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Short communication

Gradient high-performance liquid chromatography for the simultaneous determination of chlorogenic acid and baicalin in plasma and its application in the study of pharmacokinetics in rats

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Abstract

A novel HPLC–UV method was developed for the simultaneous determination of two major active components in Yinhuang injection, chlorogenic acid and baicalin, in rat plasma. Extracted from the plasma samples with methanol–acetonitrile (3:1, v/v), the two compounds were successfully separated using a C_{18} column with a gradient elution composed of 15 and 54% methanol–acetonitrile (1:1, v/v) in 0.2% (v/v) phosphoric acid water solution (pH 2.0). The flow-rate was set at 1 ml min^{-1} and the eluent was detected at 327 nm for chlorogenic acid, 278 nm for baicalin. Puerarin and rutin were used as the internal standards for chlorogenic acid and baicalin, respectively. The method was linear over the range of 0.388–12.4 μg ml^{-1}, 0.485–124 μg ml^{-1} for chlorogenic acid and baicalin, respectively. The correlation coefficient for each analyte was above 0.998. The intra-day and inter-day precisions were better than 7 and 9%, with the relative error ranging from −9.5 to 7.3% and from −4.2 to 1.8%. The limit of detection (LOD) and the limit of quantification (LOQ) for chlorogenic acid and baicalin in plasma were 0.194, 0.122, 0.388 and 0.485 μg ml^{-1}, respectively. This assay has been successfully applied in the pharmacokinetic study of chlorogenic acid and baicalin in vivo through intravenous administration of Yinhuang injection to rats.
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Keywords: Chlorogenic acid; Baicalin; HPLC; Pharmacokinetics

1. Introduction

Chinese patent medicine is prepared with natural Chinese crude drug under the guidance of the theory of traditional Chinese medical sciences. Because of the low toxicity, good therapeutic efficacy and convenience for administration, Chinese patent medicines are becoming more and more popular nowadays. However, due to the complexity of active components and the deficiency of conclusive drug mechanism, it is difficult for people from other countries to adopt Chinese patent medicine. Therefore, it requires us to strengthen the study of the mechanism of drug effects to further extent.

Yinhuang injection, prepared from *Flos Lonicerae* and *Radix Scutellariae*, is widely used to treat acute tonsillitis, upper respiratory infection, febris, tussis, pharyngalgia, etc., with therapeutic effect of relieving fever, deintoxication and relieving sore-throat [1]. Chlorogenic acid (ChA) and baicalin (Bc) are the major active components of *Flos Lonicerae* and *Radix Scutellariae*, respectively.

Several papers have described HPLC methods for the pharmacokinetic studies of ChA [2–4] or Bc [5–7] independently. In addition, several previous papers [8–10] have described HPLC methods for the simultaneous determination of Bc and its aglycone, baicalein in plasma. As both ChA and Bc are the major active component of Yinhuang injection, it is essential to develop a method to determine both of them in plasma samples for the purpose of studying the mechanism of action. However, no method has previously been described for the simultaneous determination of ChA and Bc in vivo in literature.

In the current study, we developed a gradient high-performance liquid chromatographic method for the simultaneous determination of ChA and Bc in Yinhuang injection in rat plasma. In addition, we applied the developed method successfully in the simultaneous pharmacokinetic study of ChA and Bc following intravenous administration of Yinhuang injection in rats.

