailed Student’s \( t \)-test was employed for a two-group comparison. \( * \) \( P < 0.05 \) was considered as different, and ** \( P < 0.01 \) was considered as significant difference.
Results

The stability of TAMC

The content of different concentrations of TAMC was unchanged in the in situ SPIP in rats. This illustrated that no drug had been attached to the intestinal wall and the tubes in the experiments (data not shown). Moreover, there was no statistically significant difference between the samples at 0 h and 3 h in perfusion fluid of different pH and blank perfusates at 37 °C. Therefore, the stability of TAMC was not affected by external factors.

Site-dependence of TAMC absorption in an in situ SPIP of rats

The absorption parameters of TAMC in four intestinal segments are illustrated in Fig. 2. It was demonstrated that the order of Ka for TAMC was duodenum = jejunum = ileum > colon; the same as the order of Papp for TAMC. There were no obvious differences among duodenum, jejunum, and ileum for absorption parameters (P > 0.05). Accordingly, the duodenum was chosen as absorptive segment in the following experiments.

Concentration-dependence of TAMC absorption in an in situ SPIP of rats

Fig. 4 (A) Ka of TAMC, single compound, and mixture compounds in an in situ SPIP in rats. *P < 0.05 vs single compound. **P < 0.01 vs single compound. #P < 0.05 vs mixture compounds

Concentration-dependence of TAMC absorption in an in situ SPIP in rats

Table 1 Absorption parameters of TAMC at different concentrations (T ± s, n = 6)

<table>
<thead>
<tr>
<th>Concentration (μg·mL⁻¹)</th>
<th>Ka × 10⁶ (s⁻¹)</th>
<th>Papp × 10⁻⁵ (cm·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.68</td>
<td>1.41 ± 0.43</td>
<td>9.74 ± 1.42</td>
</tr>
<tr>
<td>19.36</td>
<td>1.43 ± 0.44</td>
<td>9.64 ± 3.10</td>
</tr>
<tr>
<td>48.40</td>
<td>1.46 ± 0.37</td>
<td>9.89 ± 2.60</td>
</tr>
</tbody>
</table>

TAMC, single compound and mixture compounds absorption in an in situ SPIP in rats

As shown in Fig. 3, the intestinal absorption parameters of TAMC were much higher than a single compound and a mixture of compounds. It can be speculated that the absorption of TAMC was promoted by other unknown ingredients.

Effects of absorption enhancers on TAMC in an in situ SPIP in rats

The accumulated amount of TAMC is shown in Fig. 4 in the presence or absence of absorption enhancers. The results showed that the cumulative absorption amount of TAMC was significantly enhanced by borneol (P < 0.01) and menthol (P < 0.05). However, neither camphor nor menthone had absorption-enhancing ability; in fact they hindered the absorption of TAMC. Meanwhile, the Papp of TAMC was increased more than 2.8- and 1.6-fold by borneol and menthol, respectively. Table 2 also shows a 1.3- and 2.0-fold depression in Papp of TAMC after co-administration with camphor and menthone, respectively. These results suggested that
borneol and menthol were able to enhance the absorption of TAMC, while camphor and menthone restrained the absorption of TAMC.

**Effects of membrane proteins on TAMC in an in situ SPIP in rats**

Verapamil, probenecid, pantoprazole, and thiamine were studied for their effects on the intestinal absorption characteristics of TAMC in an in situ SPIP in rats separately. The accumulated absorption amount of TAMC is shown in Fig. 5 in the presence or absence of membrane proteins. As shown in Table 2, Papp of TAMC was increased 1.8-fold by verapamil, while it was reduced to one half by thiamine in comparison with TAMC. However, the Ka and Papp of TAMC were essentially unchanged by probenecid and pantoprazole (Table 2). These results implicated that the membrane proteins affected the intestinal absorption behaviors of TAMC differently.

**Discussion**

There are main three mechanistic pathways for drug transport, passive transport, active transport, and membrane mobile transport. As shown in Table 1, the results indicated that the intestinal absorption of TAMC might be passive transport, and it is one of the main ways of drug absorption. Based on a comparison to TAMC and single compound with mixture compounds absorption in an in situ SPIP in rats, it could be speculated that the absorption of TAMC was promoted by other unknown ingredients.

In **in vitro** (e.g. the rat everted gut sac method or Caco-2 cell model) and **in vivo** (e.g. in situ SPIP in rat or intestinal loop) models are used to study the intestinal absorption of drugs. In **in situ SPIP** offers a simple and relevant method of permeability assessment for the true absorption properties in humans. The animal’s blood supply is kept intact in this method, thus the results would be more consistent with the **in vivo** situation than other techniques. It is the best model to mimic the **in vivo** environment, and the drugs can be continuously absorbed and quickly distributed to other tissues and organs at a sink condition. It is a common method for the assessment of drug absorption transport.

