Baicalin 6-O-β-d-Glucopyranuronoside Is a Main Metabolite in the Plasma after Oral Administration of Baicalin, a Flavone Glucuronide of Scutellariae Radix, to Rats

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Baicalin (BG) and its aglycone, baicalein (B), are strong antioxidants that exert various pharmacological actions and show unique metabolic fates in the rat. The aim of the present study was to identify major metabolite(s) besides BG in rat plasma after oral administration of BG or B. The main metabolite was detected by HPLC equipped with an electrochemical detector at a potential of +500 mV and identified as baicalin 6-O-β-d-glucopyranuronoside (B6G) by HPLC/MS/MS. When BG at a dose of 20 mg/kg was administered orally to Wistar rats, the level of B6G in plasma was higher than that of BG. Cmax and the area under the concentration–curve from 0 to 24 h (AUC0–24h) values of the plasma B6G were 1.66±0.34 µM and 19.8±3.9 µM·h, respectively, whereas those of BG were 0.85±0.065 µM and 10.0±3.1 µM·h, respectively. When B was administered, similar results were also obtained. B6G-producing activities from B were found in microsomes of both rat jejunum and liver, in spite of the low activity. Rat everted jejunal sacs formed B6G after application of B, but only in a small amount that was excreted into the mucosal side, and not the serosal side, indicating little contribution to the appearance of B6G in plasma. On the other hand, when B was injected into the rat portal vein, B6G was detected at a higher level than BG in the systemic circulation, demonstrating the hepatic contribution to the appearance of plasma B6G.

Key words baicalin; baicalein 6-O-β-d-glucopyranuronoside; metabolite; rat

Scutellariae Radix, the root of Scutellaria baicalensis Georgi (Labiatae), is used in combination with other herbs in Chinese traditional (Kampo, in Japanese) medicines, and contains baicalin (BG), 5,6,7-trihydroxyflavone-7-β-d-glucuronide; Fig. 1) as its main active constituent. BG and its aglycone, baicalein (B), have wide-ranging pharmacological effects, such as antiallergic, anti-inflammatory, antiviral, antiproliferative, and antitumor effects, and also show strong antioxidant activity. In addition, they inhibit prostaglandin E2 production, and prevent the gene expression of inducible nitric oxide synthase and cyclooxygenase-2. B also has an anxiolytic-like effect and a protective effect against amnesia.

BG has been detected in rat plasma after its oral administration, suggesting that it is absorbed directly from the gastrointestinal tract. However, in our previous study when BG was given to germ-free rats, only a small amount of BG was detected in their plasma. In addition, BG, and not B, has been detected even after oral administration of B to rats. On the other hand, many kinds of metabolites such as B-glucuronides and B-sulfates besides BG have been found in bile after oral administration of either BG or B to rats. Many kinds of B-metabolites have also been detected in bile and urine after oral administration of Scutellariae Radix extract or Chinese traditional medicines that include Scutellariae Radix. In addition, unidentified metabolites besides BG in rat plasma have been found after oral administration of Kampo medicine including Scutellariae Radix, after being hydrolyzed enzymatically using β-glucuronidase and sulfatase. However, types of metabolites besides BG that are present in plasma after oral administration of BG or B to rats are still unclear.

The aim of the present study was to identify metabolite(s) other than BG in plasma and to evaluate its pharmacokinetics after oral administration of BG or B to rats.

MATERIALS AND METHODS

Chemicals BG and B of standard grade were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The solvents used for HPLC were of special HPLC grade. All other reagents were of the best quality available commercially.

Apparatus 1H- and 13C-NMR spectroscopy were performed with a JNM LA 400 NMR spectrometer (1H, 400 MHz; 13C, 100 MHz, JEOL Co.). HPLC/MS coupled with photodiode array detection (PAD) was carried out on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with degasser, binary pump, PAD, electrospray ionization and ion-trap analyzer mass spectrometer (ESI-MS), Esquire 3000 plus, Bruker Daltonik GmbH, Bremen, Germany). Data were acquired and integrated using a ChemStation. The HPLC/MS/MS data were acquired using Esquire Control soft-
Animals and Treatment Male Wistar rats at 6 weeks of age were purchased from SLC Co. (Hamamatsu, Japan), fed standard laboratory chow with water *ad libitum*, and maintained for one week before being subjected to the experiments. The rats were maintained in the Laboratory Animal Research Center, and the animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. After an overnight fast, the rats received BG or B orally at a dose of 20 mg/kg or 12.1 mg/kg (a dose equivalent to 20 mg BG/kg), respectively. The BG was dissolved in dimethyl sulfoxide (DMSO, final concentration 6% v/v), then diluted to 12.1 mg/mL with water just before administration. Blood samples were withdrawn from the tail vein of the rats at 0, 3, 6, 9, 15, 21, 30, 45, 60, 90, and 120 min after injection, and treated as described above.