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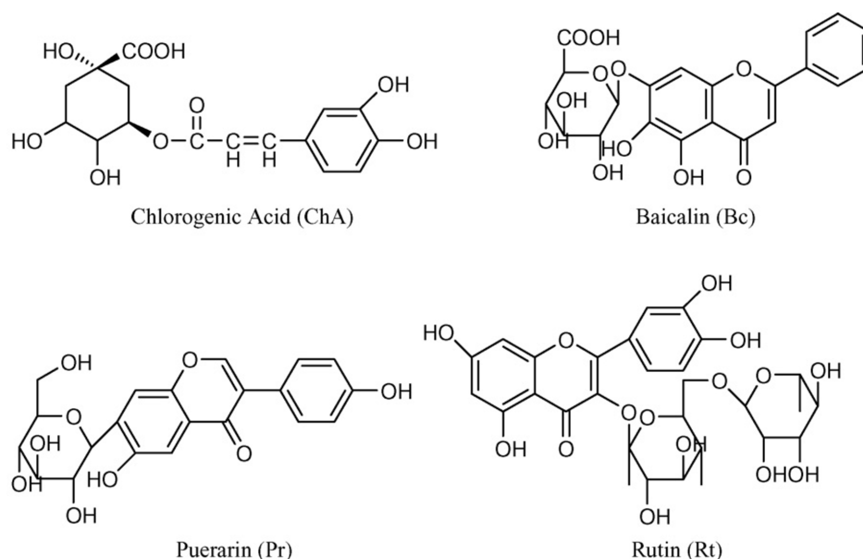

Fig. 1. Chemical structures of the two analytes, chlorogenic acid (ChA) and baicalin (Bc) and their internal standards, puerarin (Pr) and rutin (Rt).

2. Materials and methods

2.1. Reagents and chemicals

ChA and Bc, internal standard puerarin (Pr) and rutin (Rt) (Fig. 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Methanol and acetonitrile (HPLC grade) were purchased from Ludu Chemical Factory (Shanghai, China). Water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and used without further purification.

2.2. Instruments

The liquid chromatographic system was a Varian series ProStar HPLC system equipped with a ProStar 210 solvent delivery module and a ProStar 325 UV–vis detector. System control and data analyses were carried out using ProStar software (Revision 6.3, Varian). The chromatographic separation of the two compounds and internal standards was achieved by using a reversed-phase HPLC column (Polaris C_{18}-Ether, 150 mm × 4.6 mm i.d., 5 μm particle size, Varian) protected by a guard column (ChromGuard HPLC C_{18} Column, 10 mm × 3 mm, Varian).

2.3. Chromatographic conditions

The mobile phase for gradient elution consisted of two solvent systems: solvent A, 0.2% (v/v) phosphoric acid solution (pH 2.0); solvent B, methanol–acetonitrile (1:1 v/v). A gradient elution was carried out as follows: 15% (v/v) solvent B was used in the first minute, then solvent B percentage was linearly increased to 54% during 2–14 min, decreased to 15% in the 15th min and maintained for another 5 min. The total run time was 20 min including equilibration of the system. The flow-rate was 1 ml min^{-1}. The eluent was monitored by a UV detector, the detection wavelength was set at 327 nm for ChA and Pr, 278 nm for Bc and Rt. The sample injection volume was 20 μl and the column temperature was 35 °C.

2.4. Preparation of standard solutions

Solutions of ChA and Bc at 124 and 386 μg ml^{-1} were prepared separately in methanol. A stock solution containing ChA and Bc was prepared by mixing and diluting the above separate solutions with methanol to yield concentrations of 24.8 and 248 μg ml^{-1}, respectively. The internal standard solution containing 24.2 μg ml^{-1} of Pr and 22.6 μg ml^{-1} of Rt was prepared in methanol. These solutions were stored at 4 °C away from light and were found to be stable for at least 1 month. Working standard solutions for spiking plasma were freshly prepared by diluting the stock solution with methanol to appropriate concentrations.

The samples for plasma standard calibration curves were prepared by spiking the blank rat plasma (100 μl) with 100 μl of the appropriate working solution to yield the following concentrations: ChA at 0.0122–24.8 μg ml^{-1}, Bc at 0.122–248 μg ml^{-1}. Quality control samples used for the study of intra-day and inter-day accuracy and precision, extraction recovery and stability were prepared in the same way as the calibration samples.

2.5. Calibration curves

The plasma samples for standard curve were prepared as described in Section 2.6. Calibration curves were constructed by plotting the peak-area ratios of each analyte/internal standard versus analyte concentration in plasma. In order to avoid undue bias to the low concentrations of the standard curve by the high concentrations, the calibration curve of Bc was split into two ranges: 0.485–7.75 and 7.75–124 μg ml^{-1}.

