Journal of Chromatography, 575 (1992) 131–136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6220

Determination of baicalin and baicalein in rat plasma by high-performance liquid chromatography with electrochemical detection

Yoko Wakui*, Erika Yanagisawa, Emiko Ishibashi, Yutaka Matsuzaki, Shuichi Takeda, Hiromi Sasaki, Masaki Aburada and Tsutomu Oyama

Research Institute for Pharmacology, Tsumura & Co., 3586, Yoshiwara, Ami-machi, Inashiki, Ibaraki 300-11 (Japan)

(First received September 5th, 1991; revised manuscript received November 14th, 1991)

ABSTRACT

A rapid and sensitive method, using electrochemical detection, has been developed for the determination of baicalin and baicalein, the flavonoid of *Scutellariae radix*, in rat plasma. Following separation by high-performance liquid chromatography, baicalin and baicalein were oxidized at a glassy carbon electrode to permit selective electrochemical detection. Absolute detection limits were found to be 5 ng/ml from 50 μl of plasma for baicalin and 2 ng/ml from 100 μl of plasma for baicalein. The resulting assays were suitable for pharmacokinetic studies of baicalin and baicalein in rats.

INTRODUCTION

Baicalin (BG) and baicalein (B) (Fig. 1) are a flavone glucuronide and its aglycone, respectively, which are found in the root of *Scutellaria baicalensis* Georgi (Woogon), a substance frequently used in traditional Chinese and Japanese herbal medicine in the form of a "Kampo decoction" as a remedy for inflammation and allergic diseases. BG and B have been shown to have anti-inflammatory [1] and antiallergic [2] pharmacological activity and to have effects on hyperlipidemia and lipolysis [3] *in vivo* and *in vitro*. Thus, in order to elucidate the biological effects of such medicines, it is necessary to investigate the fate of BG and B in animals after oral administration; however, only their excretion into bile [4] and urine [5] have hitherto been reported.

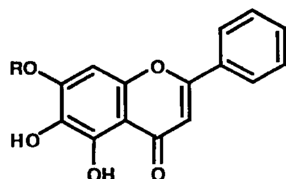

Fig. 1. Structures of baicalin (BG) and baicalein (B).

The determination of BG and B by high-performance liquid chromatography (HPLC) for the quantitative evaluation of Woogon and the quality control of pharmaceutical preparations has been reported [6–10]. However, in these procedures the detection limits were not sensitive enough to determine BG and B in plasma for pharmacokinetic studies. Many flavonoid glucosides are known to undergo microbial hydrolysis in the gut, and BG has been found to be transformed, in part, to B prior to absorption [4,11]. In order to study further the fate of BG and B, we developed new assays to determine BG and B in rat plasma in order to study their pharmacokinetics. Because of their high sensitivity, these as-

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says are suitable for the clinical pharmacokinetic study of BG and B following oral administration of Kampo decoctions to humans.

This paper describes a method for the sensitive determination of BG and B in a small amount of rat plasma, using HPLC and electrochemical detection.

EXPERIMENTAL

Chemicals and reagents

The BG and B used as standards in the assay were obtained from Wako (Osaka, Japan). The internal standards used were quercetin which was purchased from Nacalai Tesque (Kyoto, Japan) and 2,3-dihydroxynaphthalene which was purchased from Aldrich (Milwaukee, WI, USA). Other reagents used were HPLC grade or reagent grade.

Animals

Male Sprague–Dawley rats, eight weeks old (260–290 g), were obtained from Charles River Japan and were fasted overnight before being used in experiments.

Drug administration and plasma sample collection

In the case of oral treatment, BG or B was dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC) aqueous solution. Each rat received a 20 mg/kg oral dose of BG or a 200 mg/kg oral dose of B. For intravenous administration, BG or B was suspended in 2% Tween 80–saline and was injected into the caudal vein at a dose of 2 mg/kg of BG or 20 mg/kg of B. Blood samples were withdrawn from the jugular vein at appropriate intervals after administration. Plasma was separated by centrifugation and stored at −80°C until analysis.