Blood samples were withdrawn from the tail vein of the rats. Plasma was separated by centrifugation of the heparinized blood, and used immediately for measurement of BG and B or stored below −20°C for measurement of baicalein 6-O-β-d-glucopyranuronoside (B6G; Fig. 2).

An *in situ* experiment involving injection of B into the portal vein was also conducted, as reported previously. Briefly, a jejunal segment 10 cm long from a Wistar rat was everted, and the inside (serosal side) of the resulting sac was filled with 1.3 mL Krebs–Ringer bicarbonate solution containing 0.1% glucose and 1 mg/mL bovine serum albumin (BSA). The intestinal sac was then placed in 20 mL of aqueous solution containing 1 mg/mL BSA and 0.1 mm B. Aliquots of the fluids from the serosal and mucosal sides after 0, 10, 20, and 30 min of incubation at 37°C were extracted with 3 or 4 volumes of methanol under acidic conditions. The amounts of BG and B were determined immediately, and that of B6G after storage below −20°C, as described below.

Isolation of B6G After induction of anesthesia with pentobarbital, the abdominal cavity was opened *via* a midline incision and the bile duct was then cannulated with PE tubing (SP45, Natsume Co., Tokyo, Japan). The rats orally received 100 mg/kg B, which had been dissolved in DMSO (final concentration 10% v/v) and then diluted with water just before administration to a concentration of 5 mg/mL. Bile samples were collected cumulatively for 24 h in 20 mL volumes of methanol under acidic conditions. The amounts of methanol were extracted with 3 or 4 volumes of methanol with cooling, and stored below −20°C until use after acidification. The collected bile samples were evaporated to remove the methanol, and the supernatant fraction after centrifugation was adjusted to pH 4.5 with 1 M HCl. This was chromatographed on a reverse-phase column (100C18, Wako Pure Chemical Industries, Ltd.) by stepwise elution with MeOH–water (10:90, 20:80, 30:70, 50:50, 60:40) containing 3% acetic acid. The fractions that were eluted with the 50:50 and 60:40 mixture contained B6G, and subsequently preparative HPLC afforded pure B6G, which was identified by NMR and MS spectroscopic analyses in comparison with the spectroscopic data reported previously (Abe et al.). The purity was more than 95%, based on HPLC analysis.

B6G: Yellow amorphous powder. 1H-NMR (500 MHz, DMSO-d6) δ: 3.33–3.38 (3H, m, H-2′,3′,4′), 4.26 (1H, d, J=7.31 Hz, H-5′), 4.93 (1H, d, J=7.6 Hz, H-1′, anomic proton), 6.64 (1H, s, H-8), 6.96 (1H, s, H-3), 7.59 (3H, m, H-3′,4′,5′), 8.06 (2H, dd, J=7.1, 1.8 Hz), 13.03 (1H, s, 5-OH). 13C-NMR (125 Hz, DMSO-d6) δ: 163.4 (C-2), 104.2 (C-3), 182.3 (C-4), 153.1 (C-5), 128.1 (C-6), 157.7 (C-7), 94.5 (C-8), 152.6 (C-9), 104.7 (C-10), 130.7 (C-1′), 126.5 (C-2′ 6′), 129.2 (C-3′ 5′), 132.1 (C-4′), 103.7 (C-1″), 73.6 (C-2″), 76.1 (C-3″), 71.5 (C-4″), 75.6 (C-5″), 170.2 (C-6″).

Extraction of Metabolite from Plasma and Treatment with β-d-Glucuronidase Heparinized blood was collected 2 h after oral administration of B at 12.1 mg/mL and the plasma was obtained immediately by centrifugation. The plasma was first washed with ethanol–ether (1:1 by volume) after lyophilization, and then extracted with 75% ethanol. The extract was dissolved in 20 mL phosphate buffer (pH 7.5) after evaporation to dryness. The solution was then treated with β-d-glucuronidase (Sigma, from *E. coli*, 3600000 units/g solid, final 0.21 mg/mL at 37°C for 30 min with or without addition of α-saccharic acid 1,4-lactone (an inhibitor of β-d-glucuronidase, final concentration 10 mM). The reaction was stopped by addition of two volumes of methanol, and the resulting solution was subjected to HPLC.

