An Efficient Purification Method for Quantitative Determinations of Protodioscin, Dioscin and Diosgenin in Plasma of Fenugreek-Fed Mice

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Summary An efficient purification method for simultaneous recovery of polar saponins, protodioscin (PD) and dioscin (DC), and non-polar aglycon, diosgenin (DG), from plasma of mice fed diets containing seed flours of fenugreek (Trigonella foenum-graecum) was established for subsequent quantitative analysis by LC-ESI-MS/MS. Mice plasma samples were first deproteinated by addition of acetonitrile, and the supernatant was applied to a carbon-based solid phase extraction tube. After successive washing with methanol and 35% chroloform/methanol (v/v), PD, DC and DG were eluted simultaneously with 80% chroloform/methanol (v/v). The eluate was evaporated to dryness, and re-dissolved in 80% methanol (v/v). The filtered sample was analyzed with an LC-ESI-MS/MS system. After the purification procedure, recovery rates between 89.3 to 117.4% were obtained without notable ion suppression or enhancement. The use of internal standards was therefore not necessary. The utility of the method was demonstrated by analyzing plasma of mice from a fenugreek feeding study.

Key Words fenugreek, saponin, solid phase extraction, LC-MS/MS, plasma

Trigonella foenum-graecum (fenugreek) is said to originate in Southern Europe and Western Asia, and is now cultivated worldwide (1). It emits a characteristic caramel-like sweet aroma, and is one of the major spices used in curry powder. It has also long been used in Ayurvedic and Chinese medicine (2). Recently, various therapeutic effects of fenugreek seeds, such as antidiabetic and anti-cholesterol effects, have been reported (3–7). However, the strong bitterness of fenugreek seeds makes it difficult for us to consume an effective dose through foods we eat regularly.

As the bitterness is due mainly to the major fenugreek saponin, protodioscin (PD) (Fig. 1) (8, 9), enzymatic hydrolysis of PD into less bitter dicosin (DC) (Fig. 1) yields fenugreek with reduced bitterness (FRB). Recently, Uemura et al. reported that oral administration of FRB had an antidiabetic effect on obese diabetic KK-Ay mice (10). They suggested that diosgenin (DG) (Fig. 1), the aglycon of PD and DC, was the main effective substance, and that DG inhibited the transactivation of liver-X-receptor α (LXRα) with antagonist-like effect and increased the expression of peroxisome proliferator-activated receptor γ (PPARγ) mRNA (10–12).

Unprocessed bitter fenugreek seeds contain saponins mainly in the form of PD, which is hydrolyzed to DC in FRB, and when consumed, PD and DC would be hydrolyzed to DG in vivo. Hence, an efficient method for quantitative determinations of PD, DC and DG would be indispensable for absorption and pharmacokinetic studies of these compounds.

While quantitative determinations of each of these compounds in plasma have been reported independently, different sample preparation methods had to be employed for PD and DC (deproteination alone with acetonitrile) and for DG (solvent extraction alone with a mixture of hexane and ethylacetate) (13–16).

Here we report a purification method that allowed simultaneous recovery of PD, DC and DG from small plasma samples taken from KK-Ay mice used in the study of the antidiabetic effect of diets containing unprocessed bitter fenugreek or FRB.

EXPERIMENTAL

Materials. Protodioscin (PD) and dicosin (DC) were purchased from ChromaDex, Inc. (Irvine, CA). Diosgenin (DG) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ultra-pure water used in LC-MS analysis was prepared by using the Elga Stat UHQ PS Water Purifier system (Elga Ltd., High Wycombe, UK). All other chemicals were reagent or LC/MS grade.

A commercial mouse plasma (blank plasma) used for spike-recovery experiments was purchased from Kohjin Bio Co., Ltd. (Saitama, Japan).

Apparatus and analytical conditions. A Quattro Pre-
mier/XE (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source, and interfaced with a Waters Acquity UPLC (Waters), was used for the LC-ESI-MS/MS analysis. The data were processed using MassLynx Version 4.1.

