Simultaneous determination of five nucleosides and nucleobases of *Rehmannia glutinosa* Libosch. by high performance liquid chromatography

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Abstract: This study is to establish a method for simultaneously determination of five nucleosides and nucleobases, including hypoxanthine, uridine, adenine, guanosine and adenosine in *Rehmannia glutinosa* Libosch. which was collected from different regions in China. A Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) was used. Acetonitrile and 0.04 mol·L^{-1} potassium dihydrogen phosphate solution were adopted as mobile phase with gradient elution. The flow rate was 1 mL·min^{-1} and column temperature was 30 ℃. The detection wavelength was at 254 nm. The method had good linearity over the range of 1.0 − 16.0 μg·mL^{-1} (r^2 = 0.999 8), 5.0 − 80.0 μg·mL^{-1} (r^2 = 0.999 8), 1.0 − 16.0 μg·mL^{-1} (r^2 = 0.999 5), 1.25 − 20.0 μg·mL^{-1} (r^2 = 0.999 8) and 1.0 − 16.0 μg·mL^{-1} (r^2 = 0.999 8) for hypoxanthine, uridine, adenine, guanosine and adenosine, respectively. The average recoveries were between 98.8% and 100.7%. The content of hypoxanthine, uridine, adenine, guanosine and adenosine in *Rehmannia glutinosa* Libosch. from different regions was significantly different. This established method was sensitive and reliable for the quantification of five chemical constituents in *Rehmannia glutinosa* Libosch.

Key words: high performance liquid chromatography; *Rehmannia glutinosa* Libosch.; nucleoside; nucleobase; simultaneous determination

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HPLC 法同时测定地黄中的 5 种核苷和碱基的含量

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摘要: 建立 HPLC 法同时测定来自中国不同地区的地黄中次黄嘌呤、尿苷、腺嘌呤、鸟苷、腺苷等 5 种核苷类成分的含量。采用的色谱柱为 Diamonsil C_{18} 柱 (250 mm × 4.6 mm, 5 μm), 流动相为乙腈−0.04 mol·L^{-1} 磷酸二氢钾溶液梯度洗脱, 流速 1 mL·min^{-1}, 柱温 30 ℃, 检测波长 254 nm。次黄嘌呤、尿苷、腺嘌呤、鸟苷和腺苷的质量浓度分别在 1.0～16.0 μg·mL^{-1} (r^2 = 0.999 8), 5.0～80.0 μg·mL^{-1} (r^2 = 0.999 8), 1.0～16.0 μg·mL^{-1} (r^2 = 0.999 5), 1.25～20.0 μg·mL^{-1} (r^2 = 0.999 8) 和 1.0～16.0 μg·mL^{-1} (r^2 = 0.999 8) 内线性关系良好, 平均回收率为 98.8%～100.7%。结果表明, 不同地区的地黄中次黄嘌呤、尿苷、腺嘌呤、鸟苷和腺苷的含量有显著性差异。该方法准确, 重复性好, 适用于地黄药材中 5 种核苷类成分的含量测定。

关键词: 高效液相色谱; 地黄; 核苷; 碱基; 同时测定

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Rehmannia glutinosa Libosch., belonging to the family of Scrophulariaceae, is one of the most widely used traditional Chinese herb[1]. Traditionally, *Rehmannia glutinosa* Libosch. has been used to nourish Yin and invigorate the kidney in traditional Chinese medicine (TCM) with a very high medicinal value[2]. In recent decades, a great number of chemical and pharmacological studies have been done on *Rehmannia glutinosa* Libosch. The main ingredients known for *Rehmannia glutinosa* Libosch. are glycosides, sugar and amino acids[3], which were considered as the major effective constituents. In the previous literatures, many studies on the quality control of *Rehmannia glutinosa* Libosch. focused on rehmanniosides[4−6], catalpol[7−9] and oligosaccharides[10, 11]. Further study showed that nucleosides as basic ingredients in TCM demonstrated a unique and irreplaceable role in anti-virus and anti-tumor activities, which can be used as active ingredients in drugs[12]. Indeed, nucleosides are involved in the regulation and modulation of various physiological processes through purinergic and pyrimidine receptors[13, 14]. Adenosine can lower blood pressure and slow down heart rate[15]. There is a large quantity of uridine, guanosine and adenosine in the root of *Rehmannia glutinosa* Libosch. Therefore, determination of nucleosides and nucleobases is very important for pharmacological study and quality control of *Rehmannia glutinosa* Libosch. and their products. So far, there was only report on the determination of adenosine in *Rehmannia glutinosa* Libosch. by HPLC[16]. And there are no reports for the quality control of other nucleosides and nucleobases in *Rehmannia glutinosa* Libosch. This work was to establish an alternative approach for the quality assessment of *Rehmannia glutinosa* Libosch. There are five nucleosides and nucleobases, including hypoxanthine, uridine, adenine, guanosine and adenosine in the samples of *Rehmannia glutinosa* Libosch. determined by HPLC. The contents of these components in *Rehmannia glutinosa* Libosch. from different regions were also compared.

