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Absorptive profile of chlorogenic acid in rats

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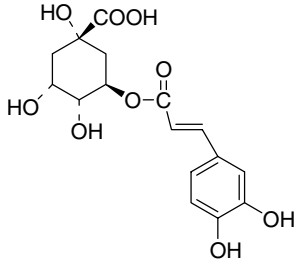
The objective of this work was to systematically investigate the absorptive profile of chlorogenic acid (CGA) in rats and to increase its intestinal absorption using absorption enhancers. The rat *in situ* intestinal perfusion technique was used to examine absorptive rate and extent of CGA. *In vitro* enzymatic drug degradation study was carried out with rat intestinal washings. Various experimental conditions such as different concentrations, extraction purity, and different absorption enhancers such as sodium taurocholate (ST), sodium lauryl sulphate (SLS), carbomer, borneol were examined. Absorption rate constants were determined by the plot of log remaining amount of drug in perfusate vs. time. During the *in vitro* enzymatic degradation study, no measurable degradation was observed. It was found that CGA could be poorly absorbed by the gastrointestinal tract. The absorptive rate of CGA is independent of the drug concentration. Absorption processes fit first order processes. Extraction of high purity is benefit to the absorption of CGA. Drug absorption extent could be increased via absorption enhancers except SLS. ST appeared to be more effective for enhancing the intestinal absorption of CGA than the other absorption enhancers. *In vivo* studies were carried out to compare the pharmacokinetic parameters of solutions with and without ST. The order of increasing CGA absorption caused by the enhancers was ST $>$ carbomer $>$ borneol $>$ SLS.

1. Introduction

Lonicerae flos, a commonly used traditional Chinese medicine (TCM), is highly prized in TCM practice for its treatment of sores, carbuncles, furuncles, swelling and affections caused by exopathogenic wind-heat or epidemic febrile diseases at the early stage (Pharmacopoeia of the People's Republic of China 2005). Chlorogenic acid (CGA), as a major phenolic compound of *Lonicerae flos*, is generally used as a marker to characterize the drug owing to its high content (no less than 1.5%) (Pharmacopoeia of the People's Republic of China 2005) and its wide range of biological activity. It can suppress the reverse transcription and polymerase chain reaction steps (Singh and Singh 1998), inhibit hepatic glucose 6-phosphatase (Arion et al. 1997), as well as modify plasma and liver concentrations of cholesterol, triacylglycerol, and minerals (Sotillo and Hadley 1997). It also plays an important role in the anti-oxidant (Salvi et al. 2001), anti-inflammatory (Miceli and Taviano 2005), anticancer (Jin et al. 2005) activity. In previous work, CGA was studied in many ways, including the separation and purification (Lu et al. 2004), the oxidative metabolism (Santos et al. 2005), the hydrolysis (Asther et al. 2005), pharmacokinetics in rat by i.v. administration (Tsai et al. 2000), etc. In TCM practice, the peroral route is considered to be the most convenient way for drug application. However, no information on the absorptive profile of CGA through the gastrointestinal tract has been reported. No method is reported to promote its absorption.

In this work, a rat *in situ* intestinal perfusion technique (Schurgers et al. 1986) was adopted to examine the uptake of CGA in the main absorption segments, including gastric and small intestine, and the change of their absorption at different concentrations and different extract purity from *Lonicerae flos*. Another objective of this study was to investigate the change of permeability when some absorptive promoters are co-administered. In addition, the results obtained from the *in situ* absorption study of CGA were employed to *in vivo* rat absorption.



Chlorogenic acid

2. Investigations, results and discussion

2.1. Drug absorption studies

CGA is a hydrophilic natural product and easily soluble in water. Its solubility in water at 25 °C is 4%. Thus, CGA solution in the Krebs-Ringer fluid and the artificial

gastric fluid (20, 40, 80 $\mu g \cdot ml^{-1}$) was used in the experiment. In control experiments performed without animals, drug loss by absorption onto or absorption into the tubes of the perfusion system was found to be insignificant. Therefore, this loss was not taken into account for data processing.

The drug was found to be stable to the enzyme of the rat gastrointestinal tract for at least 180 min as shown in Fig. 1.

