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Article

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Interindividual Variability in Metabolism of [6]-Shogaol by Gut Microbiota

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Abstract

[6]-Shogaol (6S), one of the major bioactive components in dry ginger, is attracting considerable attention because of its wide spectrum of biological activities, but its metabolic fate is still not fully understood. In the present study, the microbial metabolism of 6S was examined for the first time in *in vitro* batch fecal fermentation system and in mice. Two major microbial metabolites were detected and identified as 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol (M9) and 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one (M11). Our results indicated that reductions of the double bond and the ketone group are the major metabolic pathways of 6S by the human gut microbiota. We also observed the interindividual variability in the metabolism of M11 to M9 by human gut microbiota. In addition, we demonstrated that the glucuronidated form of 6S and its metabolites could be rapidly de-conjugated by human gut microbiota and in mice, which can be regarded as a reactive process taking place in the intestinal tract. To our knowledge, this is the first report involving the identification of the microbial metabolites of 6S in an *in vitro* fermentation system, and the first demonstration of the critical role of gut microbiota in producing the bioactive free form of 6S and its metabolites in the intestinal tract in mice.

Keywords: [6]-Shogaol, Microbiota, Ginger, Metabolism, Interindividual variation

Introduction

Ginger (*Zingiber officinale* Roscoe) is one of the most widely consumed spices and dietary condiments.[1] The health-promoting perspectives of ginger are well documented and the FDA ranked it in the generally recognized as safe (GRAS) list.[2] Consumption of ginger has been associated with many health benefits including the prevention of cancers and cardiovascular diseases,[3-6] a phenomenon mostly attributed to the presence of ginger phenolics. Shogaols and gingerols are the major bioactive phenolics present in ginger extracts, and shogaols, the dehydrated products of gingerols, are the predominant pungent constituents in dried ginger.[7]

Recently, shogaols have received increasing attention due to their stronger anticarcinogenic activity than gingerols, with [6]-shogaol (6S) being the most potent one.[8, 9] The content of 6S in powdered dry ginger is in the range of 353 to 1990 mg/kg.[10, 11] However, pharmacokinetic studies of 6S in mice, rats, and humans have revealed that the plasma concentration of 6S was low, and 6S was present in the blood circulation mainly as its metabolites.[12-16] Following a single consumption of 2 g of ginger extract containing 45 mg of 6S, very limited amount of 6S was detected with mean AUC_{0-t} less than 0.025 μg·h/L and mean C_{max} less than 0.050 μM.[17] It seems that such low contents of 6S in plasma have difficulty to reach its effective concentration. Our lab has previously established that 6S was extensively metabolized to its phase I metabolites, such as 1-(4′-hydroxy-3′-methoxyphenyl)-4-decen-3-ol (M6), 5-methoxy-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one (M7), 3′,4′-dihydroxyphenyl-decan-3-one (M8), 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol (M9), 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one (M10), 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one

(M11), and 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol (M12), and phase II metabolites, including 5-cysteinyl-M6 (M1), 5-cysteinyl-[6]-shogaol (M2), 5-cysteinylglycinyl-M6 (M3), 5-*N*-acetylcysteinyl-M6 (M4), and 5-*N*-acetylcysteinyl-[6]-shogaol (M5).[18-20] We have also proved that the phase I and thiol conjugated metabolites of 6S remain bioactive, and in the blood circulation most of them were transported in their glucuronidated forms.[19-21] We recently further revealed that the glucuronidation of 6S can largely eliminate its bioactivities.[22] Thus, mechanisms/pathways underlying observed biological effects of 6S remain unclear.

Metabolism of 6S in mice showed that 6S and its metabolites (phase I metabolites and thiol conjugates) were excreted into urine mainly in a glucuronidated form while being present in fecal samples in free forms.[18, 19] We therefore hypothesized that the gut microbiota may play an important role in the metabolism of 6S, converting inactive glucuronated 6S and its metabolites to bioactive free molecules, leading to exposure of colon tissues to a relatively high concentration of bioactive molecules, including 6S and its phase I metabolites as well as its thiol conjugates. To verify this hypothesis, we investigated the metabolism of 6S and the glucuronidated metabolites of 6S and its phase I and thiol conjugates in *in vitro* batch fecal fermentation and in mice. We demonstrated for the first time that gut microbiota played an important role in metabolism of 6S, and also accounted for conversion of inactive glucuronidated 6S and its metabolites into bioactive free molecules. Furthermore, we observed interindividula difference in metabolism of 6S by human gut microbiota.

Materials and Methods

Materials

6S (purity > 98%) was purified from ginger extract in our laboratory.[8] 1-(4'-Hydroxy-3'-methoxyphenyl)-decan-3-ol (M9) and 1-(4'-hydroxy-3'-methoxyphenyl)-decan-3-one (M11) were purified from 6S treated mouse feces as described before,[18] structures of 6S, M9, and M11 are shown in **Figure 1A**. Glucuronidated 6S (6S-G) was synthesized in our lab.[22] *β*-Glucuronidase from limpets (*Patella vulgata*), was purchased from Sigma (St. Louis, MO, USA). HPLC and LC-MS grade solvents were obtained from Thermo Fisher Scientific (St. Louis, MO, USA).