Materials and methods

Chemicals, reagents and materials Nucleosides and nucleobases, including hypoxanthine, uridine, adenine and adenosine were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Guanosine was purchased from Shanghai Tauto Biotech Co., Ltd. The purities of these compounds were determined to be more than 99.5% by HPLC. Their structures are shown in Figure 1. Acetonitrile for liquid chromatography was purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate was purchased from Sinopharm Chemical Reagent Co., Ltd. Other reagents of HPLC grade or the highest grade were commercially available. The root of *Rehmannia glutinosa* Libosch. was collected from different regions in China and authenticated by Professor Ya-jun Cui, Shanghai University of Traditional Chinese Medicine. Ten samples of Rehmannia glutinosa Libosch. (1−10) are from Shanghai, Beijing, Nanjing, Zhengzhou, Taiyuan, Shenyang, Changchun, Huhehaote, Xi'an, and Jinan, separately.

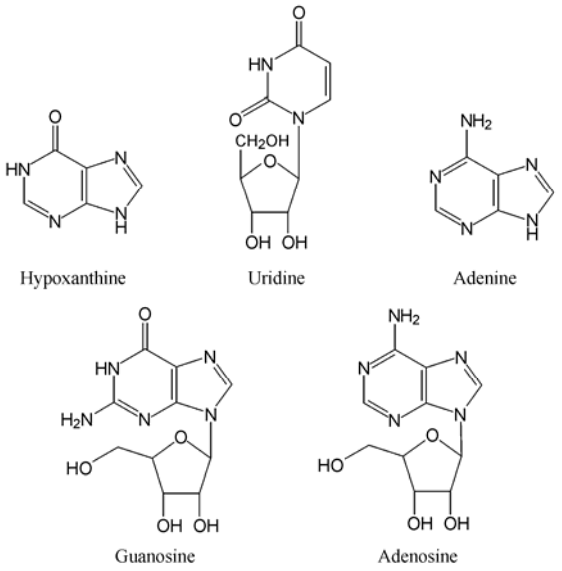

Figure 1 Chemical structures of five nucleosides and nucleobases in *Rehmannia glutinosa* Libosch.

Preparation of standard solutions A stock solution of standards containing hypoxanthine (40 μg·mL^{-1}), uridine (200 μg·mL^{-1}), adenine (40 μg·mL^{-1}), guanosine (50 μg·mL^{-1}) and adenosine (40 μg·mL^{-1}) was prepared and appropriately diluted with water-methanol (50∶50, *v/v*) to obtain a series of working standard solutions for calibration curves. The solutions were stored at 4 ℃ and left at room temperature before use.

Preparation of sample solutions The ground powder of *Rehmannia glutinosa* Libosch. from different regions were accurately weighed (1.0 g), and ultrasonic-extracted with 10.0 mL of a mixture of water-methanol (90∶10, *v/v*) for 1 h, and then the same mixture was added to compensate for the lost weight during the extraction. After extraction, the supernatant was

filtered through a 0.45 μm membrane filter before HPLC analysis.

Instrumentation and HPLC conditions Quantitative analysis was performed on a Waters 2695 HPLC system equipped with a vacuum degasser, a quaternary pump, an autosampler and a Waters 2487 UV detector system, connected to a Waters Empower software. A Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) was used for separation. Optimum separation was achieved using gradient elution with 40 mmol·L^{-1} potassium dihydrogen phosphate solution (A) and acetonitrile (B): 0 – 25 min, 0% B; 25 – 40 min, 0 – 1% B; 40 − 50 min, 1 – 5% B; 50 − 60 min, 5% B. The flow rate was set at 1.0 mL·min^{-1} and the injection volume was 20 μL. The column temperature was maintained at 30 ℃ and the UV detection wavelength was set at 254 nm for analysis.