2.6. Sample preparations

A 100 μl plasma sample was aliquoted in a centrifuge tube, then spiked with 20 μl 0.2 M hydrochloric acid by vortex mixing for 1 min. The mixture was extracted once with 800 μl methanol–acetonitrile (3:1, v/v) and 40 μl internal standard solution by vortex mixing for 2 min and centrifuged at 15,000 rpm for 15 min to separate protein from the organic phase. The supernatant (800 μl) was transferred to a clean centrifuge tube and evaporated to dryness in vacuum at 40 °C away from light. The residue was reconstituted in 200 μl of 20% methanol–acetonitrile (1:1, v/v) in 0.2% (v/v) phosphoric acid water solution (pH 2.0). After centrifuging at 15,000 rpm for 15 min, 20 μl of the supernatant was injected into the HPLC system for analysis.

2.7. Validation of the assay method

2.7.1. Specificity

The specificity of the method was investigated by comparing the chromatograms of blank plasma samples from different rats with that of blank plasma spiked with standard solution and the samples collected after intravenous administration of Yinhuang injection.

2.7.2. Precision and accuracy

The intra-day precision was determined within 1 day by analyzing five replicates of control samples at concentrations of 0.775, 3.1,12.4 $\mu g\,ml^{-1}$ for ChA and 0.97, 7.75, 124 $\mu g\,ml^{-1}$ for Bc. The inter-day precision was determined on 5 separate days for the control samples. The intra-day and inter-day precision was defined as the relative standard deviation (R.S.D.) and accuracy was determined by calculating the relative error (R.E.).

2.7.3. Recovery

Recovery was calculated by comparing the peak areas of the extracted quality control samples with that of the unextracted standard solutions containing the equivalent amount of analytes.

2.7.4. Sensitivity

The limit of detection (LOD) was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, i.e., R.S.D. less than 20% and relative error between ±20%.

2.7.5. Stability

The stability of the two analytes was determined in two ways: (1) The stability of analytes in the plasma sample stored at −20 °C was determined in 0, 1, 2, 3, 7 days. (2) The stability of the analytes after extraction from plasma and dissolving in methanol–acetonitrile (1:1, v/v) in 0.2% (v/v) phosphoric acid solution (pH 2.0) at 4 °C away from light was determined at 0, 24 and 48 h.

2.8. Assay application

Yinhuang injection was administered to five male Wistar rats intravenously with a dose of 0.5 $ml\,kg^{-1}$, equivalent to 1 $mg\,kg^{-1}$ of ChA and 10 $mg\,kg^{-1}$ of Bc. Venous blood samples were collected and centrifuged at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 420 min, respectively. The plasma sample (100 μl) was analyzed immediately or stored at −20 °C for further analysis.

3. Results and discussion

In addition to the determination of effective components in traditional preparation medicines, it is more critical to clarify the destiny of effective components in vivo—the pharmacokinetic profiles. Because there are various effective components in a single preparation, simultaneous determination of several effective components in vivo through optimizing the extraction method and chromatographic conditions should be a quick and simple way to study the pharmacokinetic properties of several effective components in one preparation. Due to the significant difference in polarity and concentration between ChA and Bc, simultaneous determination of ChA and Bc in plasma becomes difficult in a single HPLC run. Although there are literatures concerning the determination of ChA [3] and Bc [7] independently in plasma using HPLC with relatively fine sensitivity, the methods effective in determination of single effective ingredient preparation may not be appropriate for the study of the traditional Chinese preparations with two or more effective ingredients. The major contribution of the present HPLC method is to develop a simple extraction method and a gradient elution program to reduce the running time and reach satisfactory specificity and sensitivity for both ChA and Bc at the same time.