The absorptive fraction in unit time (P %) of CGA in gastric region and small intestine was $7.7 \pm 1.2\%$ and $7.9 \pm 2.2\%$ ($\bar{x} \pm s$, $n = 5$), respectively. It was shown that P % of two segments was less than 8% and had no significant difference. CGA is an organic acid and the acidic environment of artificial gastric fluid ($pH = 1.2$) could inhibit the dissociation of CGA and raise the ratio of molecular form. Therefore, the liposolubility could be raised and the amount of transmembrane transport should be increased. But P % in the gastric region was no more than that in the small intestine. The reasons may be as follows. Gastric region does not have villi and microvilli like the intestinal wall, which can significantly increase the absorptive area. In addition, some drug transport carriers may exist on the intestinal wall. Thus CGA could be absorbed throughout the main digestive tract, but the amount was relatively poor. In order to improve the gastrointestinal absorption of CGA, various absorption enhancers were studied (discussed below).

To examine the effect of concentration on intestinal permeability, perfusates at the concentrations of 20, 40, 80 $\mu g \cdot ml^{-1}$ 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 these three concentrations had no statistical difference ($P > 0.05$), and the P % at these three concentrations also had no statistical difference ($P > 0.05$). In other words, the absorptive profiles of CGA were independent of the concentration. Absorption processes fit first order processes. Passive diffusion could be the main manner of intestinal absorption. Active transport could not be excluded at these three concentrations. However, if active transport exists, the carrier should not be completely occupied. Further experiments will be carried out to examine whether active transport exist.

CGA is a highly hydrophilic substance, so its K_a in the digestive tracts is relatively low. Their absorptive half-life ($t_{1/2}$) is more than 10 h. If it was orally administered, its bioavailability would be definitely low. To improve systemic bioavailability through gastrointestinal administration, two strategies are commonly employed.

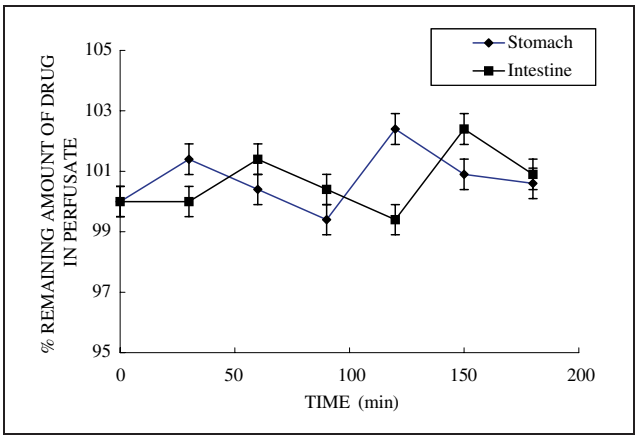


Fig. 1: Drug stability in perfusate ($n = 5$)

Table 1: Absorption parameters of CGA in different concentrations ($n = 5$, $\bar{x} \pm s$)

Concentration ($\mu g \cdot ml^{-1}$)	K_a (h^{-1})	$t_{1/2}$ (h)	P (%)
20	0.0521 ± 0.0132	13.85 ± 2.85	7.70 ± 3.39
40	0.0525 ± 0.0043	13.18 ± 1.08	7.96 ± 2.20
80	0.0472 ± 0.0055	14.84 ± 1.79	5.54 ± 0.64

Table 2: Absorption parameters of CGA in different purity Extraction ($n = 5$, $\bar{x} \pm s$)

Purity (%)	K_a (h^{-1})	$t_{1/2}$ (h)	P (%)
5.50	0.0341 ± 0.0057	20.82 ± 3.66	3.68 ± 0.86
40.25	0.0338 ± 0.0023	20.60 ± 1.38	3.56 ± 0.62
99.8	0.0525 ± 0.0043	13.18 ± 1.08	7.96 ± 2.20

One is to prolong the retention time in the digestive tract and the other is to enhance the permeability of the luminal wall.

The parameters of extraction of different purity from *Lonicerae flos* are given in Table 2. The K_a of 99.8% purity was obviously higher than that of the other two groups ($P < 0.05$). The K_a of 40.25% and 5.50% purity had no statistical difference ($P > 0.05$). As for the P % of these three groups, the result was the same.

CGA is the main polyphenolic compound in *Lonicerae flos* and usually co-existed with various ingredients in extractions. The results suggested that there might be some impurities in lower purity extraction that could decrease CGA absorption. Thus, extraction of higher purity should be chosen in order to improve CGA availability.

