Additional file 2 Identification and measurement of major constituents in huangqin (*Scutellaria baicalensis* Georgi)

Introduction

Huangqin (*Scutellaria baicalensis* Georgi) is a kind of perennial herb plant that derived from Labiatae family, its dry roots are officially listed in the Chinese Pharmacopoeia (Commission, 2015). Huangqin is highly valued and widely distributed in China, Russia, Mongolia, Korea and Japan, that was first recorded in Shennong's Classic of Materia Medica (Shen Nong Ben Cao Jing) in 100 BC. In recent decades, the natural resources of huangqin was deficient, and gradually replaced by artificial cultivation, hence cause the expansion of planting area and increase of production (Yan et al., 2017).

Flavonoids usually exist in fruits, vegetables, seed and bark, with chemical structure of benzo-g-pyrone derivatives and are considered as natural antioxidants. Baicalin (BG) and wogonoside (WG) as well as their aglycones baicalein (BA) and wogonin (WO), the four major flavonoids in the extract of *Scutellaria baicalensis* Georgi, are widely accepted as the indicators of quality control for Scutellariae Radix and compound recipes containing it, because of their remarkable pharmacological effects of anti-inflammatory, antioxidant, anti-apoptotic and neuroprotective effects (Gaire et al., 2014; Woźniaka et al., 2015; Zhang et al., 2017).

In this article, an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-TOF-MS) method of simultaneous analysis of 4 constituents (BA, BG, WO and WG) was established and validated. Meanwhile, their concentrations in huangqin extract were identified and measured.

Material and methods

Reagents and chemicals

Standard substances of BA (111595-201607), BG (110715-201619), WO (111514-201605) and WG (112002-201702) were purchased from the National Institute for Food and Drug Control (Beijing, China).

HPLC-grade acetonitrile and methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water (> 18 mΩ) was obtained from a Milli-Q water purification system (Bedford, MA, USA). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, OH, USA). Other chemicals were all analytical reagents.

Huangqin (dried roots of *Scutellaria baicalensis* Georgi) was purchased from Sichuan Xinhehua Medicinal Materials Electuary Co. Ltd. (Sichuan, China), and the voucher specimens were deposited at the Herbarium of Translational Medicine Center, Hong Hui Hospital, Xi'an Jiaotong University, Xi'an, China.

Preparation of standard solutions

Stock standard solutions of BA, BG, WO and WG were prepared with methanol to eight different concentrations of 1000, 500, 200, 100, 50, 20, 10 and 5 ng/mL. All the stock standard solutions were stored at 4℃ in the refrigerator.

LC-MS instruments and operation conditions

Liquid chromatography

An SHIMAZDU 30AD System (Shimazdu Corp., Japan) was used to injected 5 μl aliquots of samples on an Acquity UPLC HSS T3 column (100×2.1 mm, i.d., 1.8 mm, Waters, USA) which was maintained at 35℃ in a column oven. The mobile phase was consisting of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.5 mL/min using a gradient elution of 2% at 0-0.5 minutes, 2-90% at 0.5-4.5 minutes, 90% at 4.5-6 minutes, 2% at 6.1-10 minutes.

Mass spectrometric conditions

A TripleTOF 5600+ mass spectrometer (AB Sciex Corp., USA) equipped with an electrospray ionization (ESI) interface was used. ESI/MS was operated in positive mode for flavones. The operating parameters were as follows: curtain gas, 30 L/h; GS1, 50 L/h; GS2, 60 L/h; ISVF, 5500V; DP, 60V; CE, 10V.

Quantitative analysis of BA, BG, WO, WG was performed by monitoring $[M+H]^+$ for analytes in TOF-MS-IDA-TOF-MS/MS (n = 4). The molecular formula, abbreviations, mass-to-charge ratio (m/z) and retention times of these 4 flavones are shown in **Additional Table 2**.

Additional Table 2 molecular formula, mass-to-charge ratio (m/z), retention times, calibration curves, correlation coefficients, linear ranges, precisions and stability in 72 hours of 4 constituents

		Baicalein (BA)	Baicalin (BG)	Wogonin (WO)	Wogonoside (WG)
Molecular formula		$C_{15}H_{10}O_5$	$C_{21}H_{18}O_{11}$	$C_{16}H_{12}O_5$	$C_{22}H_{20}O_{11}$
m/z		271.0501 - 271.0701	447.0822 - 447.1022	285.0658 - 285.0858	461.0978 - 461.1178
Retention time (t_R, minutes)		4.38	3.60	4.85	3.86
Calibration curves		y = 4044x + 75122	y = 3156x + 31674	y = 9170x + 97915	y = 3831x + 49574
Correlation coefficients (r^2)		0.996	0.999	0.999	0.998
Linear ranges (ng/mL)		5-1000	5-1000	5-1000	5-1000
Precisions	high	2.96%	3.05%	2.71%	1.44%
	medium	2.12%	0.84%	3.24%	1.68%
	low	2.75%	1.82%	1.06%	2.49%
Stability in 72 hours		2.48%	3.45%	1.07%	3.50%

Preparative method of decoction

Huangqin extract was prepared from the herb by three rounds of reflux with 75% ethanol: in the first round, the reflux time was 3 hours and the mass of 75% ethanol was 8-fold the huangqin

mass; the duration of the second round was 2 h and the ethanol mass was 7-fold; and the third round was for 1 hour with 5-fold ethanol. The extracted liquid was filtered through a double-layer absorbent gauze. The filtrate was combined, concentrated under vacuum (equal to 1 g of the herb per milliliter), and then stored in the refrigerator at 4℃.

For analysis, all the samples were diluted 10,000 times with ultrapure water before injection.