In vitro fecal batch fermentation experiments

Six healthy volunteers (30-42 y, 60-80 kg, non-smokers) were recruited in this study. They were asked to avoid consumption of any ginger-containing foods for at least 2 d prior to the study. Use of antibiotics in past 6 months was used as an exclusion criterion. Fecal samples were collected and processed as described previously.[23] The *in vitro* fermentation experiment was performed according to the method described by Gross *et al.* with slight modifications.[24] Fermentation basal medium was prepared by mixing 1000 mL of distilled water, 4 mL of Tween 80, and 2 g of peptone. The medium was then autoclaved at 121 °C for 15 min, and stored in refrigerator until use. Anaerobic condition was achieved by using sterilized medium (autoclave the medium and 'driven out' the oxygen) to prepare the fecal slurries. Briefly, 50 g of stool samples were mixed with 100 mL of medium in a sterilized stomacher bag using a Seward stomacher (Model: 400 circulator), swirled, and mixed well. The mixture was briefly centrifuged (2–3 min at 3000 rpm) to remove particulate materials. The supernatant (fecal slurry) was mixed with 35% pre-sterile glycerol, divided into 15 mL sterilized tubes, and stored at -80 °C for later use. Ten μL of 6S or M11 stock solution (10 mg/mL in DMSO), 5 mL fermentation

medium, and 0.5 mL of human fecal slurry were mixed and then divided into 7 sets of samples (~0.6 mL each) for seven time points (0, 12, 24, 48, 72, 96, and 120 h). Samples were incubated at 37 °C under anaerobic conditions and harvested at the corresponding time points. Once harvested, 450 μL of the supernatant was transferred and mixed with 900 μL of acetonitrile containing 0.2% acetic acid. Samples were centrifuged at 16,100 × g for 10 min and then 200 μL of the supernatants were transferred to vials for HPLC analysis.

Treatment of mice and sample collection

Mouse study was conducted according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina Research Campus (IACUC No. 13-014). Male CF-1 mice were purchased from Charles River (Wilmington, MA, USA) and acclimate for at least 1 week before random assignments. The mice were housed and maintained in air-conditioned quarters with a room temperature of 20 ± 2 ºC, relative humidity of 50 ± 10 %, and an alternating 12-h light-dark cycle. The mice were fed AIN-93G diet (Research Diets Inc., New Brunswick, New Jersey, USA) and water, and were allowed to eat and drink *ad libitum*. 6S in vehicle (20% DMSO in corn oil) was administered to mice by oral gavage (200 mg/kg body weight). Twenty-four hour mouse urine and fecal samples were collected in metabolic cages (5 mice per cage) after administration of vehicle (control group, n = 5) or 6S (treated group, n = 5). For the collection of the intestinal content samples, eighty male CF-1 mice were orally administered a single dose of 6S (200 mg/kg body weight). Eight mice were stochastically assigned to each group representing 0, 0.25, 0.50, 1, 2, 3, 4, 6, 8, and 24 h. Mice were sacrificed at respective time points, and the entire small intestine and colon

were immediately excised. Subsequently, the entire small intestine was cut evenly into proximal, middle, and distal segments, and the according content samples were collected and pooled together. Samples were stored at -80 ºC until analysis.

Sample preparation

For the acquisition of the metabolic profiles of the 6S treated mouse feces, 50 mg of mouse fecal sample was weighed and vigorously homogenized with 500 μL of 80% aqueous methanol (0.2% acetic acid) in a bead homogenizer (settings: 8 m/s for 30 seconds × 2, Omni Bead Ruptor 24, Omni International Incorporated, Kennesaw, GA, USA). The mixture was then centrifuged at 16,100 × g for 10 min at 4 °C, and the supernatant was diluted 10 times with 50% aqueous methanol containing 0.2% acetic acid and transferred into HPLC vials for analysis of high performance liquid chromatography-electrochemical detection (HPLC-ECD). For non-hydrolyzed urine samples, 50 μL urine sample from each group was diluted by 950 μL 50% aqueous methanol containing 0.2% acetic acid, and then centrifuged at 16,100 × g for 10 min at 4 °C. 10 μL of the supernatant was injected into the HPLC-ECD system. The intestinal and colon content samples were processed using the protocol as preparation of the mouse fecal sample above.

Enzymatic de-conjugation was performed as described previously with slight modifications.[23] In brief, 50 μL of authentic 6S-G solution (500 μM in water) or mouse urine samples (control and treated) were treated with *β*-glucuronidase (250 U) for 45 min at 37 °C, respectively. The reaction was terminated by adding an equal amount of acetonitrile containing 1% acetic acid. The mixture was then centrifuged at 16,100 × g for 10 min at 4 °C. The supernatant (200 μL) was transferred for HPLC analysis. Bacterial

de-conjugation experiments were carried out as follows. Fifty μL of authentic 6S-G solution or 6S treated mouse urine sample, 400 μL of fermentation medium (deionized water with 0.4% (v/v) Tween 80 and 0.2% (w/v) peptone), and 50 μL of human fecal slurries were vigorously mixed and incubated anaerobically for 45 min at 37 °C. The reaction was stopped by adding 500 μL of acetonitrile containing 1% acetic acid, and the mixture was then centrifuged at 16,100 × g for 10 min at 4 °C. Ten μL of the supernatant was injected to the HPLC-UV and/or HPLC-ECD system.