2.2. Effect of absorption enhancers on intestinal absorption of CGA

In case of pH 7.4 intestinal perfusate, only 8% of the drug was absorbed into the small intestine. In order to improve the intestinal absorption of CGA, various absorption enhancers such as sodium taurocholate (ST), sodium lauryl sulphate (SLS), carbomer, borneol, were studied. The absorptive rate of drugs was measured. K_a and P % calculated for each additive is given in Table 3, and the contrast of the absorptive profile is shown in Fig. 2. According to Table 3, the K_a and P % of CGA co-administered with SLS had no statistical difference with those in reference group ($P > 0.05$). The K_a of CGA co-administered with ST had significant increase, and the K_a had a positive correlation with the concentration of ST ($P < 0.05$); as for the P %, a positive correlation was also obtained ($P < 0.05$). The K_a of CGA solution containing carbomer was greater than that in the reference group ($P < 0.05$); the same P % result was obtained ($P < 0.05$). The K_a and P % of CGA co-administered with borneol were also greater than that

Table 3: Absorption parameters of CGA co-administered with absorption enhancer ($n = 5$, $\bar{x} \pm s$)

Absorption enhancer	K_a (h^{-1})	$t_{1/2}$ (h)	P (%)
Control	0.0525 ± 0.0043	13.18 ± 1.08	7.96 ± 2.20
0.5% ST	0.0926 ± 0.0128	7.59 ± 0.95	13.38 ± 1.68
1.0% ST	0.1527 ± 0.0118	4.56 ± 0.36	14.50 ± 3.22
SLS	0.0550 ± 0.0042	12.67 ± 0.96	8.01 ± 1.60
Carbomer	0.1311 ± 0.0198	5.41 ± 1.00	8.97 ± 2.80
Borneol	0.0834 ± 0.0072	8.36 ± 0.71	10.27 ± 2.23

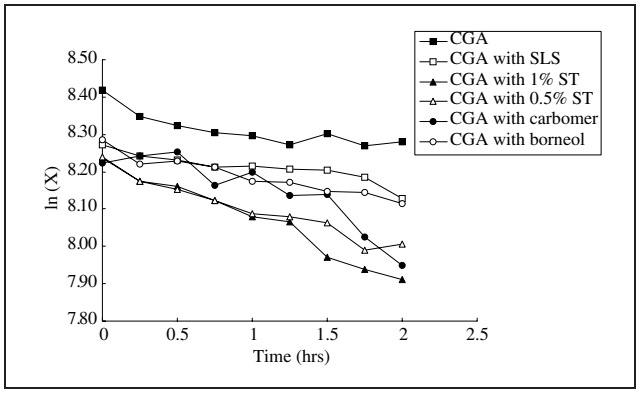


Fig. 2: The absorptive rate of chlorogenic acid co-administered with different absorption enhancer (n = 5). CGA: chlorogenic acid; SLS: sodium lauryl sulphate; ST: Sodium taurocholate

in the reference group ($P < 0.05$). The order of increasing absorption of CGA caused by the enhancers was ST > carbomer > borneol > SLS.

2.3. *In vivo studies*

In vivo studies were carried out for CGA co-administered with 1.0% ST in rats. After administration of oral solutions, the drug was observed to achieve plasma level rapidly. After 17 min, plasma concentrations of 9 $\mu g \cdot ml^{-1}$ were observed. However, in case of CGA solution without enhancer, a plasma concentration of 2.4 $\mu g \cdot ml^{-1}$ was achieved in 23 min. T_{max} values for solutions with and without 1.0% ST were 17 and 23 min, respectively, as shown in Fig. 3. K_a value for oral solution with enhancer was 0.08 min^{-1} and for oral solution without enhancer was 0.07 min^{-1}. Plasma half-lives ($T_{1/2\beta}$) for oral solutions with and without enhancer were 205 and 231 min, respectively. AUC values for oral solutions with and without enhancer were 858 and 316 $\mu g \cdot ml^{-1} \cdot min$. A significant difference between the products was found for the pharmacokinetics parameters of C_{max}, t_{max} and AUC. However, between the subjects the difference was no significant indicating that there was little subject-to-subject variation.

Our present data demonstrate that absorption enhancers especially ST were effective in enhancing the oral absorption of CGA. Hersey et al. suggested that bile salts might enhance the permeation by removing epithelial cells, which constitute a major permeability barrier (Hersey and Jackson 1987). It was also reported that bile salts interact with cell membrane to form reverse micelles, which act as transmembrane channels or mobile carriers to increase permeation of the test compound (Gordon et al. 1985).

The mechanism of carbomer is to conjugate with the calcium ion existing at the tight junction in the membrane, resulting in increase permeability of the paracellular route. In addition, the bioadhesiveness of carbomer is of great benefit to facilitate absorption (Borchard et al. 1996).