HPLC/UV and HPLC/MS Conditions HPLC separation was performed on a COSMOSIL 5C18-MS-II Waters column (150×4.6 mm i.d.; Nacalai Tesque, Inc., Kyoto, Japan).
The mobile phase contained solvents C and D, where C was water–acetic acid (95.5:0.5) and D was acetonitrile–acetic acid (95.5:0.5). The linear gradient profile was from 30 to 100% D in 20 min, and maintained at 100% D for 10.0 min. The wavelengths used for PAD detection were 205, 254, 290, 320 and 366 nm. The flow rate was 1 mL/min for HPLC and PAD detection with the column kept at 40°C. A splitter was connected between the PAD and MS detectors, which reduced the flow rate to 0.2 mL/min for MS detection. The electrospray ESI-MS was operated in the negative ion mode with a fragmentation amplitude of 1.5, trap drive level at 50%, scanning range of m/z 50 to 1000, and capillary voltage of 4000 V. High-purity nitrogen (99.99%) was used as a drying gas to evaporate the solvent at a flow rate of 9 L/min and a temperature of 350°C. Nitrogen was used as the nebulizer gas at 40 psi.

Uridine 5′-Diphosphate Glucurononate Glucuronosyltransferase (UGT) Activity Microsomes from rat liver and jejunum were prepared, and the UGT activities toward B as an acceptor for hepatic and jejunal microsomes were measured as described previously, except for use of HPLC to determine the acceptor for hepatic and jejunal microsomes were measured as described previously, except for use of HPLC to determine the acceptor for hepatic and jejunal microsomes, which was isolated from rat bile after oral administration of B and identified by NMR spectroscopic analysis. In addition, HPLC/MS/MS analyses identified the unknown peak in the plasma extract with a retention time of 5.1 min as B6G by comparing its HPLC/MS (Fig. 2), HPLC/MS2 (Fig. 3) and HPLC/MS3 with those of the standard compound. Both the blood sample and the standard compound displayed the [M−H]− ion at m/z 445 by negative mode ESI-MS and the [M−H−glucuronic acid]− ion at m/z 269 in by MS2.

**Plasma B6G and BG after Oral Administration of BG or B** After oral administration of BG to rats, it was detected in their plasma as described previously, and B6G was also detected at higher concentrations than BG (Fig. 4), being a glucuronide form of B. Moreover, the unknown peak was coincident with that of B6G, which was isolated from rat bile after oral administration of B and identified by NMR spectroscopic analysis. In addition, HPLC/MS/MS analyses identified the unknown peak in the plasma extract with a retention time of 5.1 min as B6G by comparing its HPLC/MS (Fig. 2), HPLC/MS2 (Fig. 3) and HPLC/MS3 with those of the standard compound. Both the blood sample and the standard compound displayed the [M−H]− ion at m/z 445 by negative mode ESI-MS and the [M−H−glucuronic acid]− ion at m/z 269 in by MS2.

**RESULTS**

**Identification of B6G as the Main Plasma Metabolite** When the extract of rat plasma 2 h after oral administration of B was subjected to HPLC, a new peak besides that of BG was detected using the ECD at a potential of +500 mV. This peak disappeared upon treatment of the extract with β-d-glucuronidase to give a B peak, and the disappearance was inhibited by addition of α-saccharic acid 1,4-lactone (an inhibitor of β-d-glucuronidase), suggesting that the new peak was a glucuronide form of B. Moreover, the unknown peak was coincident with that of B6G, which was isolated from rat bile after oral administration of B and identified by NMR spectroscopic analysis. In addition, HPLC/MS/MS analyses identified the unknown peak in the plasma extract with a retention time of 5.1 min as B6G by comparing its HPLC/MS (Fig. 2), HPLC/MS2 (Fig. 3) and HPLC/MS3 with those of the standard compound. Both the blood sample and the standard compound displayed the [M−H]− ion at m/z 445 by negative mode ESI-MS and the [M−H−glucuronic acid]− ion at m/z 269 in by MS2.