Apparatus and liquid chromatographic conditions for BG and B determination

For BG determination, the HPLC system consisted of a constant-flow, double-piston pump LC-6A (Shimadzu, Kyoto, Japan), equipped with an automatic injector (SIL-6B autoinjector, Shimadzu), a C-R4AX computing integrator (Shimadzu), and a Bioanalytical Systems LC-4A amperometric detector, with a glassy carbon working electrode and an Ag/AgCl reference electrode (Eicom, Kyoto, Japan). The column used was a stainless-steel Nova-Pak (150 mm × 3.5 mm I.D.) (Waters Assoc., Milford, MA, USA) packed with 4-μm spherical particles of octadecylsilane (C_{18}). The mobile phase consisted of 0.1 *M* phosphoric acid–acetic acid–tetrahydrofuran (800:80:57, v/v/v). The column temperature was maintained at 50°C and the flow-rate was 1.2 ml/min. For determination of B, the HPLC instrumentation and column were the same as for BG determination. Only the flow-rate and the constituents of the mobile phase were changed: the mobile phase was 0.2 *M* phosphoric acid–methanol–tetrahydrofuran (500:250:34, v/v/v) and the flow-rate was 0.8 ml/min.

Sample preparation of BG

A solid-phase isolation Bond Elut C_{18} cartridge (Analytichem, Harbor City, CA, USA) was initially prepared by flushing with 2-propanol, 20% acetic acid–propanol, methanol, water and 50 m*M* sodium phosphate buffer (pH 6.5). A 100-μl volume of 2,3-dihydroxynaphthalene (50 ng/ml, internal standard), 50 μl of plasma, and 500 μl of 50 m*M* sodium phosphate buffer (pH 6.5) were passed through the cartridge. The cartridge was flushed with 2 ml of water and 1 ml of 0.15 *M* phosphoric acid, and the components of interest were eluted with 2 ml of 20% acetic acid–propanol. The eluent was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 100 μl of the mobile phase. An aliquot (20 μl) was injected into the HPLC column. The sample for the calibration curve was prepared in a similar manner, except that a stock solution of BG was added to the blank plasma at concentrations ranging from 5 to 2000 ng/ml of plasma. The peak-area ratio of BG to the internal standard was plotted against a concentration of BG spiked to obtain a standard curve.

Preparation of a low-level sample of B by solid-phase isolation method

A Bond Elut PH cartridge (Analytichem) was

initially prepared by flushing with 2-propanol, 20% acetic acid–2-propanol, methanol, water and 50 mM sodium phosphate buffer (pH 4.0). A 100-μl volume of quercetin (50 ng/ml, internal standard), 500 μl of the above buffer and 100 μl of plasma were passed through the cartridge. The cartridge was flushed with 2 ml of 0.15 M phosphoric acid and 1 ml of water, and the components of interest were eluted with 2 ml of 20% acetic acid–2-propanol. The eluent was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 50 μl of 0.15 M phosphoric acid–methanol and 50 μl of the mobile phase. An aliquot (20 μl) was injected into the HPLC column. The sample for the calibration curve was prepared in a similar manner, except that a stock solution of B was added to the blank plasma, at concentrations ranging from 2 to 50 ng/ml of plasma. The peak-area ratio of B to the internal standard was plotted against a concentration of B spiked to obtain a standard curve. This method was used for the determination of B in rat plasma after oral administration of B.

Sample preparation of B by liquid–liquid extraction method

The interfering peak from plasma was removed by the solid-phase isolation method, but the recovery of B at high concentrations was not sufficient. A liquid–liquid extraction method was chosen for B at high concentrations, such as plasma levels in the rat after intravenous administration of B. A 100-μl volume of quercetin (250 ng/ml, internal standard) was added to 50 μl of plasma, after which 200 μl of 2 M hydrochloric acid and 3 ml of ethyl acetate were added. The resulting mixture was vigorously shaken for 15 min. Centrifugation at 1500 g for 5 min provided a clear organic layer. This organic layer was transferred to another tube, and the residue was again extracted with 3 ml of ethyl acetate. The combined organic layers were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 500 μl of 0.15 M phosphoric acid–methanol and 500 μl of the mobile phase. An aliquot (20 μl) was injected into the HPLC column. The sample for the calibration curve was prepared at concentrations ranging from 20 to 5000 ng/ml. The peak-area ratio of B to quercetin was plotted against the corresponding concentration of B spiked to obtain a standard curve for B.