3.1. Specificity

Since ChA and Bc have maximum UV absorptions at different wavelengths, in order to obtain the highest sensitivity for each component, two different wavelengths of 327 and 278 nm were chosen, respectively. The polarity of ChA is largely different from that of Bc, and their concentrations vary greatly (1:10). Consequently, using single internal standard method may not be appropriate to determine both ChA and Bc simultaneously. Puerarin and rutin were selected as the internal standards based on our investigation of a series of chemicals. Since there existed interference ingredients in the preparation that have the same chromatographic behavior as ChA, the desired effective separation of ChA ($R > 1.5$) cannot be achieved using solvent A 0.2% (v/v) phosphoric acid water solution) and solvent B (simple methanol or acetonitrile). In this study, a gradient elution of methanol–acetonitrile 1:1 (v/v) in 0.2% (v/v) phosphoric acid water solution (pH 2.0) was used to achieve complete separation of ChA ($R \geq 1.8$). The representative HPLC profiles of a blank plasma sample (A), a blank plasma sample spiked with standard solution (B) and plasma sample at 2 h after intravenous administration of Yinhuang injection (C) were shown in Fig. 2. No interference was observed under the assay conditions. The

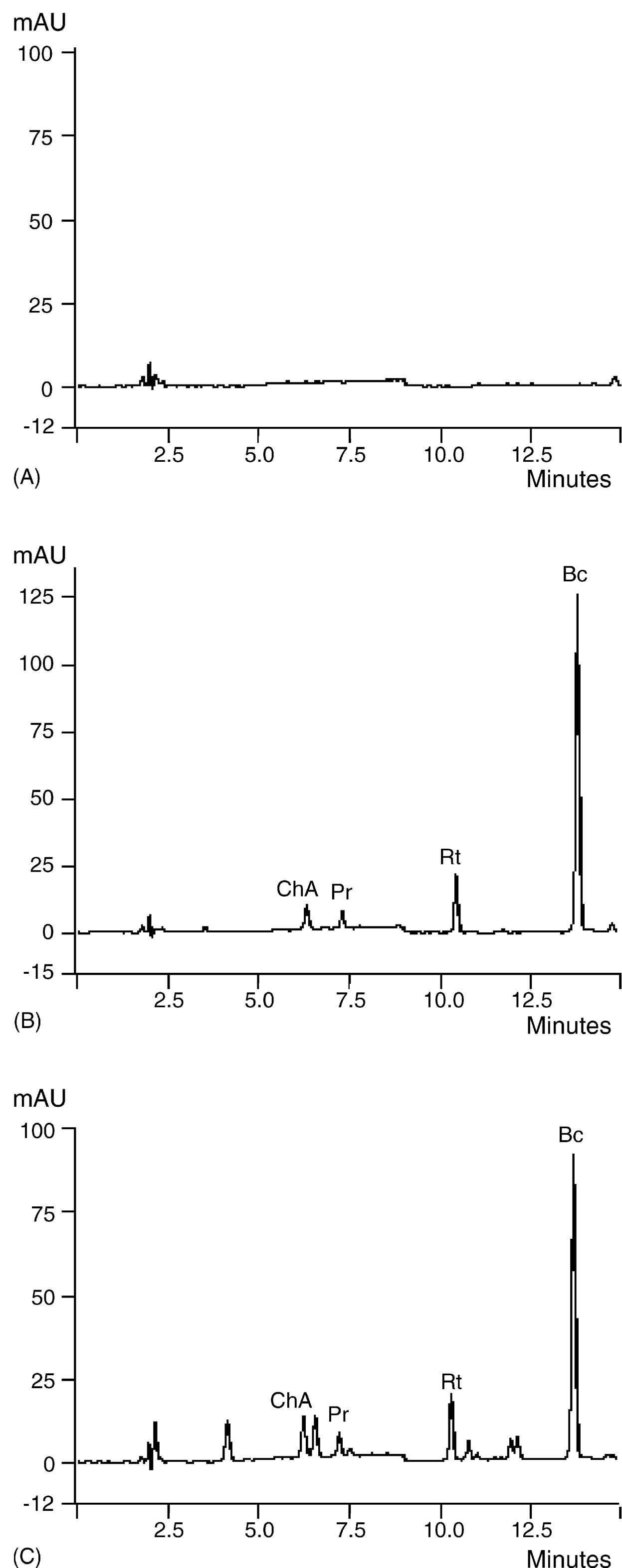

Fig. 2. Chromatograms of rat plasma samples. (A) Blank plasma; (B) plasma spiked with ChA, Bc and their internal standards Pr and Rt; (C) plasma sample at 2 h after intravenous administration of Yinhuang injection.

peaks of the analytes in the plasma were identified by comparing their retention time with that of the standard and further confirmed by their on-line UV spectra. Typical retention times were as follows: ChA, 6.3 min; Pr, 7.3 min; Rt, 10.4min; Bc, 13.7 min.