HPLC and LC-MS analysis

An HPLC-ECD system (ESA Laboratories, Chelmsford, MA) consisting of two ESA-Model 584 HPLC Pump, an ESA-Model 542 autosampler, an EAS-Model 526 UV detector, and an 8-channel ESA CoulArray electrochemical detector (ECD) was used. The separation was performed on a Gemini C_{18} column (150 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase A consisted of 30 mM sodium phosphate buffer containing 1.75% acetonitrile and 0.125% tetrahydrofuran (pH 3.35), and mobile phase B consisted of 15 mM sodium phosphate buffer containing 58.5% acetonitrile and 12.5% tetrahydrofuran (pH 3.45). It is important to note that glucuronidated 6S and its metabolites, without free phenolic hydroxyl group in structures, will not exhibit ECD response. Thus, for the measurement of 6S-G, HPLC-UV with wavelength at 280 nm was used with the following gradient: 20% B from 0-3 min, 20-55% B from 3 to 11 min, 55-65% B from 11 to 13 min, and 65-100% B from 13 to 40 min, then held consistent for 5 min. The column was then re-equilibrated with 20% solvent B for 5 min. The flow rate was set at 1.0 mL/min, and the injection volume of each sample was 10 μL. The retention time (R.T.) of 6S-G and 6S were 8.5 and 21.5 min, respectively. A HPLC-ECD method,

same as HPLC-UV method, was used for metabolites profiling of the mouse urine, fecal, and fecal batch incubation samples. The eluent was monitored by the ESA CoulArray ECD with potential settings at -100, 0, 100, 200, 300, 400, and 500 mV. Data for **Figure 1B** was from the channel set at 300 mV. LC-MS analyses were carried out according to our previous method with slight modification.[20] In brief, a Thermo-Finnigan spectra system consisting of an Ultimate 3000 degasser, an Ultimate 3000 RS pump, an Ultimate 3000 RS column compartment, and an LTQ Velos Pro ion trap mass spectrometer (Thermo Electrom) coupled with an electrospray ionization (ESI) source, was used. The ESI interface was operated in positive ion polarity mode. The voltage on the ESI interface was maintained at approximately 4.5 kV and ESI capillary temperature set at 300 ^{o}C. Nitrogen gas was used as the sheath gas and auxiliary gas which was set at 30 and 5 unites, respectively. The collision energy was set at 35 with isolation width of 2 Da for MS^2. Chromatographic separation was performed using a Gemini C18 column (5 μm, 3.0 mm i.d.× 150 mm, Phenomenex, Torrance, CA, USA) with a flow rate of 0.3 mL/min. The column was eluted with 100% solvent A (5% aqueous methanol with 0.2% acetic acid) for 3 min, followed by linear increases in B (95% aqueous methanol with 0.2% acetic acid) to 40% from 3 to 12 min, to 85% from 12 to 30 min, to 100% from 30 to 35 min, and then with 100% B from 35 to 40 min. The column was then re-equilibrated with 100% A for 5 min. Data acquisition and analysis were performed using Xcalibur 2.2 version (Thermo Electron, San Jose, CA, USA).

Results and discussion

[6]-shogaol is metabolized into M9 and M11 by the gut microbiota with interindividual difference

6S was incubated with fecal slurries to yield microbial metabolites, which were analyzed by HPLC-ECD and LC-MS. **Figure 1** shows the representative HPLC-ECD chromatograms for 6S and its microbial metabolites (obtained from subject-1). 6S was degraded progressively as incubation time increased. Compared to controls (time point: 0 h), a new peak (R.T. 24.5 min) came out after 12 h of incubation, and then increased to reach up to maximum at time point of 48 h. Another new peak (R.T. 22.5 min) appeared starting from 24 h of incubation. These two peaks were identified as 1-(4'-hydroxy-3'-methoxyphenyl)-decan-3-ol (M9) and 1-(4'-hydroxy-3'-methoxyphenyl)-decan-3-one (M11) by comparing their retention times and tandem mass data with those of the authentic standards. Of which, the molecular weight of M11 at m/z 279 $[M+H]^+$ is 2 Da higher than that of 6S. Whereas, M9, with molecular ion at m/z 281 $[M+H]^+$, is a ketone group-reduced product of M11, demonstrating that reductions of the double bond (C=C) and the ketone group are the major metabolic pathways of 6S by microbiota. We have previously reported M9 and M11 as the metabolites of 6S in liver microsomes.[20] To the best of our knowledge, this is the first report of M9 and M11 as the microbial-derived metabolites of 6S. As a matter of fact, bio-reduction of C = C bonds in active drugs and other chemicals by crucial reductases, found in a variety of microorganisms (e.g. yeasts, bacteria, and lower fungi), animals, and plants, has been recently reviewed.[25] For example, human Δ7-dehydrocholesterol reductase [EC 1.3.1.21] reduces the double bond at C7-C8 of 7-dehydrocholesterol to yield cholesterol.[26] In addition, the human gut microbiota possesses an impressive armory of enzymes to use and convert dietary constituents, and reduction reaction is not uncommon. For instance, two bacterial strains that were isolated from human feces, *Slackia equolifaciens* and *Adlercreutzia*

equolifaciens, were identified to be able to reduce the double bond of resveratrol to dihydroresveratrol.[27] In another study, isoflavones, daidzein and genistein, were also found to be converted into dihydrodaidzein and dihydrogenistein, respectively, by human gut bacterial species of *Clostridium sp. HGH6* through reduction of double bond between C-2 and C-3.[28] However, there are rare reports of the microbial enzyme-mediated reduction of ketone groups. It is a subject of future studies to identify the bacterial strains to metabolize 6S to M9 and M11.

