Comparative stomach tissue distribution profiles of four major bio-active components of Radix Astragali in normal and gastric ulcer mice

Xiaohua Liu¹, Zhiyuan Xue¹, Bo Wang¹,², Ying Wang¹, Mengting Zhang¹, Shilan Feng*¹⁴

¹School of Pharmacy, Lanzhou University, PR China, ²Gansu Entry-Exit Inspection and Quarantine Bureau Central Laboratory of Technical Center, P.R. of China

Numerous studies have demonstrated that Radix Astragali can inhibit gastric ulcers in mice. Anhydrous ethanol (0.01 mL/g) administered to mice by intragastric infusion can induce gastric ulcer injury. This study was performed to compare the stomach tissue distribution profiles of four major bioactive constituents of Radix Astragali (calycosin-7-O-β-d-glucoside, calycosin, ononin and formononetin) after oral administration of extract of Radix Astragali (ERA) in normal and gastric ulcer mice. The abundance of Radix Astragali constituents was determined using an ultra-pressure liquid chromatograph with a photodiode array detector (UPLC-PDA), after which histograms were drawn. In comparison with normal mice, the contents of calycosin-7-O-β-d-glucoside, calycosin, ononin and formononetin in the stomach tissue samples of gastric ulcer mice showed significant differences at the selected time points (P < 0.05). The abundance of each of the four tested constituents in the normal groups was higher than that of the gastric ulcer groups. This study provides an empirical foundation for future studies focused on developing clinical applications of Radix Astragali.

Keywords: Gastric ulcer mice. Radix Astragali. Tissue distribution. UPLC.

INTRODUCTION

Tissue distribution studies are vital for understanding the major target sites of phytochemicals and interpreting their disposition invivo (Chang et al., 2012). Considering the beneficial effects of certain phytochemicals on human health, detailed invivo disposition studies of phytochemicals used in traditional Chinese medicine are crucial (Chang et al., 2012). Calycosin-7-O-beta-d-glucoside, ononin, calycosin, and formononetin are major bio-active constituents of Radix Astragali (Qi et al., 2006; Huang et al., 2009; Singh et al., 2010; Chen et al., 2011; Gu, Wang, Fawcett, 2004). Two reports demonstrated that calycosin-7-O-beta-d-glucoside, ononin, calycosin, and formononetin contribute to the strong anti-gastric ulcer activity of Radix Astragali (Liu et al., 2014a; Liu et al., 2012), while other studies assessed the pharmacokinetics and tissue distributions of these compounds in rats (Liu et al., 2014a; Liu et al., 2014b; Liu et al., 2015).

However, previous experiments assessing the bio-active constituents of Radix Astragali have focused on normal animals and excluded pathological models. Many disease conditions can alter drug tissue distribution patterns and pharmacokinetics (Shi et al., 2014a; Shi et al., 2014b; Tian et al., 2010). Few studies have assessed the tissue distribution profiles of the bio-active constituents of Radix Astragali in animals afflicted with gastric ulcers in comparison with normal animals. Studying differences in the stomach distribution of the bio-active constituents of Radix Astragali in normal mice and gastric ulcer mice could reveal the mechanism of action by which Radix Astragali produces an anti-gastric ulcer effect.

Therefore, this study was conducted to compare the stomach tissue distribution profiles of four major...
bio-active constituents of Radix *Astragali*, two pairs of flavonoid glycosides (calycosin-7-O-beta-d-glucoside and ononin) and their corresponding aglucones (calycosin and formononetin), in normal and gastric ulcer mice after oral administration of extract of Radix *Astragali* (ERA).

**MATERIAL AND METHODS**

**Chemicals and reagents**

*Astragalus propinquus* were collected from Min County, Gansu Province, China. Voucher specimens (No.2017-0708) were identified by Dr. Zhigang Ma (Department of Pharmacognosy, Lanzhou University, Lanzhou, China) and deposited at the Institute of Traditional Chinese Medicine and Natural Medicines, School of Pharmacy, Lanzhou University, China.

