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Short communication

High-performance liquid chromatographic method for simultaneous determination of baicalein and baicalein 7-glucuronide in rat plasma

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Abstract

A simple HPLC method with ultraviolet detection was developed for the simultaneous determination of baicalein and its major metabolite, baicalein 7-glucuronide (baicalin) in rat plasma. Following solid phase extraction with HLB cartridge, the analytes were separated using a gradient mobile phase consisting of methanol–acetonitrile–phosphate buffer. The flow-rate was set at 1 ml/min and the eluent was detected at 320 nm. The method is linear over the studied range of 1–10 and 0.05–1 μg/ml for baicalein and baicalin, respectively. The intra-day and inter-day variations of the analysis were less than 6.38%, with relative error ranging from −3.6 to 6%. The limit of quantification in plasma was 0.05 μg/ml for both baicalein and baicalin. The developed method proves to be an improved and more reliable method because of its capability for determining baicalein and baicalin simultaneously in a single chromatographic run.

Keywords: Baicalein; Baicalin

1. Introduction

Flavonoids have aroused increasing awareness because of their potential health beneficial effect [1]. Baicalein (B), a flavonoid found in the root of *Scutellaria baicalensis*, has been widely used in traditional Chinese medicine for a long time [2]. Baicalin (BG), the 7-*O*-glucuroinide of baicalein, is the predominant metabolite found in the blood after administration of baicalein to rodents [3]. The reported pharmacological effects of both B and its metabolites, BG, include anti-inflammatory [4], anti-allergic [5], anti-oxidative [6], antibacterial effect [7].

As both B and BG are bioactive, it is essential to develop a method to determine all of them in plasma samples. Back in early 1990s, separated HPLC systems and plasma sample preparation methods for B and BG were already reported by Wakui et al. [8]. But it is not convenient and quite sample consuming. Recently, Lai et al. developed a HPLC method to determine B only in rat plasma and its conjugated metabolites were quantified by detecting the increased concentration of B in plasma after 8-h treatment of β-glucuronidase/sulfatase [9]. However, as conjugated metabolites of B include not only baicalein 7-*O*-glucuronide but also the glucuronides of baicalein at 6-OH and/or 5-OH position, baicalein sulfates as well as baicalein glucuronidated and sulfated multi-conjugates [10]. Such enzymatic hydrolysis approach is not specific for the determination of BG concentration in plasma since it cannot distinguish individual conjugates.

In the present study, we have developed a reliable and specific HPLC method for the simultaneous determination of B and BG in rat plasma after solid phase extraction. In addition, we applied the developed method to the simultaneous determination of rat plasma levels of B and BG following intravenous administration of B.

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2. Materials and methods

2.1. Reagents and chemicals

Baicalein and Baicalin were purchased from Aldrich Chem Co. 6-Hydroxyflavone (6-HF) used as an internal standard (IS) was from Indofine Chemical Co. Waters Oasis® hydrophilic–lipophilic-balanced (HLB, 1cc) copolymer extraction cartridges were purchased from Waters. Acetonitrile (HPLC grade) and methanol (analytical grade) were obtained from Labscan (Labscan Asia, Thailand). All reagents were of analytical grade and used without further purification. Distilled and deionized water was used for the preparation of all solutions.

2.2. Instruments

The HPLC system is composed of Waters 600 controller (pump), Waters 717 auto sampler and Waters 2487 dual wavelength detector. The chromatographic separation of B, BG and internal standard was achieved by using a reversed-phase HPLC column (BDS reversed phase column, 25 cm × 4.6 mm i.d.; 5 μm particle size, Thermo Hypersil) connected with a guard column (Delta-Pak C_{18} Guard-Pak, Waters).

2.3. Chromatographic conditions

The mobile phase, consisting of a mixture of methanol, acetonitrile and 20 mM sodium dihydrogen phosphate buffer (pH 4.6), was run using a linear gradient elution program as shown in Table 1. The flow-rate was 1 ml/min, and the total running time was 25 min. The auto-sampler was set at 10 °C. The detection wavelength was set at 320 nm for B and BG.

