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Determination of glabridin in human plasma by solid-phase extraction and LC–MS/MS

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Abstract

Glabridin is a major flavonoid included specifically in licorice (*Glycyrrhiza glabra* L.), and has various physiological activities including antioxidant and anti-inflammatory effects. We have developed and validated an analytical method for determination of glabridin in human plasma by solid-phase extraction (SPE) and LC–MS/MS. Glabridin was extracted from plasma by SPE using a C8 cartridge and analyzed by LC–MS/MS using mefenamic acid as an internal standard (IS). The analyte were separated by a C18 column on LC, and monitored with a fragment ion of *m*/*z* 201 formed from a molecular ion of *m*/*z* 323 for glabridin and that of *m*/*z* 196 from *m*/*z* 240 for IS during negative ion mode with tandem MS detection. The lower limit of quantitation (LLOQ) of glabridin was 0.1 ng/mL in plasma, corresponding to 1.25 pg injected on-column. The calibration curves exhibited excellent linearity ($r > 0.997$) between 0.1 and 50 ng/mL. Precision and accuracy were <17 and <±7% at LLOQ, and <11 and <±5% at other concentrations. Glabridin was recovered >90%, and was stable when kept at 10 °C for 72 h, at −20 °C until 12 weeks, and after three freeze-thaw cycles. This is the first report on determination of glabridin in body fluids by the selective, sensitive, and reproducible method.

Keywords: Glabridin; Licorice; *Glycyrrhiza glabra* L.; Flavonoid; Human plasma; Solid-phase extraction; LC–MS/MS; Determination; Validation

1. Introduction

Licorice, the root of the leguminous *Glycyrrhiza* plant species, has been used for over 4000 years since ancient Egyptian time, and is one of the most frequently employed botanicals in foods and traditional medicines [1]. Glabridin is a major flavonoid included specifically in licorice (*Glycyrrhiza glabra* L.), and has antioxidant activity [2–7], anti-*Helicobacter pylori* activity [8], estrogen-like activity [9], and antinephritic and radical scavenging activities [10]. In addition, it exhibits inhibition of serotonin re-uptake [11], and inhibits melanogenesis and inflammation [12]. Glabridin is thus a biologically active compound that occurs in a natural plant. Although some reports [7,13,14] have presented analytical methods for determination of glabridin in licorice and its extracts using high-performance liquid chromatography (HPLC), no report has been made of an analytical method for determination of glabridin in body fluids of animals or humans.

It was recently reported that licorice flavonoid oil (LFO), which consisted of licorice hydrophobic flavonoids in medium-chain triglycerides (MCT) and whose glabridin content was adjusted to 1.2% (w/w), exhibited abdominal fat-lowering and hypoglycemic effects in obese diabetic KK-A^y mice [15]. Although LFO contains some hydrophobic flavonoids from licorice *G. glabra* L., glabridin is the most abundant flavonoid among all licorice flavonoids in LFO, and is the major bioactive compound. It is therefore considered a major marker compound for bioavailability of LFO. In our preliminary animal study, plasma glabridin concentrations were approximately 10–40 ng/mL in rats after oral administration of LFO, which is

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equivalent to the administration of glabridin at 12 mg/kg body weight. These findings were obtained using a modified high-performance liquid chromatography method described in our previous report [16], for which the lower limit of quantitation (LLOQ) was 10 ng/mL glabridin in plasma. A more sensitive analytical method is needed for determination of glabridin in human plasma in clinical trials. In this study, we developed and validated an analytical method for determination of glabridin in human plasma using solid-phase extraction (SPE) and LC–MS/MS.

2. Experimental

2.1. Chemicals and materials

Glabridin (purity > 97.0%), mefenamic acid (purity > 99.0%), methanol (HPLC grade), and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Solid-phase Sep-Pak Vac C8 cartridges (500 mg, 3 cc) were purchased from Nihon Waters K.K. (Tokyo, Japan), and Centricut Super Mini filters (0.45 μm) from Kurabo Industries, Ltd. (Osaka, Japan). Pure water was prepared by passing through an EQG-10L System (Millipore Corporation, Bedford, MA). Normal heparinized human plasma was purchased from Nippon Bio-Supp. Center (Tokyo, Japan).

2.2. Standard solutions

A stock solution (250 μg/mL) of glabridin was prepared by dissolving 5 mg in 20 mL of methanol, and working standard solutions of glabridin were prepared by diluting the stock solution with methanol to 5000, 4000, 500, 200, 50, 20, 10, 5, and 1 ng/mL. A stock solution of internal standard (IS) was prepared by dissolving 5 mg of mefenamic acid in 20 mL of methanol, and a working IS solution by diluting the stock solution with methanol to 50 ng/mL. These solutions were stored at 3–9 °C.