The analytical column used was an Acquity UPLC BEH C18 Column (1.7 µm, 3 mm × 100 mm, Waters). The mobile phase for PD analysis consisted of 0.1% v/v aqueous formic acid (A) and methanol (B). The gradient program was as follows: a linear gradient of B 60–100% between 0 and 20 min, an isocratic elution of B 100% between 20 and 25 min, and finally an isocratic elution of B 60% between 25.1 and 30 min (a total run time of 30 min). The mobile phase of DG analysis consisted of 0.2% v/v aqueous formic acid (A) and methanol (B). The gradient program was as follows: an isocratic elution of B 80% between 0 and 15 min, a linear gradient of B 80–100% between 15 and 20 min, an isocratic elution of B 100% between 20 and 25 min, and finally an isocratic elution of B 80% between 25.1 and 30 min (a total run time of 30 min). Each analytical condition was determined for improving ionization efficiency and avoiding ion suppression. The injection volume was 5 µL. The autosampler temperature was maintained at 5˚C. The column was maintained at 40˚C and was eluted at 0.3 mL/min. Electrospray ionization (ESI) parameters were as follows: ionization, positive ion mode; capillary voltage, 3.5 kV; desolvation gas temperature, 400˚C; desolvation gas flow, 800 L/h; source temperature, 130˚C; cone gas flow, 50 L/h; collision gas flow, 0.2 mL/min. The fragmentation transitions for multiple ion monitoring (MRM) were \( \frac{m}{z} 1032.0 \rightarrow \frac{m}{z} 869.0 \) (for PD), \( \frac{m}{z} 891.6 \rightarrow \frac{m}{z} 891.6 \) (for DC), and \( \frac{m}{z} 415.4 \rightarrow \frac{m}{z} 271.2 \) (for DG). The cone voltage and collision energy were set at 35 V and 10 V for PD, 90 V and 10 V for DC, 28 V and 16 V for DG. The dwell time was 0.2 s.

**Standard curves.** Standard solutions (1.6, 3.1, 6.3, 12.5, 25, 50, 100, and 200 ng/mL) were prepared by dissolving each authentic compound in 80% methanol, and were subjected directly to LC-ESI-MS/MS analysis as described above. Standard curves for PD, DC and DG were constructed based on the measured peak areas. They were all linear over the concentration range from 1.6 to 200 ng/mL with correlation coefficients higher than 0.99. The lower limits of quantification for PD, DC and DG were 1.6 ng/mL.

**Solid phase extraction experiment.** To 100 µL of 100 ng/mL standard solution, 200 µL of deionized water and 450 µL of acetonitrile were added, and the mixture was vortexed thoroughly. After centrifugation at 13,800 × g for 10 min, the solution was applied to a graphitized-carbon solid phase extraction (SPE) tube (Supelclean ENVI-Carb, 100 mg, Supelco, Bellefonte, PA) which had been conditioned sequentially with 1 mL of chloroform and 1 mL of methanol. The column was then eluted with 1 mL of methanol, 1 mL of 35% chloroform/methanol (v/v), 1 mL of 80% chloroform/methanol (v/v), and 1 mL of chloroform. Each fraction was dried with a centrifugal evaporator, and re-dissolved in 100 µL of 80% methanol. After passing through a filter (0.2 µm), the solution was subjected to LC-ESI-MS/MS analysis.

**Spike-recovery experiment.** Commercial mouse plasma (blank plasma) was spiked with PD, DC and DG to make the final concentrations of 10 ng/mL and 100 ng/mL as follows. A calculated amount of the standard solution of PD, DC and DG was added to 100 µL of the
plasma, and the volume was brought up to 300 μL with deionized water. To the spiked plasma samples, 450 μL of acetonitrile was added. The mixture was then vortexed, and centrifuged at 13,800 × g for 10 min. The supernatant was purified with the graphitized-carbon SPE tube. The tube was washed with 1 mL of methanol, 1 mL of 35% chloroform/methanol (v/v), and then eluted with 1 mL of 80% chloroform/methanol (v/v). The 80% chloroform/methanol fraction was dried, re-dissolved in 80% methanol, filtered and subjected to LC-ESI-MS/MS analysis as described above. The recovery rate was calculated by dividing the peak area of spiked plasma sample by the peak area of the corresponding standard solution and multiplying by 100.

Fenugreek feeding study. Fenugreek was obtained from House Foods Corp. (Osaka, Japan). Fenugreek with reduced bitterness was prepared as follows. Fenugreek seeds were boiled for 5 min, and then cooled to 40°C. An enzyme (Spezyme CP; Genencor, Palo Alto, CA) was added to the boiled seeds, which were then mashed. The paste was incubated at 55°C until the bitterness of the paste was sufficiently reduced. The enzyme was deactivated by heating at 90°C. The paste was freeze dried and crushed into powder (10).

Four-week-old male KK-Ay/Ta Jcl mice, an obese diabetic model, were purchased from CLEA Japan, Inc. (Tokyo, Japan). All the mice were kept in individual cages in a temperature-controlled room at 24±1°C and maintained on a 12 h light/dark cycle. All the animals were fed a commercial diet (CRF-1, Charles River Laboratories Japan, Inc., Kanagawa, Japan) during 1 wk of adaptation, and were given any one of a control HFD (D12492; Research Diets, New Brunswick, NJ) (control group), the control HFD supplemented with 2% w/w fenugreek (Fenugreek group), or the control HFD supplemented with 2% w/w FRB (FRB group) for 4-wk feeding period. Six mice were used in each group. The energy intake of all the mice was adjusted by pair feeding. At the end of the feeding period, anesthetized animals were killed by cervical dislocation after an overnight fast, and blood and liver tissue samples were collected from each animal and stored at −20°C until use. The animal care procedures and methods were approved by the Animal Care Committee of Kyoto University.