Results and discussion

1 Optimization of the extraction method

The extraction conditions of five nucleosides and nucleobases were investigated and optimized. Firstly, the method for the extraction was investigated, such as refluxing and ultrasonication. The amount of analytes extracted by ultrasonication was more than that extracted by refluxing. Therefore, ultrasonic extraction was selected. To achieve the maximal extraction efficiency for all analytes in *Rehmannia glutinosa* Libosch., solvent of extraction (water, methanol and ethanol), concentration of solvent (0%, 5%, 10%, 15%, 20%, 25% and 30% methanol aqueous solution), solvent volume (5 mL, 10 mL, 15 mL and 20 mL) and time of extraction (0.5 h, 1 h, 1.5 h, 2 h) were also tested, separately. It was found that methanol, 10% methanol aqueous solution, 10 mL and 1 h were the optimal extraction conditions.

2 Optimization of HPLC conditions

A series of mobile phases were investigated to obtain an optimal separation and analytical conditions for HPLC analysis, including methanol-water, acetonitrile-water, acetonitrile-0.1% formic acid, acetonitrile-ammonium acetate and acetonitrile-potassium dihydrogen phosphate. It was observed that the most favorable separation resolutions of all analytes could be achieved when potassium dihydrogen phosphate was used in the mobile phase. Then, the effect of different concentrations including 5, 10, 20, 30 and 40 mmol·L^{-1} of potassium dihydrogen phosphate on the separation of nucleosides and nucleobases was investigated, successively. The best separation was obtained when using acetonitrile-40 mmol·L^{-1} potassium dihydrogen phosphate as mobile phase with gradient in this study.

3 Method validation

The linearity, precision, accuracy, repeatability and stability were determined to validate the analysis method. The correlation coefficient (r^2 > 0.999 0), which was obtained from the linear regression equation for the calibration curve, indicated appropriate relevance between concentrations and peak areas within the tested ranges. The relative standard deviation (RSD%) was taken as an evaluation of precision, repeatability and stability for the analytes of the developed method. The overall variations of five analytes were less than 3.6% (RSD%). And the overall recovery of 98.8%−100.7% for the analytes showed good accuracy of the developed method.

3.1 Linearity, range and limits of detection The stock standard solution of the five analytes was appropriately diluted with 50% aqueous methanol to obtain a series of working solutions. And then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The linearities of the five analytes are listed in Table 1. As a result, the correlation coefficients (r^2 > 0.999 0) of the analytes indicated good linearity over the whole concentration ranges studied. Limits of detection (LOD) and quantification (LOQ) for each analyte, which were determined at a signal-to-noise ratio (*S/N*) of about 3 and 10, respectively, are also listed in Table 1.

3.2 Precision Intra-day and inter-day variability were chosen to evaluate the precision of the method. Intra-day precision was examined with the mixture standard solutions during a single day. The inter-day precision was determined over three consecutive days. The relative standard deviation (RSD%) values, which was utilized to evaluate variations, varied from 0.44% to 3.6% for intra- and inter-day assays for all the analytes (Table 1).

3.3 Repeatability Repeatability was examined by injecting six samples prepared with the same sample preparation procedure as mentioned above. The relative standard deviation (RSD%) are listed in Table 1.

3.4 Stability For the stability test, the sample solution was analyzed within 24 h at room temperature. The RSD% values varied from 0.3% to 3.4% (Table 1). It indicated that the investigated samples were stable enough for the routine analysis within a day at room temperature.

3.5 Recovery To evaluate the accuracy of the method, the recovery test was carried out by measuring the amount of each compound recovered from the samples which mixed with the standard solution at high, middle and low concentration levels in three replicates. The recoveries were between 98.8% and 100.7%. Average recoveries for hypoxanthine, uridine, adenine, guanosine and adenosine were 99.2%, 99.3%, 100.7%, 98.8% and 99.3%, respectively. And the RSD% values of each concentration level for hypoxanthine, uridine, adenine, guanosine and adenosine were 1.1%, 1.5%, 1.8%, 1.5%, 1.7%, respectively. The high recoveries illustrated good accuracy of the method.

4 Sample analysis

The developed HPLC method was applied to the determination of five nucleosides and nucleobases in *Rehmannia glutinosa* Libosch. from ten different regions (1−10), and the contents of the analytes were shown in Table 2. The contents of the five compounds varied substantially among the samples because of different climates and geographical environment.

Conclusions

A simple, sensitive and reliable HPLC method was successfully employed to the quantification of five nucleosides and nucleobases in *Rehmannia glutinosa* Libosch. from different regions (Figure 2). It can be used as the basis of the quality analysis of this widely used herb in the future.