2.3. Sample preparation

SPE of glabridin from human plasma was performed using Sep-Pak Vac C8 cartridges. Human plasma, 0.5 mL, in a glass tube was mixed with 0.5 mL of water and 50 μL of methanol or glabridin standard solution by a mixer for approximately 10 s. The mixed solution was loaded onto a Sep-Pak Vac C8 cartridge, which was sequentially conditioned with 2 mL of methanol and 2 mL of 5% (v/v) methanol/water. The cartridge was sequentially rinsed with 2 mL of 5% (v/v) methanol/water and 2 mL of 40% (v/v) methanol/water, and analyte was eluted with 2 mL of methanol. The eluate with 50 μL of IS solution added was evaporated under a nitrogen stream at 40 °C. The residue was dissolved in 400 μL of methanol/water (85:15, v/v) by mixing for approximately 10 s, sonicating for approximately 1 min, and re-mixed for approximately 10 s. After filtration with a Centricut Super Mini filter, the analyte sample was kept below 10 °C until LC–MS/MS analysis.

2.4. LC–MS/MS analysis

LC–MS/MS analysis was carried out using an SIL-HTC or an LC-10A HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to an API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Samples were analyzed using a LUNA 5 μ C18(2) analytical column (5 μm, 2.0 mm × 150 mm; Phenomenex Inc., Torrance, CA) with a Hypersil ODS guard column (5 μm, 4.6 mm × 10 mm; Supelco Inc., Bellefonte, PA) attached. The column temperature was thermostated at 40 °C. Mobile phase was methanol/0.1% (v/v) formic acid (85:15, v/v) at a flow rate of 0.2 mL/min. Injection volume was 10 μL of a sample kept in an autosampler set at 10 °C. Retention time was approximately 4.2 min for glabridin, and approximately 5.9 min for IS.

Turbo ionspray tandem mass spectrometry was used negative ionization mode at an ionspray voltage of −4000 V. Air was used as heater gas set at a temperature of 600 °C and with a setting of 80 psig, and nebulizing gas with a setting of 50 psig. Nitrogen was used as curtain gas with a setting of 40 psig, and as collision gas at a pressure of setting #4. The multiplier voltage was 2000 V. The product ion was recorded using multiple reaction monitoring (MRM) negative ion detection mode. The monitor ion and collision energy were m/z 323.1 → 201.3 and −32 eV for glabridin and m/z 240.1 → 196.1 and −24 eV for IS.

Calibration samples spiked with a standard solution of glabridin in human plasma were analyzed, and peak area ratios of glabridin/IS between 0.1 and 50 ng/mL were tested to obtain a calibration equation using a $1/y^2$-weighted least-squares method for linear regression.

2.5. Validation parameters

Selectivity was evaluated by comparing blank samples and samples spiked with glabridin standard (0.1 ng/mL) for six different lots of human plasma. Linearity was evaluated by correlation coefficients and accuracy of samples at 0.1, 0.5, 2, 5, 20, and 50 ng/mL glabridin. Intra-day reproducibility was evaluated by precision and accuracy of determination of five samples each at 0.1, 5, and 50 ng/mL glabridin, and inter-day reproducibility by repeating this procedure on three different days. Recovery was evaluated by comparing three samples each at 0.3 and 40 ng/mL glabridin when adding standard solutions to plasma before and after SPE. Stability was evaluated by comparing three samples each at 0.3 and 40 ng/mL glabridin, when analyte samples were stored in an autosampler set at 10 °C for 72 h, and when plasma samples were freeze-thawed three times and stored in a freezer set at −20 °C for 2, 4, 8, and 12 weeks.

3. Results/discussion

3.1. Development of the analytical method

In order to extract glabridin from human plasma by SPE, we initially tested recovery of glabridin on various SPE cartridges such as the Sep-Pak C8, C18, tC18, CN, and PS-2. In the preliminary test, an SPE cartridge was rinsed with water after