Borneol is often used in TCM to promote absorption. Generally it can activate phospholipase and increase cyclohexanehexol triphosphate. Thus, Ca^{2+} in intracellular fluid could be liberated and the tight junction dependent on the concentration of Ca^{2+} could be opened up. The opening of the tight junction and the increase of cellular shunt transport are the important reasons for the enhancement of the low absorptive rate of CGA.

SLS is reported to enhance the permeability by dissolving intestinal mucosa, liberating protein and phospholipid, and by decreasing the activity of lactate dehydrogenase and mitochondrial lactate dehydrogenase (NerurKar et al. 1996). It also can increase the solubility of drugs. However, solubilization of surfactant on the hydrophilic drug is weak. A prolonged contact with surfactant could result in degeneration of intestinal mucosa, interference with blood circulation, and contraction of intestinal wall, which could hinder instead of promote drug absorption (TaneKaz 1975). Thus, in our study, SLS cannot enhance the absorption of CGA.

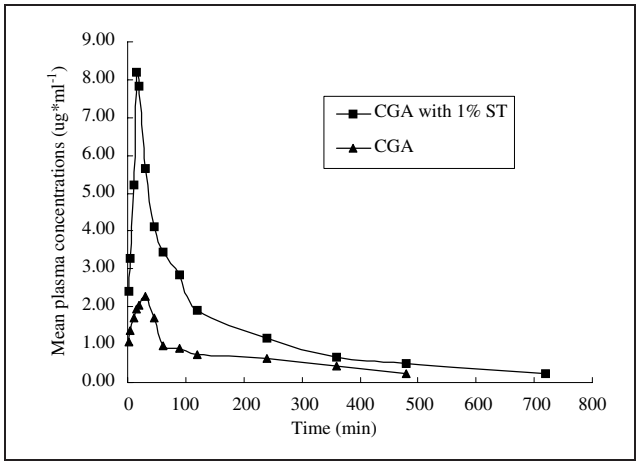


Fig. 3: Mean plasma concentration of chlorogenic acid from the solutions with and without 1% sodium taurocholate (n = 5). CGA: chlorogenic acid; ST: sodium taurocholate

3. Experimental

3.1. *Materials*

Standard CGA was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P. R. China). Extracts from *Lonicerae flos* (containing 99.8%, 40.2%, 5.5% chlorogenic acid, respectively) were gifts from Chenguang Research Institute of Chemical Engineering (Chendu, China). ST, SLS, carbomer, borneol were of analytical grade and obtained from Chendu Reagent Company (Chendu, China). Phenolsulfonphthalein (PSP) was purchased from Shandong Research Institute of Chemical agent (Shandong, China). HPLC grade acetonitrile was obtained from FisherChemicals, New Jersey, USA. Water was prepared in ultra pure water system (UPA, Chongqi, China). All other chemicals used in the study were of analytical grade at least.

The Sichuan University Animal Ethical Experimentation Committee, according to the requirement of the National Act on the Use of Experimental Animal (People's Republic of China), approved all procedures of the animal study. Male Wistar rats (180–220 g) were obtained from the Laboratory Animal Center at West China School of medicine, Sichuan University.

3.2. *Preparation of perfusates*

CGA, in different concentrations and purity, was measured accurately and dissolved in Krebs-Ringer buffer (intestinal perfusate, pH 7.4) or artificial gastric juice (gastric perfusate, pH 1.2), then transferred into a 100 ml volumetric flask. Prior to the experiment, PSP was added as a nonabsorbable marker to monitor water transport across the intestinal segment.

As for absorption enhancers, optimum concentrations of absorption enhancers were used for the present study. CGA with different absorption enhancers, including 0.5% and 1.0% ST, 0.05% carbomer, 0.012% borneol and 0.02% SLS, was dissolved into the Krebs-Ringer buffer to obtain desired concentration.

3.3. *Animal studies*

The rat *in situ* intestinal perfusion experiment was performed using recirculating perfusion (Schurgers et al. 1986). Male Wistar rats, weighing about 180–220 g, were anesthetized with intraperitoneal injection of sodium pentobarbital solution (40 $mg \cdot kg^{-1}$) following overnight fasting. Then the rat was placed on a flat plate under a heating pad to maintain body temperature throughout the experiment. A midline abdominal incision was made to expose the small intestine, with care being taken not to interfere with blood flow. After small intestine was rinsed with isotonic saline (37 °C), the glass tubings connected to silicone tubing were cannulated into both ends of the small intestine and secured with suture thread. Then, the intes-

tine 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^{-1} 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^{-1}. The perfusate was recollected into a reservoir, which was maintained at a temperature of 37 ± 0.5 °C throughout the course of an experiment. The effluent perfusate samples were collected quantitatively at predetermined time interval. Meanwhile, the PSP blank solution (20 μg · ml^{-1} PSP; intestinal perfusate without CGA) was added quantitatively into the perfusate. Each perfusion experiment lasted for 120 min.