**Fig. 3. Chromatogram and MS2 Spectra at m/z 445 for the Blood Sample and the Standard Compound (B6G)**

**Fig. 4. Plasma Concentration Profiles of B6G (■) and BG (◆) after Oral Administration of BG at a Dose of 20mg/kg to Rats** Each point represents the mean±S.D. of three different experiments. *p<0.05, (vs. BG group).
significantly higher at 3, 5 and 7h. The $C_{\text{max}}$ and $AUC_{0-24h}$ of B6G were 1.66±0.34 $\mu M$ and 19.8±3.9 $\mu M$·h, respectively, at $T_{\text{max}}$ 4.00±2.00h, whereas those of BG were 0.853±0.065 $\mu M$ and 10.0±3.1 $\mu M$·h, respectively, at $T_{\text{max}}$ 2.67±1.15h (Table 1). Both values of B6G were significantly higher than those of BG. Moreover, after oral administration of B to rats, B6G and BG were also detected in plasma (Fig. 5) with a $C_{\text{max}}$ of 3.48±1.13 and 3.22±1.59 $\mu M$ at $T_{\text{max}}$ 0.887±0.964h, and with an $AUC_{0-24h}$ of 12.2±2.5 and 7.35±3.75 $\mu M$·h, respectively (Table 2). Also in the case of B administration, the concentration of B6G was higher than that of BG, but not significantly.

**B6G Formation from B by Rat Jejunal and Hepatic Microsomes** UGT activities toward B as an acceptor were measured in microsomes of rat jejunum and liver. As reported previously,13) BG-forming activities in both types of microsomes were almost the same. Moreover, B6G-forming activities were also detected in both types of microsomes, although these were only one eighth and one third of the BG-forming activities, respectively (Table 3). Thus, the B6G-forming activities were 3 fold higher in the liver than in the jejunum. These results suggest that the appearance of B6G in rat plasma after oral administration of BG or B may be derived from B6G formed in the jejunum and/or liver.

**Little Intestinal Contribution to the Appearance of Plasma B6G** When B was applied on the outside (mucosal side) of rat everted jejunal sacs, the amount of B6G formed was quite low in comparison with that of BG. Moreover, most (more than 95%) of the B6G formed was excreted to the outside, and not to the inside (serosal side) (Fig. 6), being similar to the pattern of BG formation.14) On the other hand, an appreciable amount of applied B was transferred to the inside. These results suggest that the contribution of the intestine to the appearance of B6G in plasma after oral administration of BG or B is negligible.

**Plasma B6G after Injection of B into the Portal Vein**

When B at a dose of 1.2 mg/kg was injected in the portal vein of Wistar rats in situ, BG was detectable at a considerable level ($C_{\text{max}}$ 0.173±0.093 $\mu M$ and $AUC_{0-120min}$ 2.16±0.40 $\mu M$·min) in the systemic circulation (Fig. 7, Table 4), as reported previously in the case of SD rats and Eisai hyperbilirubinemic rats.15) Moreover, B6G was also detected at a higher level ($C_{\text{max}}$ 0.516±0.079 $\mu M$ and $AUC_{0-120min}$ 18.0±9.7 $\mu M$·min) in the systemic circulation (Fig. 7, Table 4), being 3-fold and 8-fold higher, respectively, than the corresponding values for BG. These results indicate that, in the rat, B6G like BG is produced from B in the liver and then excreted into the systemic circulation.

**DISCUSSION**

When administered orally, BG, a predominant flavone glucuronide present in Scutellariae Radix, shows a unique metabolic fate in rats. It is poorly absorbed, but absorbed as the aglycone B, transformed by intestinal bacteria in the gastrointestinal tract, and then restored to its original form in rat tissues.16) Accordingly, even when B is administered orally, BG, and not B, is detected in the systemic circulation of rats.17) On
the other hand, a surprisingly large amount of BG, which is excreted from intestinal epithelial cells via glucuronidation, is also recovered in the intestinal tract after administration of B,\(^1\), indicating an important role of the intestine in the first-pass effect of absorbed B. Moreover, when B is injected into the portal vein of rats, BG, and not B, is also detected in the systemic circulation,\(^1\) indicating an important role of the liver in the appearance of circulating BG. In addition, when BG or B is administered orally to rats, large amounts of various B-glucuronide species besides BG are recovered in bile,\(^1\) indicating the glucuronidation of absorbed B to form several kinds of B-glucuronides in the liver, and suggesting their excretion into the systemic circulation, in addition to their excretion into bile. In fact, the existence of unidentified B-glucuronides, as well as BG, in the rat systemic circulation after oral administration of Kampo medicine containing Scutellariae Radix has been reported.\(^6\) In the present study, we detected for the first time B6G at a higher level than BG in the rat systemic circulation after oral administration of BG or B, and investigated the pharmacokinetics of B6G and the contribution of the liver to the appearance of this metabolite.