2.4. Preparation of standard solutions

Stock solutions of B and BG (1 mg/ml) were prepared separately by dissolving the appropriate amount of each authentic sample in methanol. The working solutions containing both B and BG were prepared by mixing and diluting the above separate solutions with methanol and phosphate buffer (pH 2.5) comprising 1% ascorbic acid (50:50 (v/v)) to yield concentrations of 150, 100, 50, 20, 10, 5, 2, 1, and 0.5 μg/ml, respectively.

Table 1
HPLC mobile phase for simultaneous detection of BG, B and 6-hydroxyflavone

Time (min)	Flow rate (ml/min)	Percentage of each eluent (%)		
		Methanol	ACN	Buffer[a]
0	1	5	15	80
5	1	8	35	57
12	1	0	60	40
18	1	5	15	80
20	1	5	15	80
25	1	5	15	80

[a] 20 mM sodium dihydrogen phosphate buffer (pH 4.6).

Standard solution of IS was prepared by dissolving the appropriate amount of 6-HF in a solution containing methanol and phosphate buffer (pH 2.5) comprising 1% ascorbic acid (50:50 (v/v)) to yield a concentration of 10 μg/ml.

The samples for plasma standard calibration curves were prepared by spiking the blank rat plasma (100 μl) with 10 μl of the appropriate working solutions to yield the following concentrations of B and BG: 15, 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/ml, respectively. Specific quality control samples representing low, medium and high concentration were 0.1, 1, 5 and 10 μg/ml for both BG and B.

2.5. Calibration curves

The plasma samples for standard curve were prepared as described in Section 2.4. Calibration curves were plotted by the peak-area ratios of each analyte/internal standard versus concentrations of analyte in plasma. In order to avoid undue bias to the low concentrations of the standard curve by the high concentrations, the calibration curves of both BG and B were split into two ranges: 10.0–1.0 μg/ml and 1.0–0.05 μg/ml.

2.6. Sample preparations

The Oasis® HLB cartridge was preconditioned with 1 ml of methanol, followed by 1 ml of 25 mM sodium dihydrogen phosphate buffer (pH 2.5). In 100 μl of plasma sample, 10 μl of 50% methanol in phosphate buffer (pH 2.5) comprising 1% ascorbic acid [11] and 50 μl of internal standard (6 HF, 10 μg/ml) were added. The sample was then diluted with 1 ml of 35% methanol in 25 mM sodium dihydrogen phosphate buffer (pH 2.5) containing 1% ascorbic acid. After vortexing for 15 sec and centrifuged at 16,000 × *g* for 10 min, the supernatant was loaded on the preconditioned HLB cartridge. The cartridge was flushed with 1 ml of 25 mM sodium dihydrogen phosphate buffer (pH 2.5) followed by 2 ml of 50% methanol in 25 mM sodium dihydrogen phosphate buffer (pH 2.5) containing 1% ascorbic acid. The analytes were eluted from the cartridge by 1 ml methanol. The eluent was then evaporated to dryness in a Centrivap concentrator, and the residue was reconstituted with 100 μl of 35% methanol in 25 mM sodium dihydrogen phosphate buffer (pH 2.5) containing 1% ascorbic acid. An aliquot of 50 μl was injected into the HPLC column.

2.7. Validation of the assay method

2.7.1. Specificity

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

2.7.2. Precision and accuracy

The intra-day precision was determined within one day by analyzing five replicates control samples at concentrations of 0.1, 1, 5 and 10 μg/ml. The inter-day precision was determined on five 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 to 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 of −20 to 20%.

2.7.5. Stability

Freeze–thaw stability of the plasma samples was evaluated by exposing quality control samples to three freeze–thaw cycles before sample preparation. The stability of the samples in auto-sampler was evaluated by analyzing extracted quality control samples after being placed in the auto-sampler at 10 °C for 24 h.

2.8. Assay application

B was administrated to five male Sprague Dawley rats intravenously with bolus dose of 10 mg/kg. Venous blood samples were collected and centrifuged at 1, 3, 5, 10, 12, 14, 16, 20, 60, 120, 180, 240, 360, 480 and 600 min, respectively. The plasma sample was stored at −80 °C until analysis.