Calycosin-7-O-beta-d-glucoside (111920), calycosin (111530), formononetin (111703) and hesperidin (110721, internal standard, IS) were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Ononin was purchased from Shanghai Sunzo Biotech Co., Ltd. The purity of each compound was determined to be higher than 98% by normalization of the peak area detected with high-performance liquid chromatography (HPLC-Waters 2695).

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Distilled water was further purified using a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals, including methanol, were of analytical grade. All solvents and samples were filtered through a 0.22-µm filter membrane before injection into the UPLC(Waters H Class).

**Instruments and analytical conditions**

Chromatographic separations were performed using a Waters ACQUITY™ UPLC equipped with a Fluoro-Phenyl column (2.1 × 50 mm, 1.7 µm). The mobile phases consisted of 0.3% (v/v) formic acid in water (A) and acetonitrile (B). A gradient elution was performed as follows: 20–40% (v/v) B for 0–3 min, followed by 40–64% B for 3–6 min (flow rate, 0.3 mL/min). An aliquot of 10 µL of each sample was injected into the instrument. The run time was 6 min (Liu *et al.*, 2014a; Liu *et al.*, 2014b; Liu *et al.*, 2015).

**Animals**

Kunming mice (18–22 g) were obtained from the Laboratory Animal Center of Lanzhou University (Lanzhou, China, SCXK(gan)-2013-0002). The animals were allowed limited food and unlimited water for 48 h before the experiment. The animals were maintained at an ambient temperature of 22–25 °C with relative humidity of 60%. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of Lanzhou University (Lanzhou, China), and the protocol was approved by the Animal Ethics Committee of that institution.

**Preparation of the extract of plant material**

About 300 g of the plant material was extracted three times with 95% alcohol for 1h each time. The alcohol was recycled under reduced pressure in a rotary evaporator at 60 °C, and the resulting material was dried and stored at -20 °C. The extract suspension was made by combining the dried plant material, water and 0.3% polysorbate -80.

**Tissue distribution study**

Normal and gastric ulcer mice (males and females) were divided into four groups after oral administration of ERA (4.0 g/kg) for 7 days.

Anhydrous ethanol (0.01 mL/g) administered by intragastric infusion can induce gastric ulcer injury (Liu *et al.*, 2012; Liu *et al.*, 2014b). Various stomach tissue samples were collected at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 12, and 24 h after the final ERA administration. Tissues samples were rinsed with normal saline solution to remove contaminants, blotted with paper towels, and stored at -80 °C.
Determination of the concentration of four compounds in ERA

To calculate the administered doses of each compound, their concentrations in *Astragalus membranaceus* from Min County in Gansu Province were quantitatively determined (Liu et al., 2014a). The abundance of calycosin-7-O-beta-d-glucoside, ononin, calycosin and formononetin in *Astragalus membranaceus* was 52.5, 17.10, 2.88 and 1.90 mg/g, respectively.

Preparation of standard and quality control samples

The stock standard solution, which contained a mixture of calycosin-7-O-beta-d-glucoside (6.50 μg/mL), ononin (2.06 μg/mL), calycosin (2.00 μg/mL), and formononetin (1.16 μg/mL), was prepared in methanol. The IS stock solution (0.29 mg/mL) was also prepared in methanol. The IS concentration was 0.029 μg/µL in each working solution and quality control samples. Calibration standards for the stomach tissue samples were prepared by spiking 200 mg of blank tissue; QC samples at three concentrations were prepared in the same manner. Calibration tissue samples were prepared by spiking aliquots of the stock solutions into drug-free blank stomach tissue samples to obtain final concentrations in the range of 6.5–6500 ng/mL for calycosin-7-O-beta-d-glucoside, 1.03–2060 ng/mL for ononin, 4.00–2000 ng/mL for calycosin, and 5.80–1160 ng/mL for formononetin. Quality control (QC) samples were also prepared in the same way (6.5, 650, and 6500 ng/mL for calycosin-7-O-beta-d-glucoside; 1.03, 103, and 2060 ng/mL for ononin; 4.00, 400, and 2000 ng/mL for calycosin; 5.8, 580, and 1160 ng/mL for formononetin). Standard calibration and QC samples were stored at -20 °C. All solutions were stored at 4 °C. For the standard curve, the ratios of the areas of the chromatographic peaks (analytes/IS) as ordinate variables were plotted versus the compound concentration along the abscissa.