RESULTS AND DISCUSSION

HPLC conditions

Since BG and B contain catechols in their molecular structure, sensitive and selective electrochemical detection is possible. The cyclic voltammogram of BG, determined by cyclic voltammetry (CV-1B, Bioanalytical Systems), showed that the peak potential of the oxidation wave was +640 mV *vs.* an Ag/AgCl reference electrode. For a highly sensitive analysis without loss of selectivity and reproducibility, the value +650 mV (5 nA full scale) was adopted for this procedure. Electrochemical detection is favoured in a mobile phase of relatively low organic content, and for this reason a mixture of distilled water, acetic acid, and a small amount of tetrahydrofuran was used as the mobile phase. Typical chromatograms are shown in Fig. 2. The peak potential of the oxidation wave of B was determined as +570 mV by cyclic voltammetry. To reduce the intensity of the interfering peak considerably, B was detected at a potential of +450 mV (2 nA full scale) *vs.* Ag/AgCl. Figs. 3 and 4 show typical chromatograms of drug-free plasma, B-spiked plasma, and plasma obtained from a rat that was administered B. Because of the great difference in polarity between B and BG, it was necessary to use ion-pair HPLC to detect the two compounds simultaneously; however, when using an electrochemical detector it is difficult to get sensitive determinations using ion-pair reagents because of the increase of noise levels in the baseline. We therefore examined reversed-phase HPLC conditions using two kinds of mobile phase system to determine BG and B.

Extraction conditions

Despite the high resolving power of HPLC and the selectivity of the detector, plasma must be purified prior to the determination of BG. Since deproteinization with methanol or extraction by

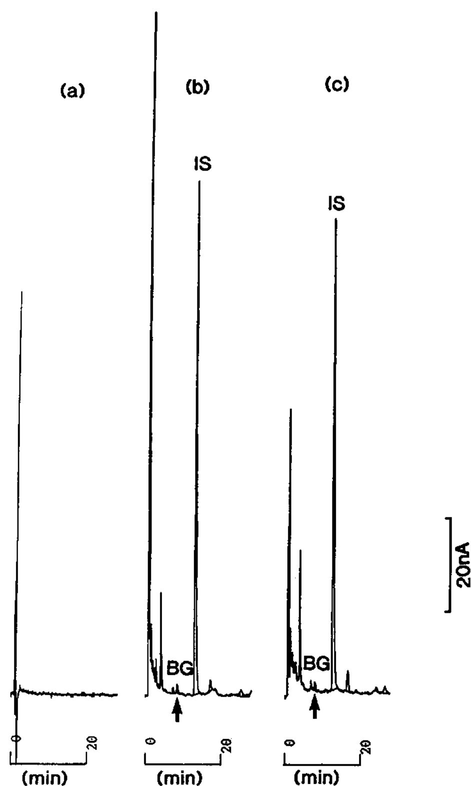

Fig. 2. Chromatograms of rat plasma. (a) Drug-free plasma; (b) plasma spiked with 10 ng/ml BG (100 pg of BG); (c) plasma obtained form a rat 32 h after oral administration of BG (20 mg/kg). Column, Novapak C_{18}; flow-rate, 1.2 ml/min; injection volume, 20 μl; applied potential, +650 mV, 5 nA full scale; recorder attenuation, × 32.