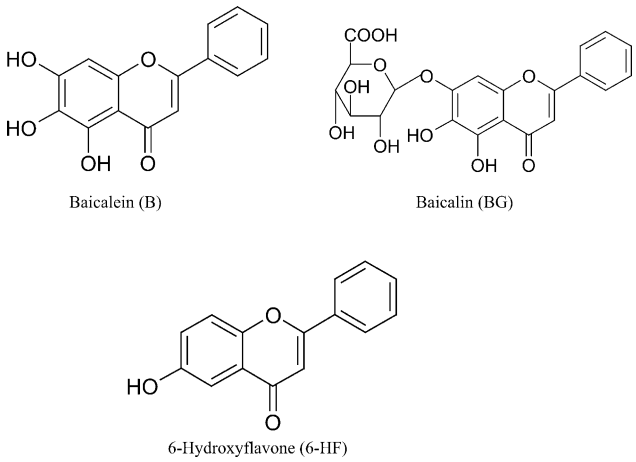

Fig. 1. Chemical structure of Baicalein (B), Baicalin (BG) and 6-hydroxyflavone (6-HF).

3. Results and discussion

Because of the significant difference in polarity between B and BG, simultaneous determination of B and BG in plasma with good sensitivity becomes difficult in a single HPLC run. Therefore, most of the published method, e.g. Wakui et al., used separate HPLC methods with two isocratic mobile phases to determine B and BG individually. The major contribution of the present HPLC method is to employ a gradient elution program to reduce the HPLC running time and give satisfactory specificity and sensitivity for both B and BG at the same time. In addition, both liquid–liquid extraction and protein precipitation had been tried for sample preparations in the current study. However, serious interferences to both analytes, especially BG, were observed in the chromatograms. Hence, solid phase extraction was employed for sample preparation Fig. 1.

Table 2
Linearity of calibration curve, intra-day, inter-day accuracy and precision for determination of B and BG in plasma

Compounds	Nominal concentration (μg/ml)	Intra-day (n = 5)			Inter-day (n = 5)			Linearity	
		Determined concentration (mean ± S.D. (μg/ml))	Precision (%R.S.D.)	Accuracy (%R.E.)	Determined concentration (mean ± S.D. (μg/ml))	Precision (%R.S.D.)	Accuracy (%R.E.)	Range (μg/ml)	R^2
BG	10.0	9.80 ± 0.12	1.18	−2.0	9.99 ± 0.29	2.88	−0.1	10.0–1.0	0.9999
	5.0	5.00 ± 0.07	1.40	0.0	5.04 ± 0.10	1.94	0.8		
	1.0	0.964 ± 0.017	1.77	−3.6	0.983 ± 0.029	2.92	−1.7	1.0–0.05	0.9999
	0.10	0.105 ± 0.002	1.62	5.0	0.102 ± 0.006	5.92	2.0		
LOQ	0.05	0.052 ± 0.004	6.99	4.0	0.050 ± 0.006	11.2	0.0		
B	10.0	9.79 ± 0.12	1.25	−2.1	10.0 ± 0.2	2.32	0.0	10.0–1.0	0.9999
	5.0	4.93 ± 0.03	0.67	−1.4	5.03 ± 0.15	2.89	0.6		
	1.0	1.00 ± 0.01	1.14	0.0	1.03 ± 0.03	2.68	3.0	1.0–0.05	0.9998
	0.10	0.106 ± 0.007	6.38	6.0	0.105 ± 0.005	5.18	5.0		
LOQ	0.05	0.049 ± 0.002	3.35	−2.0	0.050 ± 0.003	6.20	0.0		