Sample preparation

For tissue samples, 20 μL of the IS solution and 5.0 mL of methanol were added to small slices of tissue, which were subsequently homogenized using an IKA T25 homogenizer. The tissue homogenates were centrifuged at 14,000 × g for 10 min, after which the supernatant was transferred to another tube and evaporated to dryness in a water bath at 45 °C under a nitrogen stream. The residues were reconstituted in 200-μL aliquots of the UPLC mobile phase and centrifuged at 14,000 × g for 10 min at 4 °C. The supernatants (10 μL) were injected into the UPLC system.

RESULTS AND DISCUSSION

Conditions for UPLC-PDA

Under the optimized chromatographic conditions, satisfactory retention times were obtained for the analytes and IS by UPLC. Calycosin-7-O-beta-d-glucoside, calycosin, ononin, formononetin, and the IS were successfully separated within 6 min in the standard mixture, as well as in the samples, which revealed that the separation method had high detection sensitivity and analysis efficiency. Figure 1 shows the chromatograms from the quantification of calycosin-7-O-beta-d-glucoside, calycosin, ononin, formononetin, and the IS.

Method validation

Specificity and Linearity and Lower Limit of Quantitation (LLOQ)

No interference was observed at the selected retention times for either the analytes or the IS in the stomach tissue homogenate samples in Figure 1. The UPLC method exhibited good specificity.

The linear regression equation, correlation coefficient and LLOQ of calycosin-7-O-beta-d-glucoside, calycosin, ononin, and formononetin are shown in Table I.
FIGURE 1 - Chromatograms of UPLC (254nm)1-sample chromatogram of stomach tissue homogenate in mice, 2-chromatogram of calycosin-7-O-<beta>-D-glucoside 3-chromatogram of ononin, 4-chromatogram of calycosin, 5-chromatogram of formononetin, 6-chromatogram of the herbs in Radix Astragali P1- calycosin-7-O-<beta>-D-glucoside, P2 - ononin, P3- calycosin, P4- formononetin

TABLE I - Standard curves, linear ranges, correlation coefficients and lower limit of quantification of four compounds in biological samples

| Components                        | Calibration curve | \( r^2 \) | Linear range (ng) | LLOQ (ng) |
|-----------------------------------|-------------------|---------|------------------|-----------|
| Calycosin-7-O-<beta>-D-glucoside  | \( Y=1.535X-21.87 \) | 0.9928  | 6.50-6500        | 6.50      |
| Ononin                            | \( Y=9.195X-21.76 \) | 0.9955  | 1.03-2060        | 1.03      |
| Calycosin                         | \( Y=1.387X-2.68 \)   | 0.9931  | 4.00-2000        | 4.00      |
| Formononetin                      | \( Y=16.840X-60.93 \) | 0.9963  | 5.80-1160        | 5.80      |

Precision and accuracy

The method showed good precision and accuracy with good intra-day and inter-day precision. All results were within the accepted variable limits shown in Table II.

TABLE II - Precision and accuracy of four compounds in the low, middle and high QC samples

| Bio-samples                        | Intra-day(n=6) | Inter-day(n=3) |
|------------------------------------|----------------|----------------|
|                                    | Precision (RSD%) | Accuracy (mean%) | Precision (RSD%) | Accuracy (mean%) |
| Calycosin-7-O-<beta>-D-glucoside  | 65             | 95.16          | 4.07            | 103.77          |
| 650                                | 3.56           | 104.36         | 10.32           | 93.48           |
| 6500                               | 9.04           | 92.69          | 4.33            | 95.09           |
(continues on the next page...)
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Extraction recovery and matrix effect

The extraction recoveries and matrix effects of calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin in mouse stomach matrices are shown in Table III.