The rat *in situ* gastric perfusion experiment was performed using the closed loop method. The rat was prepared as mentioned above. Firstly, the stomach was exposed and the incision was made at each end. After the stomach was rinsed clearly with artificial gastric juice (37 °C), the cardiac orifice was ligated by suture thread. Then, the gastric perfusate (4 ml) was injected into the stomach followed by the ligation of the pyloric orifice. The stomach was kept in the abdomen throughout the experiment. Each experiment lasted for 120 min.

Separate experiments were conducted to measure the loss of analyte from the solution due to adsorption by system components (tubing, pump or glasswares) or due to volatilization. Each perfusion solution was circulated for 120 min through the system without the rat included. The reservoirs were sampled at 30 min intervals to measure disappearance of analyte with time.

3.4. In vitro enzymatic degradation study with rat gastrointestinal washing

Isotonic Krebs-Ringer buffer without drug, pH 7.4, was perfused through the rat small intestine for 3 h. The perfusate at the end of the experiment was collected for further use. For stomach, artificial gastric juice was used instead to inspect the influence of enzyme in the gastric region. A certain amount of CGA stock solution was mixed with solutions above and vortexed. Then the mixture was incubated at 37 °C, and 100 μl samples were withdrawn at predetermined time intervals. The drug's stability is represented with its remained percentages after the experiment.

3.5. Analytical procedure

The concentration of CGA was determined by reversed phase HPLC, and all the samples were filtered through a 0.45 μm membrane filter. An amount of 20 μl of each filtrate was injected into a HPLC column (Dikma Diamonsil^{m} C_{18} column, 5 μm, 150 × 4.6 mm) for immediate assay. The signal was monitored at 322 nm. Mobile phase prepared with acetonitrile and phosphate buffer (pH 3.0, adjusted by glacial acetic acid) (10 : 90, v/v). The flow rate was maintained at 1 ml · min^{-1}.

3.6. Data analysis

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

$$\ln X = \ln X_0 - K_a \cdot t \quad (1)$$

t is the sampling time, X_0 and X are the drug's initial and remaining quantity in the perfusate, respectively. Regression 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:

$$t_{1/2}\,(h) = 0.693/K_a \quad (2)$$

The Absorptive Fraction in Unit Time (P %) was calculated as follows:

$$P\,\%\,(h^{-1}) = (C_0V_0 - C_tV_t)/C_0V_0 \cdot t \cdot 100\% \quad (3)$$

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

The analysis of variance (ANOVA) was adopted to evaluate the sum of variances about absorptive parameters among multiple groups. If statistical difference exists, multiple comparison tests were employed to evaluate the difference between any two groups.

3.7. In vivo studies

Bioavailability studies were conducted in a group of ten rats divided randomly into two subgroups of five each. Each group received the extract from *Lonicerae flos* (containing 99.8% CGA) and that with enhancer chosen from the *in situ* experiment for comparative studies. The rats were weighed and the dose to be administered was calculated.

The controlled group received CGA solution (0.2 μg · kg^{-1}) by intragastric administration. The tested group received the same oral solution containing 1% ST.

Blood was collected from the jugular vein in heparinized centrifuge tubes at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, 360, 480, 720 min after administration. The samples were centrifuged immediately and plasma was stored at −20 °C until the time of analysis. To 100 μl of plasma sample, 20 μl internal standard solution (puerarin, 100 μg · ml^{-1}) and 20 μl methanol-phosphoric acid (0.2%) (20 : 80) was added in a stopper test tube. This was mixed with 100 μl trichloroacetic acid (10% solution) and then vortexed for 2 min to precipitate proteins. After centrifugation at 10000 × g for 10 min, the supernatant was analyzed using HPLC. The mobile phase consisted of acetonitrile/phosphate buffer (pH 3.0) at a flow rate of 1.0 ml · min^{-1}. The analysis was done at a wavelength of 322 nm. The parameters employed to evaluate were C_{max}, t_{max} and AUC values. AUC and other parameters such as K_a and plasma half-lives ($t_{1/2\beta}$) were computed from observed plasma concentration against time profile.

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