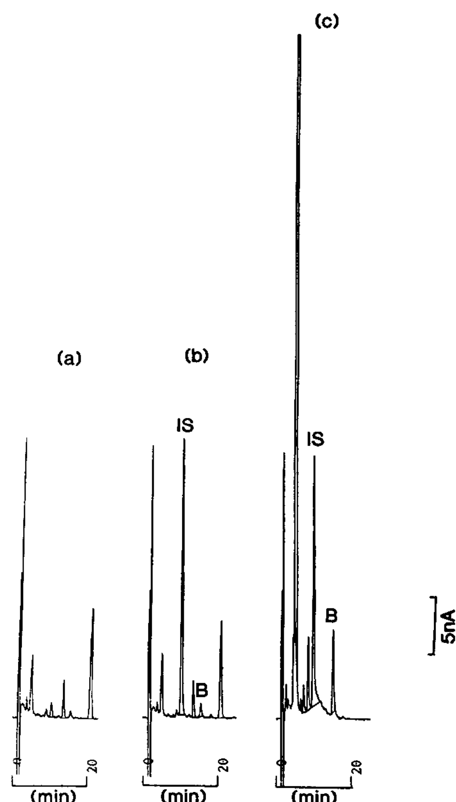

Fig. 3. Solid-phase isolation chromatograms of rat plasma. (a) Drug-free plasma; (b) plasma spiked with 1 ng/ml B (20 pg of B); (c) plasma sample obtained from a rat 8 h after oral administration of B (200 mg/kg). Column, Novapak, C_{18}; flow-rate, 0.8 ml/min; injection volume, 20 μl; applied potential, +450 mV, 2 nA full scale; recorder attenuation, × 32.

other organic solvents was not sufficient to thoroughly separate BG from interfering substances, Bond Elut C_{18} was used to remove the interfering peak from the chromatogram. The recovery of BG added to plasma was rather low under the conditions: the recovery of BG-spiked plasma at each concentration was as follows (mean ± S.D.): at 10 ng/ml, 68.8 ± 7.5%; at 100 ng/ml, 67.1 ± 3.8%; at 2000 ng/ml, 71.0 ± 5.4%. However, the sensitivity and reproducibility were suitable for clinical studies, with a detection limit of 5 ng/ml from 50 μl of rat plasma, based on a signal-to-noise ratio of 2. For the extraction of B from plasma two different methods were adopted, depending on the route of the administration, which affected the concentration of B. In this study we used a solid-phase isolation method for orally administered B and a liquid–liquid extraction method for intravenous B. The recovery of B spiked to plasma (mean ± S.D.) by the solid-phase isolation method was 117.1 ± 10.9% at 2 ng/ml, 99.2 ± 8.6% at 5 ng/ml and 88.4 ± 0.5% at 50 ng/ml. The recovery by the liquid–liquid extraction method was 90.5 ± 7.0% at 20 ng/ml, 95.5 ± 1.4% at 200 ng/ml and 98.1 ± 2.7% at 1000 ng/ml.

Linearity and precision

Calibration curves were prepared from quadruplicate or quintuplicate plasma samples spiked with BG ranging from 5 to 2000 ng/ml or with B ranging from 2 to 5000 ng/ml. In all cases,

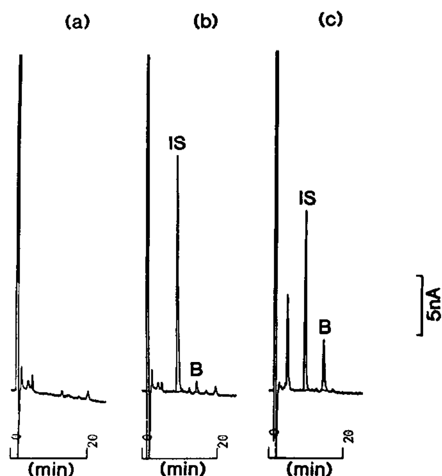

Fig. 4. Liquid–liquid extraction chromatograms of rat plasma. (a) Drug-free plasma; (b) plasma spiked with 200 ng/ml B (20 pg of B); (c) plasma obtained from a rat 2 h after intravenous administration of B (20 mg/kg). All HPLC conditions were the same as those described in Fig. 3.

the calibration curves were linear; least-squares analysis of the calibration curves yielded the following characteristics: for BG, $y = 586.71x + 2.68$, $r = 0.997$; for B, by the solid-phase isolation method, $y = 34.8x - 0.9$, $r = 1.000$; and for B, by the liquid–liquid extraction method, $y = 396.9x + 9.0$, $r = 1.000$ (y = concentration of BG or B in ng/ml; x = ratio of peak area; r = correlation coefficient). To evaluate the reproducibility of the system and the method, intra-assay precision studies were conducted. The coefficient of variation (C.V.) ranged from 4.6 to 8.6% for BG and from 0.6 to 8.8% for B (Tables I, II and III).