**TABLE III** - Extraction recovery and matrix effect of four compounds in stomach tissue homogenates of mice

| Bio-samples | Concentration (ng·mL⁻¹) | Intra-day(n=6) | Inter-day(n=3) |
|-------------|-------------------------|----------------|----------------|
|             |                         | Precision (RSD%) | Accuracy (mean%) | Precision (RSD%) | Accuracy (mean%) |
| Ononin      | 1.03                    | 9.98            | 101.16          | 9.11            | 97.32           |
|             | 103                     | 4.32            | 94.37           | 5.44            | 103.96          |
|             | 2060                    | 5.93            | 108.37          | 8.69            | 91.08           |
| Calycosin   | 4.00                    | 4.98            | 91.19           | 8.29            | 98.30           |
|             | 400                     | 9.98            | 93.14           | 8.86            | 99.04           |
|             | 2000                    | 5.86            | 97.25           | 9.06            | 92.31           |
| Formononinet| 5.80                    | 6.69            | 90.49           | 2.01            | 95.66           |
|             | 580                     | 7.17            | 100.43          | 9.01            | 92.88           |
|             | 1160                    | 11.49           | 96.73           | 7.18            | 99.32           |

Stability

The stability of the method was determined under different conditions using low, moderate, and high concentrations of compounds. Short-term stability was tested after storing the samples at room temperature for 12 h or at 4 °C for 24 h. Freeze-thaw stability was tested by freezing the samples at -80 °C overnight and then thawing them at 20 °C, for a total of four cycles. Long-term stability was tested after storing the samples at -80 °C for 1 month.

Calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin were stable in mouse stomach tissue for at least four freeze/thaw cycles (Table IV). Moreover, all investigated compounds were stable for at least 4 weeks at -80 °C.
| Condition       | Compounds                          | Spiked (ng·mL⁻¹) | Found   | Average percentage | RE (%) |
|-----------------|------------------------------------|-----------------|---------|--------------------|--------|
| Ambient, 12h    | calycosin-7-O-β-D-glucoside        | 6.50            | 6.50±0.2| 100                | 0.1    |
|                 |                                    | 650             | 647±1.5 | 98                 | -1.5   |
|                 |                                    | 6500            | 6489±2.2| 99                 | -0.5   |
|                 | ononin                             | 1.03            | 1.05±1.3| 101                | 0.7    |
|                 |                                    | 103             | 101±0.8 | 96                 | -1.2   |
|                 |                                    | 2060            | 2057±1.4| 100                | -3.4   |
|                 | calycosin                          | 4.00            | 3.98±0.9| 97                 | -0.4   |
|                 |                                    | 400             | 395±1.6 | 96                 | -0.9   |
|                 |                                    | 2000            | 1998±1.2| 97                 | -1.5   |
| -20°C, 30 days  | calycosin-7-O-β-D-glucoside        | 6.50            | 6.56±0.5| 99                 | 1.3    |
|                 |                                    | 650             | 653±1.5 | 98                 | 1.5    |
|                 |                                    | 6500            | 6494±2.6| 96                 | -2.5   |
|                 | ononin                             | 1.03            | 1.06±2.3| 101                | 1.3    |
|                 |                                    | 103             | 100±2.6 | 97                 | -1.1   |
|                 |                                    | 2060            | 2052±2.8| 96                 | -3.7   |
|                 | calycosin                          | 4.00            | 4.02±3.1| 99                 | 0.6    |
|                 |                                    | 400             | 396±1.7 | 98                 | -0.6   |
|                 |                                    | 2000            | 1999±0.4| 100                | -0.2   |
|                 | formononetin                       | 5.80            | 5.76±0.8| 96                 | -2.3   |
|                 |                                    | 580             | 583±1.9 | 99                 | 3.4    |
|                 |                                    | 1160            | 1157±3.6| 97                 | 4.8    |
| Freeze-thaw cycle | calycosin-7-O-β-D-glucoside      | 6.50            | 6.48±0.9| 99                 | -0.6   |
|                 |                                    | 650             | 649±0.2 | 100                | -0.2   |
|                 |                                    | 6500            | 6484±3.2| 96                 | -3.5   |
|                 | ononin                             | 1.03            | 1.06±2.1| 101                | 3.8    |
|                 |                                    | 103             | 102±0.6 | 99                 | -0.7   |
|                 |                                    | 2060            | 2062±1.4| 97                 | 2.4    |
|                 | calycosin                          | 4.00            | 3.98±1.9| 96                 | -0.5   |
|                 |                                    | 400             | 398±0.9 | 98                 | -0.4   |
|                 | formononetin                       | 5.80            | 5.82±0.6| 99                 | 2.3    |
|                 |                                    | 580             | 579±0.3 | 100                | -0.4   |
|                 |                                    | 1160            | 1156±3.8| 96                 | -3.9   |
Tissue concentrations of calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin following oral ERA administration