Although BG and B in rat blood have been detected with high sensitivity using an ECD at a potential of +100 mV on HPLC,\(^12,13\) B6G had never been detected under the same conditions. However, in the present study, B6G was detectable at a potential of +500 mV on the ECD as a new and sharp peak, which disappeared upon treatment with β-glucuronidase to give a B peak, in rat plasma after oral administration of BG or B. This disappearance of the peak upon addition of β-glucuronidase was repressed by addition of α-saccharic acid 1,4-lactone, an inhibitor of β-glucuronidase. These results indicate that the peak is B-glucuronide. Furthermore, the fact that the retention time of the peak was coincident with that of a B6G standard isolated from bile and that HPLC/MS data for the plasma sample were also coincident with those of the standard (Figs. 2, 3) confirmed that the new peak was B6G.

The plasma concentration profile of B6G (Fig. 4) after oral administration of BG showed three peaks, which seemed to be due to that B was produced by hydrolysis of BG mainly in stomach (the first small peak at 0.5–1 h) and in cecum (the second broad peak around 4 h) and then by hydrolysis of many kinds of B-glucuronides\(^11\) excreted in bile during enterohepatic circulation (the third broad peak around 12 h). Also in the plasma concentration profile of BG (Fig. 4) a small peak at 0.5–1 h and two shoulders (at 2–6 h and around 12 h) were observed. On the other hand, the plasma concentration profiles of B6G and BG (Fig. 5) after oral administration of B showed large peaks at 0.5–1 h, the same as the time of the first small peaks after oral administration of BG. The plasma concentration profiles of B6G (Figs. 4, 5) after oral administration of BG and B showed that the levels of B6G were higher than those of BG, indicating that B6G is the main metabolite in the plasma. In fact, in each rat and at each time point, the level of B6G was always higher than that of BG (data not shown). However, the profile patterns of B6G and BG were similar, suggesting that both were produced in the rat body and transferred to the systemic circulation in a similar manner. BG is formed from B and absorbed at almost the same rate in the intestine and liver,\(^19\) but the liver plays an important role in the appearance of BG in the rat systemic circulation.\(^3\)

The present study showed that B6G was also formed in the intestine and liver of rats (Table 3). However, B6G production activity in the intestine was one third lower than that in the liver, and even the small amount of B6G formed in the intestine was excreted into the intestinal lumen, and not into blood (Fig. 6), similar to BG,\(^19\) indicating that the contribution of the intestine to the appearance of B6G in the systemic circulation was negligible. On the other hand, when B was injected into the portal vein, a considerable amount of B6G was detected in the systemic circulation at a higher level than BG (Fig. 7), demonstrating the liver contributed to the appearance of B6G, as is the case for BG. Thus, B6G was formed in the liver from absorbed B, and then excreted mainly into blood, and not into bile. The excretion of B6G into bile after oral administration of BG or B is fairly low in comparison with other B-glucuronides such as B 6,7-di-β-glucopyranuroside, 6-O-β-glucopyranuronosyl-B 7-O-sulfate, or BG.\(^14\) Accordingly, B6G seems to be a much poorer substrate of multidrug resistance-associated protein 2 (Mrp2, Abcc2) located on the apical canalicular membrane of rat hepatocytes,\(^20\) whereas BG is a good substrate for Mrp2.\(^17,18\) This could explain the higher level of B6G than that of BG in the systemic circulation (Fig. 4, 5, 7), despite the lower B6G production activity in the liver, being one third that of BG (Table 3). B6G seems to be excreted into blood by transporter(s) located on the sinusoidal membrane, such as Mrp3, which may have higher affinity for B6G than for BG.

In humans, B6G is also formed in intestinal and hepatic microsomes (our unpublished data). Accordingly, B6G seems to appear in human blood after ingestion of Scutellariae Radix in combination with other herbs. However, no pharmacological effects of B6G are yet known, and it is expected that future studies will yield more information about the properties of B6G.

In summary, B6G has been detected for the first time as a major metabolite besides BG in plasma after oral administra-

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**Table 4. Pharmacokinetic Parameters of Plasma B6G and BG after Injection of B into the Portal Vein of Rats**

| Compound | $T_{max}$ (h) | $C_{max}$ (µM) | $AUC_{0-20\text{min}}$ (µM·min) |
|----------|---------------|----------------|----------------------------------|
| B6G      | 8.00±1.73**   | 0.516±0.079**  | 18.0±9.7                         |
| BG       | 3.00±0.00     | 0.173±0.093    | 2.16±0.40                        |

Values are expressed as mean±S.D., n=3. *p<0.05, **p<0.01, B6G vs. BG.
tion of BG or B to rats. Pharmacokinetic studies showed that the level of B6G was higher than that of BG, and that the plasma B6G was derived from that formed in the liver, even though the production of B6G was low in comparison with BG.

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