3.2. Sample preparations

In this study, several organic solvent systems, such as acetoacetate, *n*-butanol, acetoacetate–methanol and methanol–acetonitrile with different composition ratios were tested for deproteinization and extraction of ChA and Bc from rat plasma. Among them best extraction recovery and precision were observed when a mixture of methanol and acetonitrile (3:1, v/v) was chosen.

During our assay, the direct injection method using supernatant after protein elimination was tested at first, but the LOQs of two components proved exceedingly high due to the dilution effect. On the other hand, the strong solvent effect led to low column efficiency and poor peak shape, which was of no benefit to the determination of two components, especially the detection of ChA. Through trials, the satisfactory column efficiency and peak shape were obtained by evaporating the supernatant to dryness in vacuum and reconstituting the residue in 20% methanol–acetonitrile (1:1, v/v) in 0.2% (v/v) phosphoric acid solution (pH 2.0).

3.3. Linearity and sensitivity

Calibration curves of the test compounds were linear over the low and high concentration range with $r^2 > 0.998$ for both the two analytes (Table 1). The limit of detection (LOD) and the limit of quantification (LOQ) for ChA and Bc in plasma was determined to be 0.194, 0.122 and 0.388, 0.485 $\mu g\,ml^{-1}$, respectively (Table 1).

3.4. Accuracy and precision

Analytical accuracy and precision data are shown in Table 2 and are expressed as mean detected concentration, relative standard deviation (R.S.D.) and relative error (R.E.). The precisions of ChA and Bc calculated as the relative standard deviation (R.S.D.) at low to high concentrations were better than 7 and 9% for intra-day and inter-day assays, respectively. The accuracies of ChA and Bc calculated as the relative error (R.E.) of inter-day and intra-day at low to high concentration were within the range from −9.5 to 7.3% and from −4.2 to 1.8%.

3.5. Recovery

ChA and Bc and their internal standards are prone to oxidization and ChA is easily decomposed in sunlight, therefore it is difficult to detect these components simultaneously with general sample preparation method. According to literature [9,11], ascorbic acid was tested as anti-oxidant but the anti-oxidant efficiency was not apparent and seemed to interfere with the determination of ChA. ChA and Bc were relatively stable under

Table 1
Linearity and sensitivity of detection for chlorogenic acid (ChA) and baicalin (Bc) in rat plasma

Analyte	Range (μg ml^{-1}l)	Regression equation	Correlation coefficient(r^2)	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
ChA	0.388–12.4	$Y = 0.502c - 0.082$	0.9993	0.194	0.388
Bc	0.485–7.75	$Y = 0.191c - 0.005$	0.9984	0.122	0.485
	7.75–124	$Y = 0.206c - 0.314$	0.9998		

Table 2
Extraction recoveries and intra-day and inter-day (5 separate days) accuracy and precision for the determination of chlorogenic acid (ChA) and baicalin (Bc) in rat plasma

Analyte	Nominal concentration (μg ml^{-1})	Mean recovery (%) ($n = 5$)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
			Mean detected concentration (μg ml^{-1})	Precision R.S.D. (%)	Accuracy R.E. (%)	Mean detected concentration (μg ml^{-1})	Precision R.S.D. (%)	Accuracy R.E. (%)
ChA	0.775	74.2	0.709	6.9	−8.5	0.702	7.0	−9.5
	3.1	81.4	2.81	2.7	−9.5	2.84	4.6	−8.4
	12.4	82.8	13.00	1.6	4.8	13.31	3.0	7.3
Bc	0.97	96.9	0.987	5.0	1.8	0.929	8.6	−4.2
	7.75	90.0	7.50	2.9	−3.3	7.45	8.1	−3.9
	124	92.8	122.9	1.1	−0.9	119.5	5.4	−3.7

acidic conditions [12,13] and adding 20 μl 0.2 M hydrochloride acid into the plasma sample achieved satisfactory recovery result after careful investigation of different acids with different concentrations.