On the other hand, interindividual differences were observed on the metabolic rate of 6S to M9 and M11. M11 appeared after 12 h of incubation in all six subjects, while M9 was not able to be found in fecal slurries collected from subjects 3 and 5. Subjects 1, 2, and 6 produced significant amount of M9 after 96 h of incubation while only trace amount of M9 was detected from subject 4 (**Figure 2**). Since M9 was found to be the ketone group-reduced product of M11 and was generated after the formation of M11, we hypothesized that M11 could be metabolized to M9 by gut microbiota, and different subjects have different ability to produce M9. To test this hypothesis, M11 was incubated with human fecal slurries collected from subjects 3 (non-producer) and 6 (producer) up to 120 h, respectively. The samples were analyzed by HPLC-ECD as well as LC-MS. **Figure 3** showed that M11 could be converted into M9 by fecal bacteria from subject 6, rather than subject 3, which is consistent with results from fecal batch fermentation experiments of 6S with subjects 3 and 6. Interindividual variability on the biotransformation of polyphenols into their microbial metabolites has been recognized as an essential part of personalized nutrition approaches.[29-34] Our results demonstrate that the composition of gut microbiota could influence the metabolic fate of 6S, and the

226 enzymes that are used to reduce the ketone group are different from those for the
227 reduction of the double bond. Even we observed the interindividual variability in
228 metabolizing 6S to M9 and M11, especially M11 to M9, we would like to acknowledge
229 that it is unlikely to find a culture medium to mimic the growth of gut bacteria *in vivo*,
230 and the medium used in this study may not be suitable for long-term incubation. It will be
231 a plausible strategy to further analyze the microbial composition between different
232 subjects and thereof identify the specific colonic bacteria responsible for the metabolism
233 of 6S to M11 and M11 to M9. This may provide useful information to link gut microbial
234 diversity with the biotransformation and bioactivity of 6S.

235 **Critical role of gut microbiota in the production of bioactive free forms of [6]-**
236 **shogaol and its metabolites in mice**

237 To test our hypothesis that the glucuronidated 6S and glucuronidated forms of 6S
238 metabolites can be metabolized into their free forms by the gut microbiota, authentic 6S-
239 G was incubated with *β*-glucuronidase and human fecal slurry, respectively. The results
240 are shown in **Figure 4**. Glucuronidated 6S could be completely converted into 6S after
241 incubation with either *β*-glucuronidase or human fecal slurry, indicating that gut
242 microbiota indeed hydrolyzed the glucuronide moiety from 6S-G. In a separate study, 6S-
243 treated mouse urine, containing the mixture of glucuronidated forms of 6S and its
244 metabolites, was incubated with *β*-glucuronidase and human fecal slurry, respectively. We
245 found that both *β*-glucuronidase and human fecal slurry effectively broke down
246 glucuronide bonds and released a similar metabolic profile (**Figure 5**). The presence of
247 6S and its metabolites, M1-M12, were confirmed by comparing their respective retention
248 times and tandem mass data with those of the authentic standards, as described

previously.[18-20, 35] The profiles of metabolites were also similar to that from 6S-treated mouse fecal samples, suggesting a β-glucuronidase-like activity of the gut microbiota. In general, plant components and/or drugs, are treated as xenobiotics by the body, and subsequently conjugated to form glucuronides in the small intestine and liver. Part of glucuronidated conjugates is released into the gut through the enterohepatic circulation, and then subjected to the de-conjugation process by the microbial β-glucuronidase to release bioactive free form molecules, which may be further metabolized by gut microbiota as well.[30] Two different bacterial β-glucuronidase genes have been described from the human gut microbiota, the gus gene and the BG gene.[36-38] Both genes were quite well represented in Firmicutes from the human gut.[36] It is a topic of future studies to identify the bacterial strains that are responsible to metabolize the glucuronidated metabolites of 6S and its phase I and thiol-conjugated metabolites.

Metabolite profiles of 6S and its metabolites in the mouse intestinal tract

6S has been shown to have great potentials to be used as a colon cancer prevention regent.[3] We have previously demonstrated that 6S could effectively inhibit the growth of human colon cancer cells, and was an Nrf2 activator.[8, 39, 40] In addition, we also reported that the free form of phase I metabolites and thiol conjugates of 6S remained bioactive in cancer cells and had low toxicity in normal cells.[35] Recently, we found that the glucuronidation of 6S largely eliminated cell cytotoxicity of 6S against human colon cancer cell lines HT-116 and HT-29, and also suppressed Nrf2-inducing activity of 6S.[22] These findings suggested that glucuronidation is probably one of the main factors affecting bioavailability and *in vivo* biological activities of 6S. In general, glucuronidations take place in the small intestine and liver, leading to the formation of the

glucuronidated metabolites, which are either excreted by kidneys through urination or undergo the enterohepatic circulation to release back to the intestinal tract. This process may cause the lower concentrations of intact parent compounds and free metabolites of them in the blood circulation. Studies have revealed that ginger phytochemicals upon oral administration underwent enterohepatic re-circulation in mice.[41] We hypothesize that in GI tract, especially in the colon, the glucuronidated forms of 6S and its metabolites (phase I and thiol-conjugated metabolites) can be de-conjugated and/or further metabolized by gut microbiota.