loading plasma spiked with glabridin, while other procedures were similar to those in the sample preparation described in the Section 2. These analyte samples were analyzed by HPLC with a UV monitor (282 nm), using a modified version of the method described in our previous report [16]. Recovery of glabridin was in the following order: C8 > PS-2 > tC18 > C18 ≫ CN. Next, we examined rinsing of C8 cartridge with 5, 10, 20, and 40% (v/v) methanol/water. Although amounts of impurities from plasma decreased depending on the methanol content of the rinse solution, recovery of glabridin was higher with rinsing with 5 and 10% (v/v) methanol/water than with 20 and 40% (v/v) methanol/water. It appeared that recovery of glabridin would be decreased by steep change of polarity in a C8 cartridge with higher methanol content in the rinse solution. We therefore examined a two-step rinse with 5% (v/v) methanol/water and subsequent 40% (v/v) methanol/water, and achieved higher recovery (>90%) of glabridin with lower amounts of impurities from plasma. If it is assumed that glabridin binds to plasma proteins, it might be speculated that the first step of the two-step rinse released glabridin from binding protein and that the second step of the two-step rinse washed out protein while retaining glabridin on the C8 cartridge. The two-step rinse is therefore considered an efficient procedure for extraction of glabridin from human plasma, whether glabridin binds plasma proteins or not. Consequently, we therefore adopted the SPE method using a C8 cartridge with two-step rinse to extract glabridin from plasma.

The lower limit of quantitation of glabridin was 10 ng/mL in plasma with HPLC–UV analysis, using a modified version of the method described in our previous report [16]. In order to increase the sensitivity of glabridin determination, we first tried electrochemical detection (ECD) on HPLC analysis. The LLOQ of glabridin was improved to 1 ng/mL in plasma by HPLC–ECD analysis, but this method was labor-intensive and time-consuming. We therefore performed LC–MS/MS analysis to obtain higher sensitivity with simple technique. When a standard compound of glabridin (molecular weight 324.37) was preliminarily analyzed by LC–MS, an ion peak at *m*/*z* 325 was detected in positive ion mode, and at *m*/*z* 323 in negative ion mode. On preliminary analysis by LC–MS/MS, two fragment peaks were detected with different collision energies: from the ion peak of *m*/*z* 325 on positive ion mode, fragment peaks of *m*/*z* 123 and 189 were detected at collision energies of 25 and 27 eV, respectively, while from the ion peak of *m*/*z* 323 on negative ion mode fragment peaks of *m*/*z* 135 and 201 were detected at collision energies of −28 and −30 eV, respectively. Next, human plasma samples with and without glabridin spiking were treated by SPE and analyzed by LC–MS/MS under the above-mentioned conditions. On comparison of chromatograms of blank plasma and plasma sample spiked with glabridin, higher sensitivity of detection of glabridin was obtained with detection of the peak of *m*/*z* 201 from 323 in negative ion mode.

Mefenamic acid exhibited features similar glabridin on LC–MS/MS analysis, with retention time and monitored fragment ion of 5.9 min and *m*/*z* 196, respectively, for the former and 4.2 min and *m*/*z* 201 for the latter. We therefore selected mefenamic acid as IS for this analytical method. However, mefenamic acid was added to samples after SPE, since recovery of mefenamic acid on SPE differed from the relative high recovery (>90%) of glabridin on SPE.

The presumed structures of monitored ions for glabridin and IS are shown in Fig. 1. After optimization, we determined the conditions for LC–MS/MS analysis described in the Section 2. The LLOQ of glabridin was improved to 0.1 ng/mL in plasma, corresponding to 1.25 pg injected on-column, and the sensitivity of LC–MS/MS analysis was 100 times that of HPLC–UV analysis.

3.2. Validation of the analytical method

The analytical method we developed was evaluated for selectivity (0.1 ng/mL), linearity (0.1–50 ng/mL,), reproducibility (0.1, 0.5, and 50 ng/mL), recovery, and stability (0.3 and 40 ng/mL). Reproducibility was judged from precision and accuracy in intra- and inter-day assays.