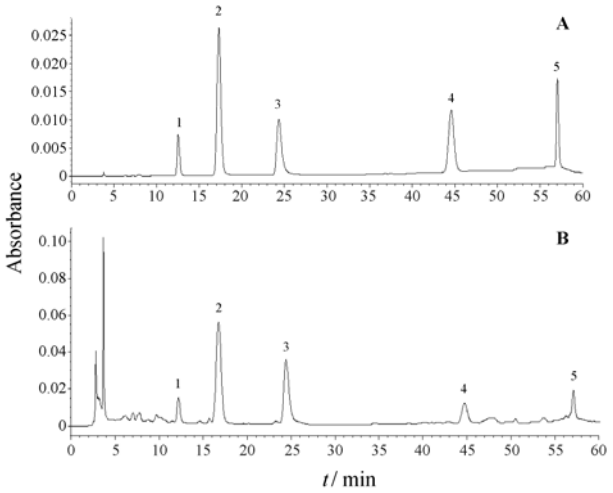

Figure 2 Typical chromatograms of standard chemicals (A) and *Rehmannia glutinosa* Libosch. (B). 1: Hypoxanthine; 2: Uridine; 3: Adenine; 4: Guanosine; 5: Adenosine

Comparing the results of the analyses, the content of uridine was found to be much more than that of the other four compounds. It was also revealed that the content of the same compound in different cultivated regions of *Rehmannia glutinosa* Libosch. was significantly different.

Table 1 Regression equation, linear range, detection limits of the method, and data of precision, repeatability and stability

Constituent	Regression equation	Correlation coefficient /R^2	Linearity range /μg·mL^{-1}	LOD /μg·mL^{-1}	LOQ /μg·mL^{-1}	Precision (RSD%)		Repeatability (RSD%)	Stability (RSD%)
						Intra-day	Inter-day		
Hypoxanthine	$Y = 7.150\times10^4X + 1.048\times10^3$	0.999 8	1.0−16.0	0.05	0.10	1.8	0.59	1.8	1.0
Uridine	$Y = 4.026\times10^4X + 4.552\times10^3$	0.999 8	5.0−80.0	0.10	0.30	1.3	1.3	0.62	0.3
Adenine	$Y = 9.909\times10^4X - 3.248\times10^4$	0.999 5	1.0−16.0	0.06	0.20	0.47	0.50	0.52	2.3
Guanosine	$Y = 4.869\times10^4X - 1.836\times10^4$	0.999 8	1.25−20.0	0.10	0.30	3.6	2.5	2.5	2.6
Adenosine	$Y = 6.794\times10^4X + 3.404\times10^3$	0.999 8	1.0−16.0	0.20	0.65	0.44	0.49	2.6	3.4

Table 2 Results of the quantification of five components in *Rehmannia glutinosa* Libosch. ($n = 3$, $\bar{x} \pm s$)

No.	Sample source	Content /μg·g^{-1}				
		Hypoxanthine	Uridine	Adenine	Guanosine	Adenosine
1	Shanghai	22.32 ± 0.03	208.5 ± 1.42	40.32 ± 0.33	26.72 ± 0.17	87.52 ± 0.85
2	Beijing	15.68 ± 0.13	374.6 ± 0.75	20.16 ± 0.09	56.64 ± 0.29	107.4 ± 0.26
3	Nanjing	26.64 ± 0.16	212.6 ± 0.32	50.72 ± 0.06	36.88 ± 0.11	112.8 ± 0.62
4	Zhengzhou	12.88 ± 0.11	219.1 ± 0.55	34.32 ± 0.30	16.48 ± 0.02	102.0 ± 0.18
5	Taiyuan	14.64 ± 0.09	212.5 ± 0.23	15.28 ± 0.02	20.24 ± 0.16	45.12 ± 0.05
6	Shenyang	19.12 ± 0.03	292.5 ± 1.08	43.28 ± 0.07	13.44 ± 0.01	113.3 ± 0.19
7	Changchun	11.12 ± 0.02	399.5 ± 0.96	50.72 ± 0.46	48.72 ± 0.06	83.76 ± 0.22
8	Huhehaote	56.96 ± 0.06	271.5 ± 1.38	68.56 ± 0.24	16.32 ± 0.10	100.9 ± 0.73
9	Xi'an	49.68 ± 0.06	219.4 ± 0.46	74.80 ± 0.61	14.92 ± 0.06	116.1 ± 0.39
10	Jinan	34.08 ± 0.25	214.2 ± 0.60	53.12 ± 0.22	44.08 ± 0.23	92.32 ± 0.82

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