To test this hypothesis, the metabolic fates of 6S and its metabolites in the mouse intestinal tract were studied. Contents from mouse small intestine (proximal, middle, and distal segments) and colon were collected as a function of time (0, 0.25, 0.5, 1, 2, 4, 8, and 24 h) after a single treatment of 6S (200 mg/kg body weight). The free 6S and its metabolites in proximal, middle, and distal segments were profiled, respectively, as shown in **Figure 6**. The peaks located into the red dotted box are free forms of 6S and its metabolites (only the major metabolite peaks were labeled). It is clear that the free 6S and free metabolites of 6S were mainly found in the distal segments and the colon after 1 h of treatment. Particularly, 6S and its major metabolites reached their maximum and maintained relatively high levels for more than 12 h in colon. In contrast, very low levels of free 6S and free metabolites of 6S were detected in the proximal and middle segments of small intestine. These findings are in agreement with the fact that different compositions of the microbial communities are colonized in different regions of the gastrointestinal tract, and generally the closer to the anus the higher densities of the microorganism are. Thus, the glucuronidated conjugates that are released to the

gastrointestinal tract through enterohepatic circulation are subjected to hydrolysis by microbial *β*-glucuronidase, resulting in exposure of the gut to high levels of bioactive free molecules, 6S and its metabolites. This may explain how the colon can gain benefits from 6S. Furthermore, the variation of the *β*-glucuronidase-producing bacterial species in human subjects would be expected to lead to considerable interindividual variation in *gus* activity and the cleavage of glucuronidated conjugates in the gut.[30]

In summary, in the present study, the microbial metabolism of 6S was investigated for the first time by using an *in vitro* batch fecal fermentation system. The reductions of the double bond and the ketone group were identified as the major metabolic pathways of 6S by the human gut microbiota. And two major metabolites, M9 and M11, were identified as the microbial derived metabolites. We also demonstrated that the glucuronides of 6S and its metabolites could be rapidly de-conjugated by human gut microbiota and in mouse GI track, releasing bioactive free forms of 6S and its metabolites. To our knowledge, this is the first report to demonstrate that gut microbiota may promote the health benefit of ginger components to the intestinal tract, and the microbiota profile may influence the health benefits of a subject received from ginger intake. Our results paved a persuadable path to understand how the colon can get health benefits from 6S.

Notes

The authors declare no competing financial interest.

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Figure legends

Figure 1. Chemical structures of [6]-shogaol and its major microbial metabolites M9 and M11 (A); Representative HPLC-ECD chromatograms of authentic [6]-shogaol, M9, and M11, and microbial metabolites of [6]-shogaol after incubation with human fecal bacteria collected from subject-1 (B).

Figure 2. Representative HPLC-ECD chromatograms of microbial metabolites of [6]-shogaol after incubation with human fecal bacteria collected from subjects 1-6 (A-F).

Figure 3. Representative HPLC-ECD chromatograms of microbial metabolites of M11 after incubation with human fecal bacteria collected from subjects 3 and 6 (A and B).

Figure 4. Representative HPLC-UV chromatograms of glucuronidated [6]-shogaol (6S-G) and its metabolites in *in vitro* incubations with human fecal slurry (A) or *β*-glucuronidase (B), or without *β*-glucuronidase and human fecal slurry (C), and the proposed pathway for the conversion of glucuronidated [6]-shogaol to [6]-shogaol.

Figure 5. Representative HPLC-ECD chromatograms of [6]-shogaol standard (10 μM) (A); urine sample from mice treated with 200 mg/kg [6]-shogaol in the absence (B) or presence (C) of *β*-glucuronidase; in the presence of human fecal slurry (D); and fecal samples from mice treated with 200 mg/kg [6]-shogaol (E). M1, 5-cysteinyl-M6; M2, 5-cysteinyl-[6]-shogaol; M3, 5-cysteinylglycinyl-M6; M4, 5-*N*-acetylcysteinyl-M6; M5, 5-*N*-acetylcysteinyl-[6]-shogaol; M6, 1-(4′-hydroxy-3′-methoxyphenyl)-4-decen-3-ol; M7, 5-methoxy-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one; M8, 3′,4′-dihydroxyphenyl-decan-3-one; M9, 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol; M10, 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one; M11, 1-(4′-hydroxy-3′-methoxyphenyl)-

decan-3-one; M12, 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol.

Figure 6. Representative HPLC-ECD chromatograms of [6]-shogaol and its metabolites (only major metabolites were labeled) in intestinal content samples collected from the proximal (A), middle (B), and distal segments (C) of small intestine, and content sample collected form colon (D). M1, 5-cysteinyl-M6; M2, 5-cysteinyl-[6]-shogaol; M3, 5-cysteinylglycinyl-M6; M4, 5-*N*-acetylcysteinyl-M6; M5, 5-*N*-acetylcysteinyl-[6]-shogaol; M6, 1-(4′-hydroxy-3′-methoxyphenyl)-4-decen-3-ol; M7, 5-methoxy-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one; M8, 3′,4′-dihydroxyphenyl-decan-3-one; M9, 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol; M10, 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one; M11, 1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-one; M12, 5-methylthio-1-(4′-hydroxy-3′-methoxyphenyl)-decan-3-ol.