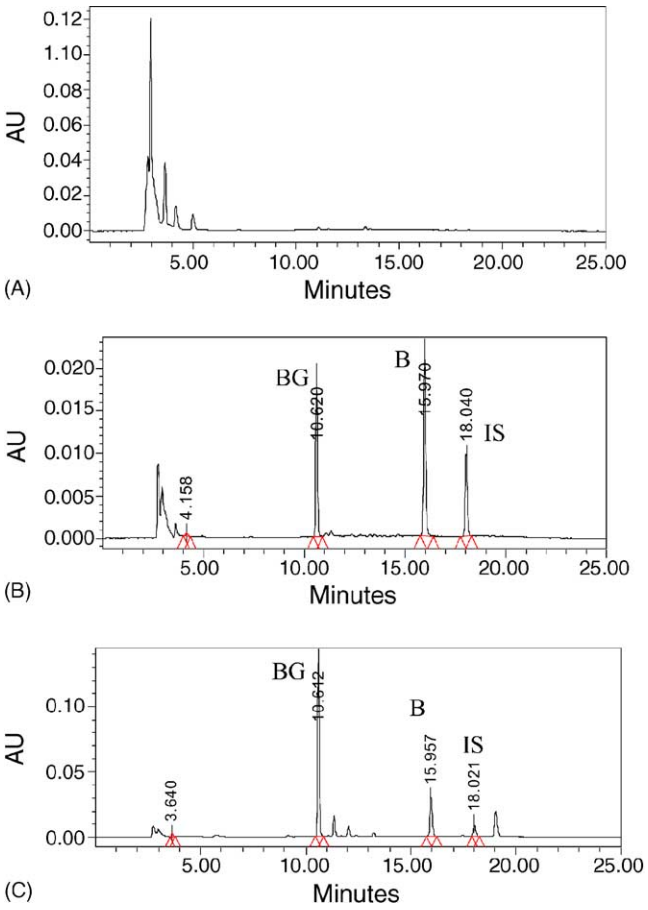

Fig. 2. Chromatograms of rat plasma samples. (A) Blank plasma; (B) blank plasma spiked with both B and BG at the concentration of 1 μg/ml; (C) plasma sample obtained 10 min after intravenous administration of B (10 mg/kg) to a rat.

3.1. Specificity

The representative chromatograms of blank plasma, plasma samples spiked with B and BG, and a plasma sample obtained from a rat following iv administration of B (10 mg/kg) were shown in Fig. 2. Under the assay condition, no interference from the endogenous plasma was observed at the retention time of both the analytes and the internal standard.

Table 3
Stability of BG and B in plasma after three freeze-thaw cycles as well as in prepared samples in auto-sampler for 24 h

Compounds	Nominal concentration (μg/ml)	Determined concentration (mean ± S.D. (μg/ml))	
BG	10.0	9.85 ± 0.26[a]	9.87 ± 0.22[b]
	5.0	4.87 ± 0.03[a]	5.08 ± 0.31[b]
	1.0	0.96 ± 0.02[a]	0.96 ± 0.11[b]
	0.10	0.094 ± 0.002[a]	0.092 ± 0.002[b]
B	10.0	10.4 ± 0.1[a]	9.49 ± 0.65[b]
	5.0	5.11 ± 0.03[a]	4.89 ± 0.21[b]
	1.0	1.03 ± 0.01[a]	0.937 ± 0.005[b]
	0.10	0.107 ± 0.003[a]	0.092 ± 0.004[b]

[a] In plasma after three freeze-thaw cycles.
[b] In prepared samples in auto-sampler for 24 h.

3.2. Precision, accuracy, linearity and sensitivity

The inter-day and intra-day precision and accuracy of the analytical method are listed in Table 2. The R.S.D. of both inter-day and intra-day for B and BG were below 6.38%. The accuracy calculated as the relative error of inter-day and intra-day at low, medium, high concentration were within the range of −3.6 to 6%. In addition, as shown in Table 2, calibration curves for B and BG were linear over both the low and high concentration ranges with satisfied regression coefficients. For both B and BG, the LOQ was 0.05 μg/ml and the LOD was 0.03 μg/ml.

3.3. Recovery

The recoveries of B and BG in plasma at low, medium and high concentration were determined and ranged from 79.3 to 91.0% for BG and 79.7 to 93.1% for B, respectively. Because of the weak acid nature of BG, an acidic wash buffer (pH 2.5) was chosen to flush the cartridge in order to increase the retention of BG in the HLB cartridge so as to increase its recovery. In addition, instead of using Bond Elut C_{18} as suggested by Wakui et al. [8], the present method employed Oasis® HLB