Normal and gastric ulcer mice (males and females) were divided into four groups after oral administration of ERA (4.0 g/kg) for 7 days. The methods used for these experiments were described in a previous study (Liu et al., 2012; Liu et al., 2014b). Various stomach tissue samples were collected at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 12, and 24 h after the final ERA administration. Tissues samples were rinsed with normal saline solution to remove contaminants, blotted with paper towels, and stored at -80 °C.

Statistical analysis

Statistical analyses were determined by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) comparison tests. Differences were considered significant at *P* < 0.05.

Comparison of the abundance of calycosin-7-O-β-d-glucoside in the stomachs of normal and gastric ulcer mice

As shown in Figure 2, calycosin-7-O-β-d-glucoside was widely distributed in mouse stomach tissue after oral administration of ERA. Calycosin-7-O-β-d-glucoside was detected 24 h after oral administration of ERA. The abundance of calycosin-7-O-β-d-glucoside was greater than that of ononin, calycosin and formononetin in the stomach tissue of normal and gastric ulcer mice. The distribution of calycosin-7-O-β-d-glucoside in normal and gastric ulcer mice differed markedly. The concentration of calycosin-7-O-β-d-glucoside in normal and gastric ulcer mice was greater than that of the samples from gastric ulcer mice at each time point. The highest levels of calycosin-7-O-β-d-glucoside were observed at 1.0 h in group A (gastric ulcer male mice), 1.5 h in female group B (gastric ulcer mice), 1.5 h in group C (normal male mice), and 1.15 h in group D (normal female mice). The time to maximum concentration (t<sub>max</sub>) of calycosin-7-O-β-d-glucoside in the stomach was between 1 and 1.5 h in all groups. The maximum concentration (C<sub>max</sub>) of group A (7358.97 ng/g) was lower than that of group C (47852.93 ng/g). The C<sub>max</sub> of group B (20833.23 ng/g) was lower than that of group D (35962.58 ng/g). Thus, the calycosin-7-O-β-d-glucoside concentrations of the normal groups were greater than those of the gastric ulcer groups. The different concentrations of calycosin-7-O-β-d-glucoside in the stomach tissue samples from the normal and gastric ulcer mice suggest that calycosin-7-O-β-d-glucoside had anti-gastric ulcer effects.
FIGURE 2 - Contents of four compounds in mice stomach tissue homogenate (*P < 0.05 compared to normal mice.).

A- the gastric ulcer male mice, B-the gastric ulcer female mice, C- the normal male mice, D- the normal female mice.
**Comparison of the abundance of ononin in the stomachs of normal and gastric ulcer mice**

The stomach distribution of ononin in the normal and gastric ulcer groups differed markedly (Figure 2). The ononin concentration of the normal mice was greater than that of the gastric ulcer mice at each time point. The $t_{\text{max}}$ of ononin was observed at 1.0 h in group A, 1.5 h in group B, 1.75 h in group C, and 1.15 h in group D. The $t_{\text{max}}$ of ononin in the stomach was between 1 and 1.75 h in all groups. The $C_{\text{max}}$ of group A (1982.58 ng/g) was lower than that of group C (4668.13 ng/g). The $C_{\text{max}}$ of group B (2076.84 ng/g) was lower than that of group D (7042.47 ng/g). The ononin concentrations of the normal groups were greater than those of the gastric ulcer groups. The different concentrations of ononin in the stomach tissue samples from the normal and gastric ulcer mice suggest that ononin had anti-gastric ulcer effects.