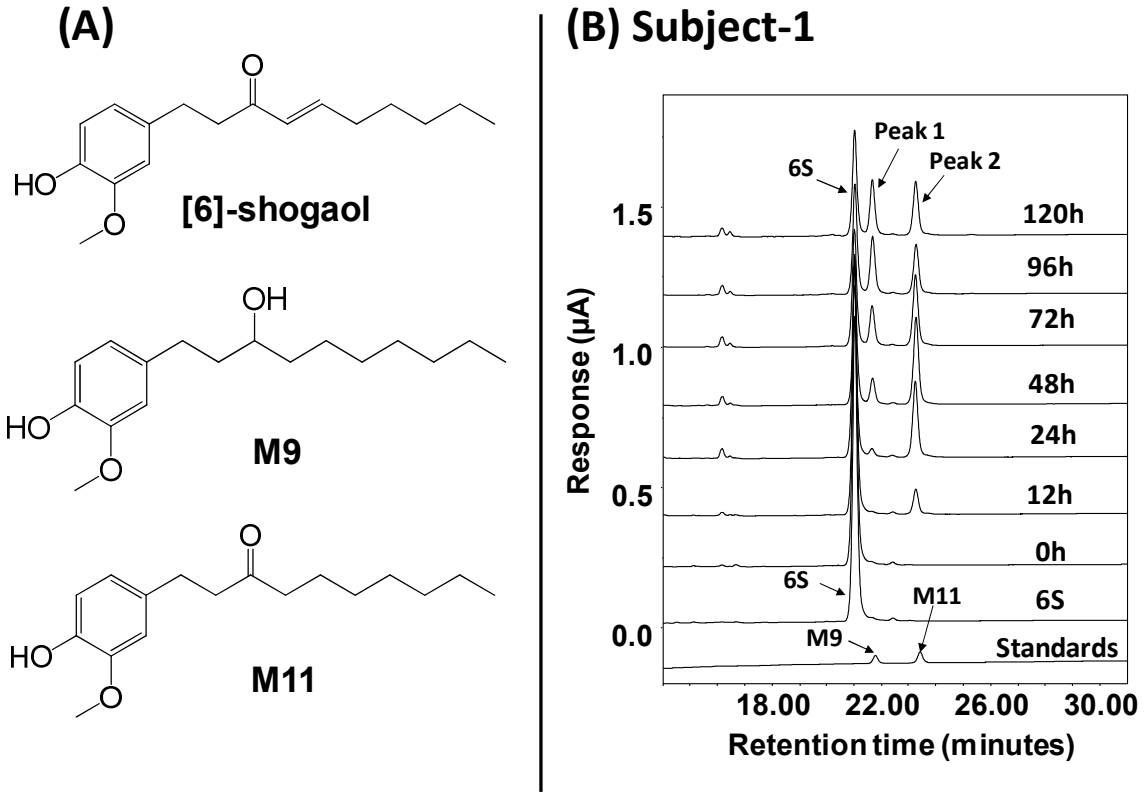
(A)
HO
O
O
[6]-shogaol
OH
HO
O
M9
O
HO
O
M11
(B) Subject-1
Peak 1
Peak 2
6S
120h
96h
72h
48h
24h
12h
0h
6S
M11
6S
M9
Standards
1.5
1.0
0.5
0.0
Response (μA)
18.00
22.00
26.00
30.00
Retention time (minutes)

Figure 1

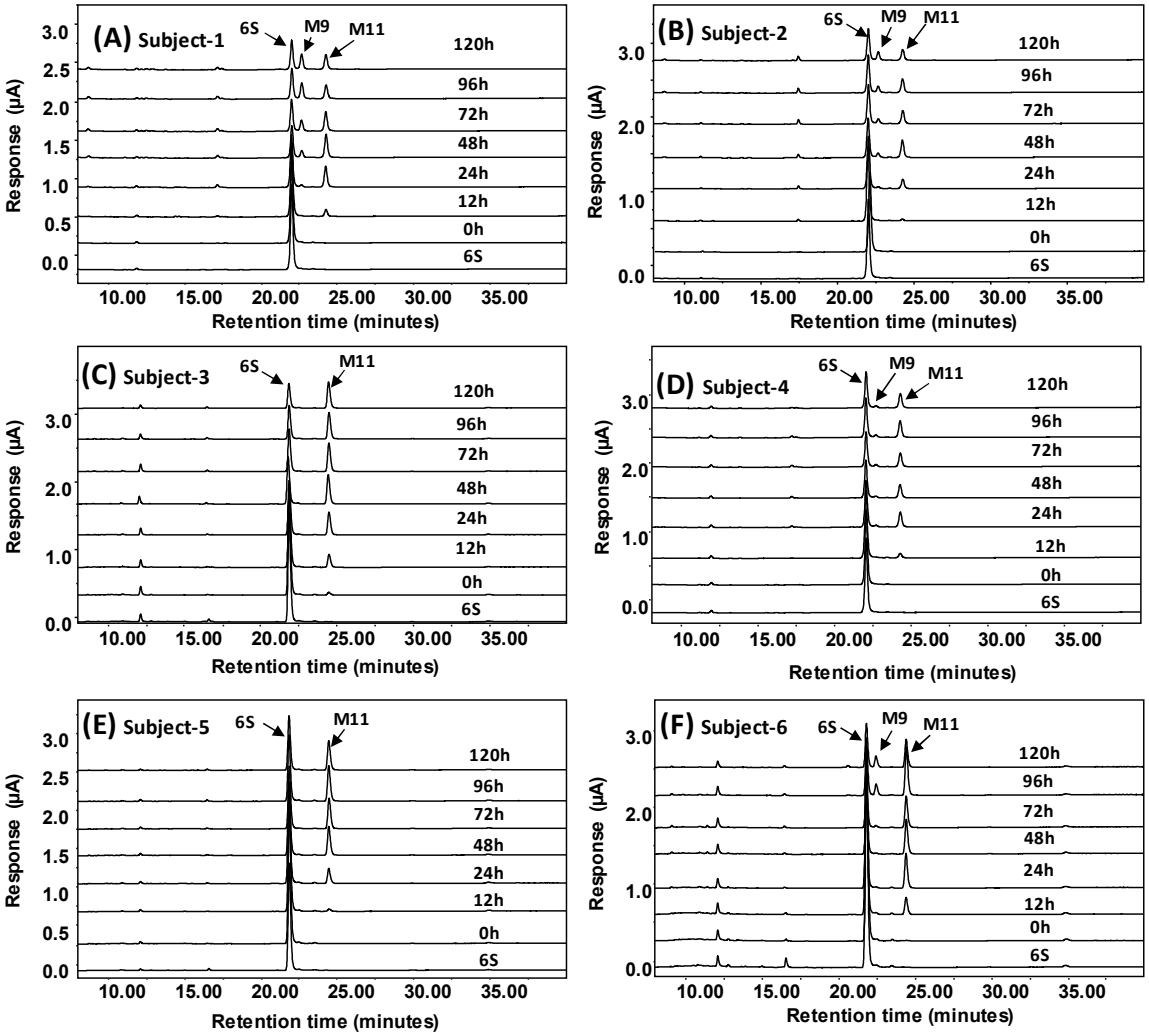
(A) Subject-1
(B) Subject-2
(C) Subject-3
(D) Subject-4
(E) Subject-5
(F) Subject-6
6S
M9
M11
120h
96h
72h
48h
24h
12h
0h
6S
Response (μA)
Retention time (minutes)
10.00
15.00
20.00
25.00
30.00
35.00