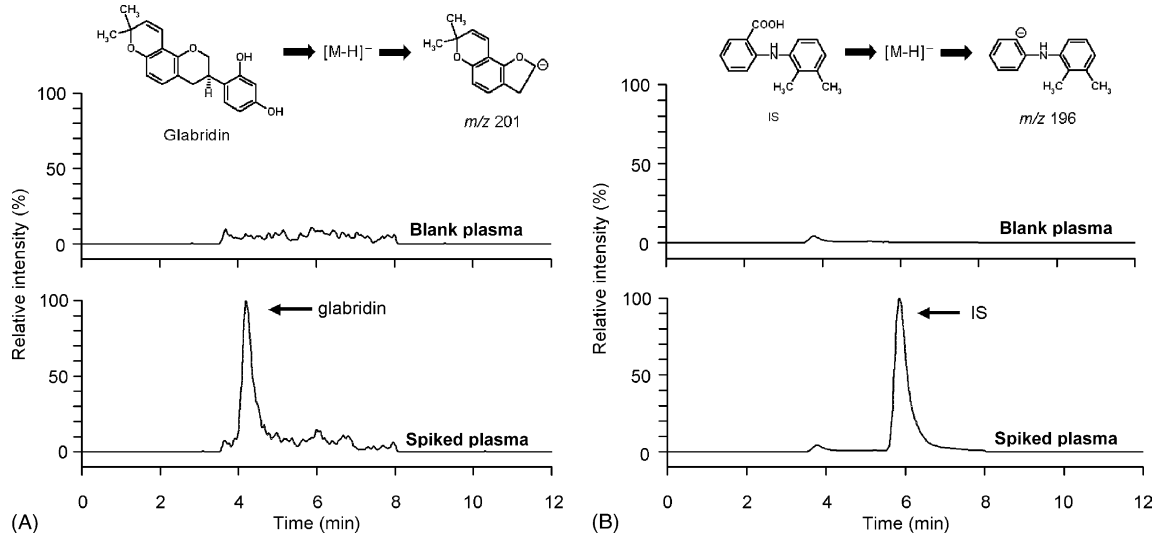

Fig. 1. Typical LC–MS/MS chromatograms of blank plasma and plasma sample spiked with 0.1 ng/mL glabridin and IS monitored at *m*/*z* 201 for detection of glabridin (A) and at *m*/*z* 196 for detection of IS (B). The presumed structures of monitored ions for glabridin and IS are shown at the top of each chromatogram.

Table 1
Intra-day reproducibility of determination of glabridin in human plasma

Added concentration[a] (ng/mL)	Measured concentration[b] (mean ± S.D., ng/mL)	Precision (R.S.D., %)	Accuracy (% deviation)
0.1	0.1050 ± 0.01731	16.5	6.1
0.5	4.751 ± 0.1091	2.3	−4.0
50	47.10 ± 2.692	5.7	−4.8

[a] Actual concentrations were calculated as 0.09900, 4.950, and 49.50 ng/mL.
[b] $n = 5$ of each concentration.

Table 2
Inter-day reproducibility of determination of glabridin in human plasma

Added concentration[a] (ng/mL)	Measured concentration[b] (mean ± S.D., ng/mL)	Precision (R.S.D., %)	Accuracy (% deviation)
0.1	0.1027 ± 0.01664	16.2	3.5
0.5	4.770 ± 0.4800	10.1	−3.8
50	47.93 ± 2.720	5.7	−3.4

[a] Actual concentrations were calculated as 0.09920, 4.960, and 49.60 ng/mL.
[b] $n = 5$ of each concentration on three different days (total $n = 15$).

Using six different lots of human plasma, blank plasma and plasma sample spiked with 0.1 ng/mL glabridin and IS were analyzed. Typical LC–MS/MS chromatograms are shown in Fig. 1. No peaks interfering with detection of glabridin or IS were observed in chromatograms of blank from six lots of human plasma. Compared with blank, the signal/noise (*S*/*N*) ratio of glabridin at 0.1 ng/mL in six lots of human plasma ranged from 5 to 30, and the *S*/*N* ratio of IS was above 32. This analytical method thus exhibited selectivity in determination of glabridin in human plasma.

Nine calibration curves with six concentration levels between 0.1 and 50 ng/mL were constructed on different days. The correlation coefficient (r) was 0.9993 ± 0.0006 (mean ± S.D., $n = 9$) with a range of 0.9979–0.9998. A typical calibration curve equation was $y = 0.01142x - 0.000008203$ ($r = 0.9994$), and accuracy was −1.1% at LLOQ (0.1 ng/mL) and ranged from −2.6 to 6.5% at the other concentrations (0.5, 2, 5, 20, and 50 ng/mL). This analytical method thus exhibited excellent linearity between 0.1 and 50 ng/mL glabridin in human plasma.

Five samples each at 0.1, 0.5, and 50 ng/mL glabridin were analyzed on the same day. Intra-day precision was 16.5% at LLOQ and less than 6% at the other concentrations, while intra-day accuracy was 6.1% at LLOQ and within ±5% at the other concentrations (Table 1). In the same way, five samples each at 0.1, 0.5, and 50 ng/mL glabridin were analyzed on three different days. Inter-day precision was 16.2% at LLOQ and less than 11% at the other concentrations, while inter-day accuracy was 3.5% at LLOQ and within ±4% at the other concentrations (Table 2). The FDA's guidance for industry on bioanalytical method validation [17] recommends that the criterion for precision and accuracy for acceptance at each concentration level is within 15%, except for LLOQ, where it is not more than 20%. According to these criteria, this analytical method could reproducibly measure the concentration of glabridin in human plasma.