**Comparison of the abundance of calycosin in the stomachs of normal and gastric ulcer mice**

The stomach distribution of calycosin in the normal and gastric ulcer groups differed markedly (Figure 2). The calycosin concentration of the normal mice was greater than that of the gastric ulcer mice at each time point. The greatest concentration of calycosin was observed at 1.15 h in all groups of normal and gastric ulcer mice. The $C_{\text{max}}$ of group A (576.06 ng/g) was lower than that of group C (1056.65 ng/g). The $C_{\text{max}}$ of group B (727.65 ng/g) was lower than that of group D (793.05 ng/g). The calycosin concentrations of the normal groups were greater than those of the gastric ulcer groups. The different concentrations of calycosin in the stomach tissue samples from the normal and gastric ulcer mice suggest that calycosin had anti-gastric ulcer effects.

**Comparison of the abundance of formononetin in the stomachs of normal and gastric ulcer mice**

The stomach distribution of formononetin in the normal and gastric ulcer groups differed markedly (Figure 2). The formononetin concentration of the normal mice was greater than that of the gastric ulcer mice at each time point. The highest concentration of formononetin was observed at 1.15 h in group A, 1.5 h in group B, 1.5 h in group C, and 1.15 h in group D. The $t_{\text{max}}$ of formononetin in the stomach was between 1.15 and 1.5 h in all groups. The $C_{\text{max}}$ of group A (814.27 ng/g) was lower than that of group C (2014.24 ng/g). The $C_{\text{max}}$ of group B (585.95 ng/g) was lower than that of group D (1753.69 ng/g). The formononetin concentrations of the normal groups were greater than those of the gastric ulcer groups. The different concentrations of formononetin in the stomach tissue samples from the normal and gastric ulcer mice suggest that formononetin had anti-gastric ulcer effects.

**Summary of the tissue distributions of calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin**

Taken together, these results show that the concentrations of the four major bio-active constituents of Radix Astragali in the stomachs of normal mice were greater than those of gastric ulcer mice following oral administration of ERA. Calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin combined with plasma proteins in the stomach, which reduced the abundance of free compounds in the gastric ulcer groups to a greater degree than was observed in the normal groups. The $t_{\text{max}}$ values of calycosin-7-O-β-d-glucoside, calycosin, ononin, and formononetin in the stomach were between 1 and 1.75 h.

The concentrations of calycosin-7-O-β-d-glucoside, ononin, calycosin and formononetin in ERA were 52.5, 17.10, 2.88, and 1.90 mg/g, respectively. The concentrations of calycosin-7-O-β-d-glucoside and ononin in the stomach tissue samples were greater than those of calycosin and formononetin.

Using ultra-pressure liquid chromatography with a photodiode array detector (UPLC-PDA), the stomach tissue distribution profiles of calycosin-7-O-β-d-glucoside, ononin, calycosin and formononetin were examined after oral administration of ERA to mice. The method described in this study is a new, simple, rapid, reliable, and sensitive technique for effectively separating components and impurities. All of the bio-
active components of ERA were absorbed rapidly and distributed widely in the stomach.

The stomach tissue distribution of the four tested compounds differed significantly between female and male mice in the normal and gastric ulcer groups. The abundance of each tested compound was greater in the normal groups in comparison with the gastric ulcer groups.

This study confirmed that the distribution of gastric tissue in normal mice is different from that in ulcer mice. In addition, the contents of the four tested components in ulcer mice were lower than those in normal mice, and female and male mice showed slight differences. These findings provide new directions for research exploring the role of drug treatment, as well as a reference for individualized and precise drug delivery.

A previous study showed that the four tested compounds have strong anti-gastric ulcer effects via the spectrum-effect relationship and human gastric epithelial mucosa cell proliferation (Liu et al., 2015). The amounts of the four bio-active constituents absorbed in the gastric ulcer groups were lower than those of the normal groups. Therefore, future studies should assess the clinical potential of these compounds, alone and in combination, as treatments for patients afflicted with gastric ulcers. This study provides a foundation for clinical applications of Radix Astragali and studies of the tissue distributions of its bio-active constituents in additional organs and disease models.

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