Determinations of PD, DC and DG in plasma samples from fenugreek feeding study. Frozen plasma samples from the fenugreek feeding study were thawed to room temperature prior to use. To 100 μL of the plasma, 200 μL of deionized water and 450 μL of acetonitrile were added. The mixture was purified and subjected to LC-ESI-MS/MS analysis as described in the spike-recovery experiment.

Biochemical analysis. The plasma glucose was determined using the glucose CII-test Wako (Wako Pure Chemical Industries, Ltd.). The plasma triacylglyceride (TAG) concentration was measured using the TG E-test (Wako Pure Chemical Industries, Ltd.). The plasma triacylglycerides in liver tissue were extracted using chloroform/methanol following the Bligh-Dyer method (17). The organic solvents were evaporated under nitrogen gas, and the residue was re-suspended in 1% Triton X-100. The TAG concentration was measured using the TG E-test (Wako Pure Chemical Industries, Ltd.).

RESULTS AND DISCUSSION

Solid phase extraction experiment

As shown in Fig. 2, PD, DC and DG applied on the graphitized-carbon SPE tube were well retained during sample application and washing with methanol and 35% chloroform/methanol. They were all eluted fully with 80% chloroform/methanol into one fraction. Subsequent washing with 100% chloroform eluted only negligible PD, DC and DG.

Because of the difference in polarity, determinations of PD, DC and DG required separate sample preparations. The graphitized-carbon SPE tube used in this study has an extreme affinity for both polar and non-polar organic compounds, especially for those of planar molecules. It would be the planar steroid skeleton of the aglycon of PD, DC and DG that made the simultaneous elution of these polar and non-polar compounds in one fraction possible. The optimal ratios of chloroform/methanol for washing and elution, however, had to be found by trial and error.

Spice-recovery experiment

The average recoveries of PD, DC and DG from spiked mice plasma were between 89.3 and 117.4% (Table 1). The recovery rates obtained for samples spiked at 10 ng/mL and 100 ng/mL were similar. None of the
Matheys effects (ion suppression or enhancement) often encountered in LC-MS analyses were observed. The peaks were clearly separated and assigned (Fig. 3). With these results, we concluded that internal standards such as trillin for PD, ginsenoside Rh2 for DC, and sarsasapogenin for DG, or calibration standards in blank plasma used by the previous researchers were unnecessary (13, 15, 16).

Analysis of mice plasma from the feeding study

As shown in Fig. 4, in the plasma from mice fed a diet supplemented with fenugreek seeds (Fenugreek group) or fenugreek with reduced bitterness (FRB group), appreciable amounts of DC and DG were detected, but PD was not. In the plasma from mice fed the control diet (no fenugreek), none of PD, DC nor DG was detected (data not shown). Therefore, the DC and DG detected in the plasma should be due to PD in unprocessed fenugreek and DC in FRB, which were hydrolyzed presumably by the action of intestinal microflora and absorbed through the intestinal wall as DC or DG. Deconjugation of the plasma samples with glucuronidase containing sulfatase did not affect the levels of DC or DG detected (data not shown), which suggested that DC and DG in the mouse plasma were in free form.

The levels of DC and DG detected in the plasma of the FRB group were significantly higher than those of the Fenugreek group, which suggested that DC in FRB...
was more readily absorbable than PD in the unprocessed fenugreek. However, whether PD can be absorbed through the intestinal wall as is or has to be hydrolyzed first into DC before it is absorbed is not known. Since it was reported that intravenously administered PD was not detectable in mouse plasma after 6 h (15), we could not exclude the possibility that absorbed PD had been metabolized in the plasma during the overnight fasting period.

**The effect of feeding fenugreek on plasma glucose, plasma TAG, and liver TAG levels**

While the levels of glucose and TAG in the plasma of the Fenugreek and FRB groups, as well as the liver TAG of the Fenugreek group, were not statistically different from those of the control group, liver TAG of the FRB group was significantly lower than that for the control group (Fig. 5). Moreover, the liver TAG of the FRB group tended to be lower than that of the Fenugreek group, though the difference was not statistically significant. The lower TAG levels in the liver may be attributable to the higher levels of plasma DG in the FRB group, because DG was reported to inhibit the transactivation of liver-X-receptor α (LXRα) in HepG2 cells and decreased plasma and hepatic triacylglycerides in KK-Ay mice (12). Not only DG but also DC was detected in the plasma of fenugreek-fed mice, understanding the physiological implications of which needs further investigation.

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