It is evident that absorption investigations are very important and necessary to understand the absorption processes from oral administration in different regions of the intestinal tract. In this study, it was concluded that TAMC was segmental-dependent and the upper intestine was a much better absorption site of TAMC than the large intestine. However, the results were dependent on the interplay between the physicochemical properties of the drug and the environment of the intestinal tract. The physicochemical properties include solubility, degree of dissociation, lipotropy, molecular size, and so on. The environment of the intestinal tract includes mucosal blood flow, unstirred water layer, membrane proteins, enteroenzyme, etc.

Different physiologic factors, including the membrane of slime layer, unstirred water layer, and tight junctions between cells affect the intestinal permeability of drugs. In this research, four natural absorption-promoting agents, borneol, camphor, menthone, and menthol, were added into perfusates separately. The cumulative absorption amount of TAMC was significantly improved by borneol (P < 0.01) or menthol (P < 0.05). Several mechanisms have been involved for the enhancement of absorption, including (a) increasing the membrane permeability of hydrophilic drugs, (b) inhibiting...
P-gp-mediated drug efflux, (c) accelerating the fluidity of phospholipid bilayer of cell membranes, (d) affecting the proteins in the membranes, and (f) loosening the intercellular tight junction. Because borneol and menthol are monoterpenic enhancers with a hydroxyl, it suggests that they may have similar mechanisms. Whereas, the cumulative absorption amount of TAMC declined either in the presence of camphor or menthone. It was presumed that the reasons why they restrained the absorption of TAMC were (a) combining with drugs by a special chemical bond which rendered them too big to pass along the path in the cell membranes, (b) blocking the drugs through some channels, and so on. Therefore, it is necessary to study the exact mechanism in the future. These findings indicated that the four absorption enhancers had different effects on TAMC even though they were all monoterpenes. The promotion of absorption by the bicyclic monoterpenoids was higher than by the monocyclic monoterpenes. The compound with hydroxyl could enhance absorption, while those with a carbonyl inhibited absorption.

In recent years, P-gp, MRP, and BCRP, which are mainly membrane proteins in ATP-binding cassette (ABC) transporter proteins, were mostly investigated as membrane protein transporters in the intestine. These transporters are expressed at the apical membrane of the intestine, and have the function of secreting drugs (if the drugs are ABC transporter protein substrates) into the intestinal lumen. Consequently, the absorption of drugs administered orally can be increased by inhibiting the function of ABC [23-24]. In this research, Papp and Ka of TAMC had been increased by P-gp inhibitor verapamil, which illustrated that TAMC was a substrate of P-gp. Therefore, it was possible to enhance the absorption and oral bioavailability for TAMC by inhibiting the P-gp-mediated drug efflux. Moreover, probenecid and pantoprazole did not affect the Ka and Papp of TAMC, which illustrated that TAMC was not a substrate of MRP and BCRP.

Organic cation transporters (OCT) are expressed in the tissues, which are related to absorption (e.g. lung and intestine), metabolism, and elimination (e.g. liver and kidney). Accordingly, these efflux transporters are able to transport cationic drugs into blood circulation [25-27]. Intestinal absorption of TAMC declined with the addition of thiamine compared with TAMC, which illustrated that TAMC was a substrate of OCT.

TCM have been attracting the attention of scientists as a result of its long time clinical efficacy and reliable therapeutic actions. However, there has been little information about pharmacokinetic research of Mahonia Caulis. The main active constituents of the plant are protoberberine alkaloids (PBAs), including berberine, palmatine, and jatrorrhizine. Meanwhile, most pharmacokinetic studies were conducted on the single alkaloid berberine, rather than the total alkaloids containing berberine [28-29]. In these studies, it was found that the absorption of TAMC was higher than the single compounds. Although the mechanisms of enhanced absorption have not been elucidated, unknown ingredients might play an important role in promoting absorption. There were several mechanisms for their enhancement of absorption, including improving the membrane permeability of target components in the gastrointestinal tract, inhibiting efflux transporters like P-gp, opening ion channels, accelerating the fluidity of cell membranes, loosening the intercellular tight junction, increasing solvent drag capabilities, and so on. The stronger intestinal absorption of TAMC reflected a holistic view and the compatibility of TCM.

Conclusions

In summary, the results showed that (a) the intestinum tenue was the major segment of absorption, (b) passive transport was the main absorption pattern, (c) the absorption of TAMC was faster than a single compound or a mixture of compounds, (d) the absorption of TAMC was enhanced by borneol and menthol, but inhibited by camphor and menthone, (e) TAMC might likely be a substrate of P-gp and OCT, rather than MRP and BCRP. The results suggested that enhancements of intestinal absorption of TAMC were probably due to inhibition of the P-gp-mediated drug efflux, modulating OCT-mediated transport, and altering membrane fluidity.

References

[10] Lemos C, Faria A, Meireles M, et al. Thiamine is a substrate of