Method validation

Linearity

The linearity was assessed by the correlation coefficients (r^2) of the calibration curves, which were generated by standard mixtures spiked with BA, BG, WO and WG. Peak areas of analytes were recorded and calibration curves were drawn, then regression equations of the curves and r^2 were calculated.

Precision

The precisions were evaluated by quality control samples prepared above at high- (500 ng/mL), medium- (100 ng/mL) and low-level (10 ng/mL) by consecutive 3 repeating analysis. Relative standard deviation (RSD%) was calculated as an estimate of precision.

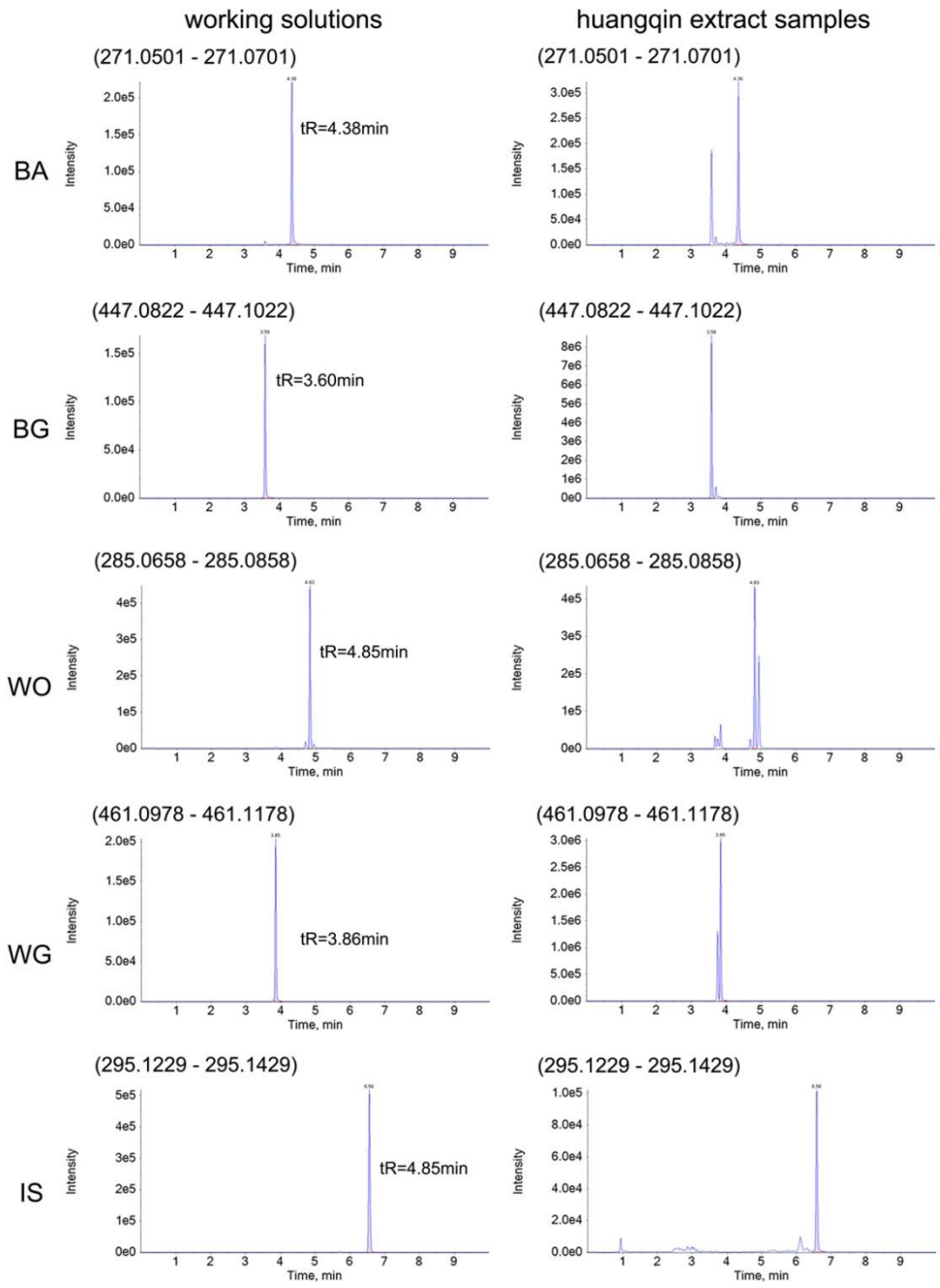
Stability

Three concentrations of samples were assayed at 24, 48 and 72 hours after huangqin extract was prepared. The averages and standard deviation (SD) values of concentrations for 4 constituents in 72 hours were calculated and RSD% was calculated as an estimate of stability.

Results

Method validation

The typical chromatograms of 4 analytes in standard solution and sample solution of huangqin extract are shown in **Additional Figure 3**.



Additional Figure 3 Typical chromatograms of 4 flavonoids and internal standard performed by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-TOF-MS) in working solution and sample of huangqin flavonoids extraction.

The calibration curves, correlation coefficients, linear ranges, precision and stability within 72 hours of the 4 analytes are shown in **Additional Table 2**. The calibration curves of 4 analytes exhibited good linearity within the selected ranges with the correlation coefficients (r^2) between 0.996 and 0.999. All the results indicated that the established method had a satisfactory precision and stability.

Concentration of 4 flavones in huangqin extract

The prepared sample of huangqin extract was injected and analyzed for 3 times. The areas of 4 analytes were recorded and concentrations of calculated with regression equations.

The concentrations of BA, BG, WO and WG in the huangqin extract were 1.68 ± 0.04 mg/g, 54.06 ± 1.07 mg/g, 0.90 ± 0.03 mg/g and 14.26 ± 0.22 mg/g, respectively.

Reference

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