Figure 2

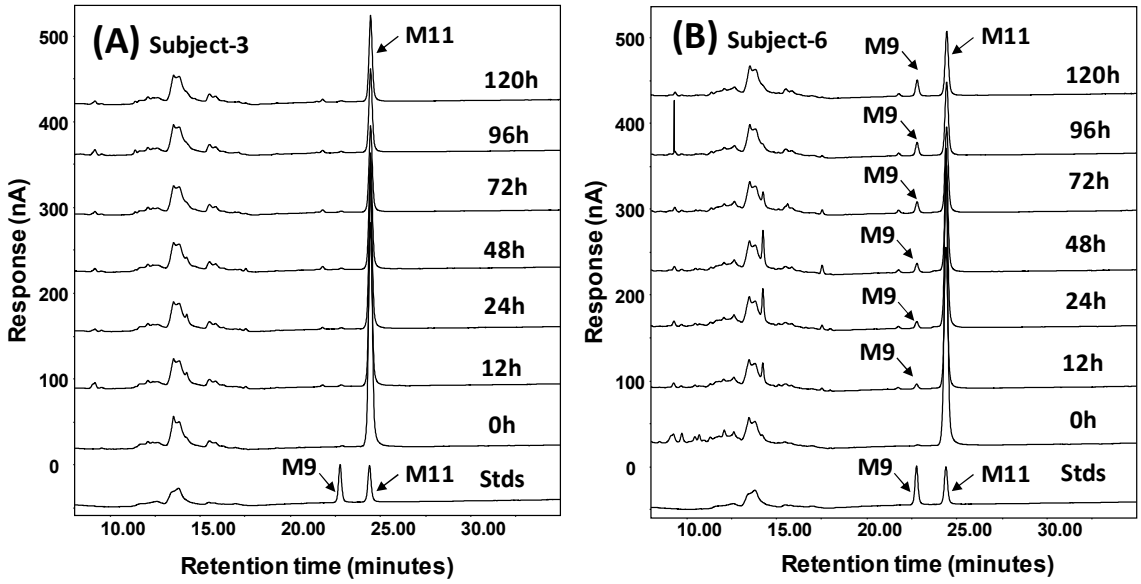
(A) Subject-3
M11
120h
96h
72h
48h
24h
12h
0h
M9
M11
Stds
Response (nA)
Retention time (minutes)
(B) Subject-6
M9
M11
120h
96h
72h
48h
24h
12h
0h
M9
M11
Stds
Response (nA)
Retention time (minutes)