The extraction recoveries of the two analytes ($n = 5$) from spiked rat plasma (Table 2) were satisfactory at low, middle and high concentrations. They varied from 74 to 83% for ChA, from 90 to 97% for Bc. Recovery of the internal standard of Pr was very consistent with a mean of 89.1% and a R.S.D. of 4.5% ($n = 15$), recovery of Rt was a mean of 95.7% and a R.S.D. of 3.1% ($n = 15$).

3.6. Stability

The stability test indicated that both ChA and Bc were stable for at least 7 days stored in plasma at −20 °C (Table 3). In addition, ChA and Bc were also stable in prepared samples for 48 h (Table 3) demonstrating the stability of the analytes in 2 days when placed at 4 °C away from light waiting to be analyzed.

3.7. Application in pharmacokinetics study

The validated HPLC-UV method has been successfully used to simultaneously determine the concentrations of ChA and Bc in plasma samples obtained after intravenous administration of 0.5 ml kg^{-1} of Yinhuang injection to rats. The obtained pharmacokinetic profiles of ChA and Bc are shown in Fig. 3. The concentration of Bc in plasma decreased quickly during 10–90 min, while the concentration at 120 min was higher than that at 90 min, which suggests there might exist enterohepatic circulation of Bc in vivo in agreement with literature [14].

The pharmacokinetic parameters were estimated by the DAS Ver1.0 (Drug and Statistics for Windows) program. A three-compartment model was used to estimate the parameters of ChA, and a two-compartment model for Bc. The established three-compartment model of ChA varies from two-compartment of ChA in literature [15], this may be in part due to the existence of unknown components in Yinhuang injection, which might influences the metabolism of ChA in vivo. However, such effect was

Table 3
The stability of ChA and Bc in plasma samples stored at −20 °C was determined during 7 days as well as in prepared samples stored at 4 °C away from light during 48 h

Analyte	Nominal concentration (μg ml^{-1})	In plasma		In prepared samples	
		Mean detected concentration (μg ml^{-1})	Variation coefficient (%)	Mean detected concentration (μg ml^{-1})	Variation coefficient (%)
ChA	0.775	0.703	7.9	0.696	2.7
	3.1	2.76	0.7	2.63	1.1
	12.4	13.24	2.0	13.07	2.6
Bc	0.97	0.954	4.1	0.942	3.5
	7.75	7.07	3.8	7.99	0.8
	124	115.5	3.2	121.17	1.9

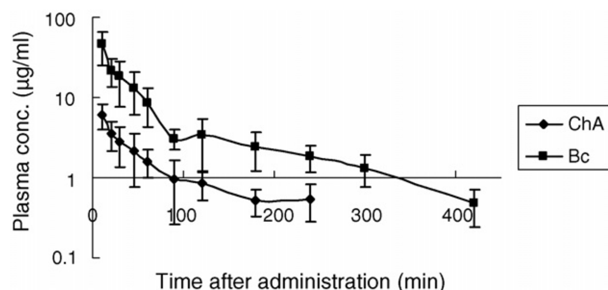

Fig. 3. Plasma concentration versus time profiles of chlorogenic acid (ChA) and baicalin (Bc) in rats ($n = 5$) following intravenous administration of Yinhuang injection at a single dose of $0.5\,mg\,kg^{-1}$, equivalent to $1\,mg\,kg^{-1}$ of ChA and $10\,mg\,kg^{-1}$ of Bc.

not observed in the metabolism of Bc and the established two-compartment model of Bc was in parallel with literature [5,14].

4. Conclusion

A gradient HPLC method for the simultaneous quantification of the plasma concentrations of two major active components, ChA and Bc, in Yinhuang injection has been developed. Puerarin and rutin were used as the internal standards, respectively. The method is not only simple and efficient with excellent accuracy, precision, reproducibility and low detection limit, but also allows for the simultaneous determination of the two major active components in plasma sample and might be applied in further pharmacokinetic studies.

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