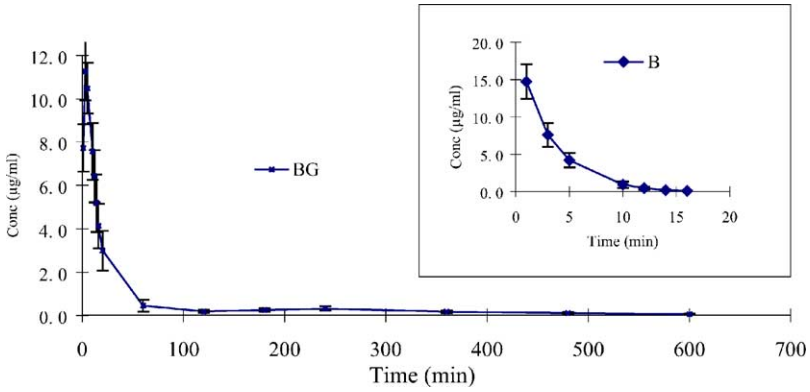

Fig. 3. Plasma concentration of B and BG vs. time profile in rats after intravenous administration of B (10 mg/kg) (n = 5).

cartridge containing water-wettable sorbent so that there is no impact of sorbent drying. Moreover, optimization of the percentage of methanol in the wash buffer indicated that 50% methanol in wash buffer could provide both clean background of the samples and satisfactory recoveries for all the analytes.

3.4. Stability

The stability experiment indicated that both B and BG were stable for at least three freeze-thaw cycles (Table 3). In addition, B and BG were also stable in the prepared samples after placing in the auto-sampler for 24 h at 10 °C (Table 3).

3.5. Application to in vivo pharmacokinetics study

The validated HPLC-UV method has been successfully applied to simultaneously determine the concentrations of B and BG in plasma samples obtained after intravenous administration of 10 mg/kg of B to rat. The resulted pharmacokinetic profiles of B and BG are shown in Fig. 3. The results demonstrated that B was quickly metabolized to BG in vivo and cannot be detected after 20 min of the dosing, whereas BG could still be detected 6 h post dosing.

4. Conclusion

A simple and specific HPLC-UV analytical method for simultaneous determination of B and BG in rat plasma has been developed. This method has been fully validated with satisfactory accuracy and adequate reproducibility. The successful application of the developed analytical method demonstrates that the current method can be readily utilized for the simultaneous determination of B and its major metabolites BG in plasma samples.

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References

[1] C.S. Yang, J.M. Landau, M.T. Huang, H.L. Newmark, Ann. Rev. Nutr. 21 (2001) 381–406.
[2] D. Yang, H. Hu, S. Huang, J.P. Chaumont, J. Millet, Zhong Yao Cai 23 (2000) 272–274.
[3] T. Akao, K. Kawabata, E. Yanagisawa, K. Ishihara, Y. Mizuhara, Y. Wakui, Y. Sakashita, K. Kobashi, J. Pharm. Pharmacol. 52 (2000) 1563–1568.
[4] T. Nakajima, M. Imanishi, K. Yamamoto, J.C. Cyong, K. Hirai, Planta Med. 67 (2001) 132–135.
[5] A. Koda, H. Nagai, H. Wada, Nippon Yakurigaku Zasshi 66 (1970) 237–247.
[6] Y. Kimura, M. Kubo, T. Tani, S. Arichi, H. Okuda, Chem. Pharm. Bull. 29 (1981) 2610–2617.
[7] I.X. Liu, D.G. Durham, R.M. Richards, J. Pharm. Pharmacol. 52 (2000) 361–366.
[8] Y. Wakui, E. Yanagisawa, E. Ishibashi, Y. Matsuzaki, S. Takeda, H. Sasaki, M. Aburada, T. Oyama, J. Chromatogr. A 575 (1992) 131–136.
[9] M.Y. Lai, S.L. Hsiu, S.Y. Tsai, Y.C. Hou, P.D. Chao, J. Pharm. Pharmacol. 55 (2003) 205–209.
[10] K. Abe, O. Inoue, E. Yumioka, Chem. Pharm. Bull. 38 (1990) 209–211.
[11] M.V. Martinez-Ortega, M.C. Garcia-Parrilla, A.M. Troncoso, Anal. Chim. Acta 502 (2004) 49–55.