TABLE I

PRECISION AND REPRODUCIBILITY OF BAICALIN DETERMINATION IN RAT PLASMA BY SOLID-PHASE ISOLATION METHOD

Added (ng/ml)	Found (mean ± S.E., n = 5) (ng/ml)	C.V. (%)	Accuracy (%)
5	9.1 ± 0.5	12.4	181.4
10[a]	12.4 ± 0.5	8.6	124.2
100	96.9 ± 2.0	4.6	96.9
500	492.5 ± 13.7	6.2	98.5
2000	2002.0 ± 64.3	7.2	100.1

[a] n = 4.

TABLE II

PRECISION AND REPRODUCIBILITY OF BAICALEIN DETERMINATION IN RAT PLASMA BY SOLID-PHASE ISOLATION METHOD

Added (ng/ml)	Found (mean ± S.E., n = 4) (ng/ml)	C.V. (%)	Accuracy (%)
2	2.0 ± 0.1	8.8	101.2
5	5.0 ± 0.2	8.6	99.9
50	50.0 ± 0.4	2.7	100.0

Gastrointestinal absorption of BG and B

The average plasma levels of BG in four or five rats after oral and intravenous administration are shown in Fig. 5. The profiles show that there was a time lag in the absorption of BG after oral administration. After the BG concentration reached the C_{max} level, its elimination was slow. The absolute bioavailability of BG, calculated from the area under the time–concentration curve (AUC), was 62%. Fig. 6 shows the profiles of B plasma levels after oral and intravenous administration. When B was administered orally, its plasma levels were very low.

TABLE III

PRECISION AND REPRODUCIBILITY OF BAICALEIN DETERMINATION IN RAT PLASMA BY LIQUID–LIQUID EXTRACTION METHOD

Added (ng/ml)	Found (mean ± S.E., n = 4) (ng/ml)	C.V. (%)	Accuracy (%)
20	27.0 ± 0.7	5.3	135.1
200	196.6 ± 1.6	1.6	98.3
1000	995.3 ± 6.7	1.3	99.5
5000	5001.0 ± 14.4	0.6	100.0

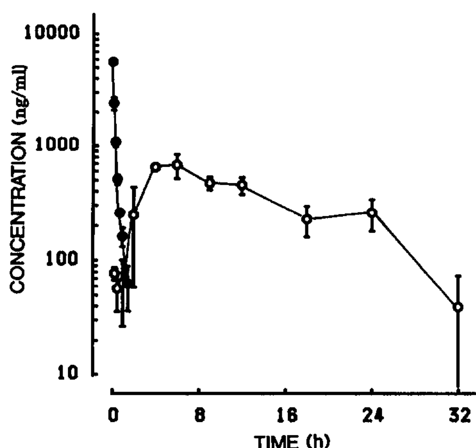

Fig. 5. Plasma levels of BG after (○) oral administration (20 mg/kg) and (●) intravenous administration (2 mg/kg) to rats. Each point represents the mean and S.E. of four or five rats.

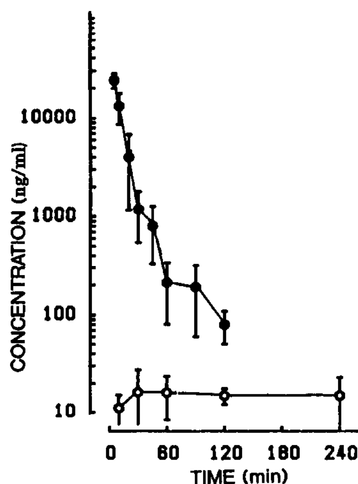

Fig. 6. Plasma levels of B after (○) oral administration (200 mg/kg) and (●) intravenous administration (20 mg/kg) to rats. Each point represents the mean and S.E. of six rats.

CONCLUSION

The use of an electrochemical detector, under optimal consant conditions, resulted in sensitive, precise and reliable measurements of BG and B in rat plasma, at concentrations as low as 5 and 2 ng/ml, respectively. This technique is a new highly sensitive method for the quantitative analysis of BG and B in biological specimens such as rat plasma. It can be used for clinical pharmacokinetic and bioavailability studies of the small amounts of BG and B that are contained in Japanese Kampo preparations.

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