Recovery of glabridin was determined by comparing human plasma samples spiked with glabridin before and after the SPE procedure. Recovery was 90.8 ± 5.91% (mean ± S.D., $n = 3$) at 0.3 ng/mL and 96.2 ± 4.99% at 40 ng/mL.

Stability of the standard solution of glabridin in methanol was evaluated prior to that of analyte samples and plasma samples. After 2-month storage, relative percentages of glabridin against the initial values were 100.5 ± 0.6% (mean ± S.D., $n = 3$) at 4 °C and 100.2 ± 0.6% at 25 °C, indicating stability for at least 2 months.

When analyte samples were kept in an autosampler set at 10 °C for 72 h, accuracy was 3.4 and 7.5% at 0.3 and 40 ng/mL (Table 3). After plasma samples spiked with glabridin at 0.3 and 40 ng/mL were freeze-thawed three times, accuracies were

Table 3
Stability of glabridin in an analyte sample at 10 °C and in human plasma stored at −20 °C and after freeze-thaw

	Added concentration[a] (ng/mL)	Measured concentration[b] (mean ± S.D., ng/mL)	Accuracy (% deviation)
Before storage			
	0.3	0.3114 ± 0.01324	4.8
	40	43.93 ± 0.3568	10.9
Stored an analyte sample			
At 10 °C for 72 h	0.3	0.3070 ± 0.01153	3.4
	40	42.57 ± 0.8637	7.5
Afterfreeze-thaw (3 times)	0.3	0.3168 ± 0.02493	6.7
	40	41.13 ± 0.07506	3.9
Stored a plasma sample			
At −20 °C for 2 weeks	0.3	0.3307 ± 0.01853	11.3
	40	40.60 ± 0.3502	2.5
At −20 °C for 4 weeks	0.3	0.2970 ± 0.01269	0.0
	40	36.70 ± 1.046	−7.3
At −20 °C for 8 weeks	0.3	0.3120 ± 0.05280	5.1
	40	37.81 ± 0.4500	−4.5
At −20 °C for 12 weeks	0.3	0.3091 ± 0.05594	4.1
	40	38.26 ± 2.951	−3.4

[a] Actual concentrations were calculated as 0.2976 and 39.60 ng/mL.
[b] $n = 3$ of each concentration at each storage condition.

6.7 and 3.9%, respectively (Table 3). When the plasma samples were stored at −20 °C for 2, 4, 8, and 12 weeks, accuracies were less than ±12% at at 0.3 ng/mL and less than ±8% at 40 ng/mL (Table 3). On the basis of the accuracies determined before storage (4.8 and 10.9% at 0.3 and 40 ng/mL, respectively) and the accuracy criteria in the FDA's guidance for industry on bioanalytical method validation [17], we judged stability to have been demonstrated based on accuracy within 15%. Glabridin was thus found to be stable in analyte samples at 10 °C for at least 72 h and in plasma at −20 °C until 12 weeks and after freeze-thawing.

Using the developed and validated analytical method, we preliminarily determined glabridin concentrations after LFO administration in order to clarify the range of quantitation of glabridin in human plasma. In this preliminary determination, three in-house volunteers were orally administered a capsule containing LFO which was equivalent to the administration of glabridin at 3 mg/man. Glabridin concentration in human plasma was 0.63 ± 0.40 ng/mL (mean ± S.D., $n = 3$) at 4 h after administration. Scalbert and Williamson reviewed dietary intake and bioavailability of polyphenols including phenolic acids and flavonoids [18], and noted that bioavailability studies in humans have shown that the maximum concentrations of intact flavonoids in plasma rarely exceed 1 μM after consumption of 19–202 mg of flavonoids such as quercetin, genistein, daidzein, and catechins. This review article suggested that bioavailability of glabridin was lower than that of the other flavonoids. However, we concluded that the analytical method for determination of glabridin in this study was sensitive enough to investigate the pharmacokinetics of glabridin.

4. Conclusions

SPE is a general procedure for extraction of analyte compounds from body fluids such as plasma and urine, and LC–MS/MS is a sensitive method of analysis. In this study, we developed an analytical method for determination of glabridin in human plasma using SPE and LC–MS/MS, and demonstrated it to be selective, precise, and accurate. This analytical method was 100-fold more sensitive than HPLC–UV analysis. Glabridin is a bioactive flavonoid included in licorice (*G. glabra* L.). Although the biological and pharmacological activities of glabridin have been investigated in a variety of studies in vitro and in vivo, no pharmacokinetic research on it has been reported. Use of this newly developed analytical method will facilitate pharmacokinetic and pharmacodynamic studies of glabridin in human as well as animals.

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