Figure 3

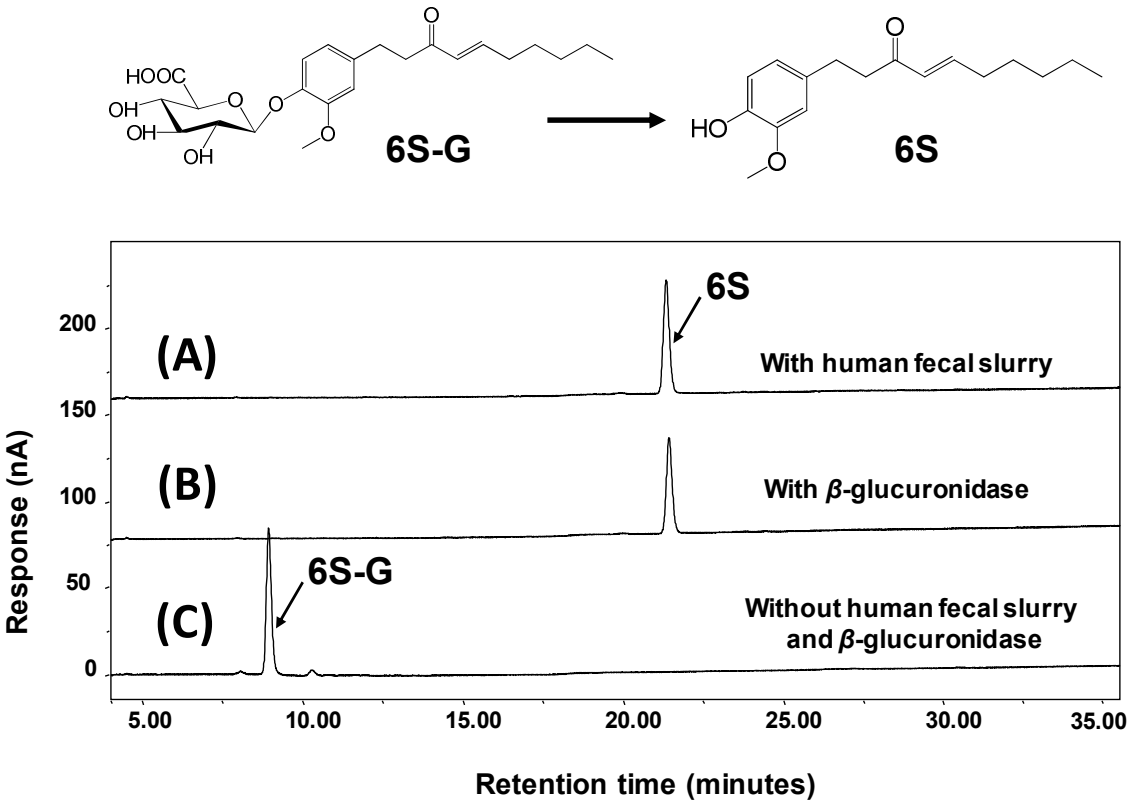
HOOC
OH
OH
OH
O
O
O
O
6S-G
HO
O
O
6S
(A)
6S
With human fecal slurry
(B)
With β-glucuronidase
(C)
6S-G
Without human fecal slurry
and β-glucuronidase
Response (nA)
200
150
100
50
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Retention time (minutes)

Figure 4

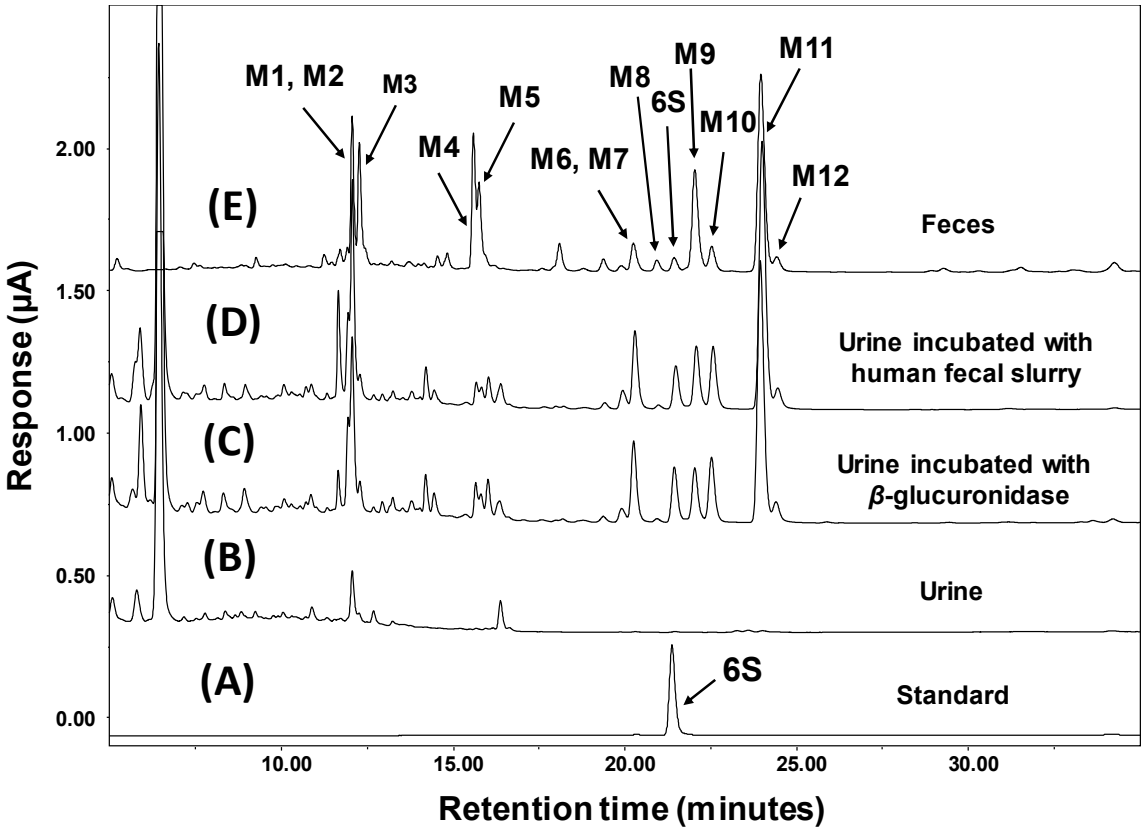
M1, M2
M3
M4
M5
M6, M7
M8
6S
M9
M10
M11
M12
(E)
Feces
(D)
Urine incubated with human fecal slurry
(C)
Urine incubated with β-glucuronidase
(B)
Urine
(A)
6S
Standard
Response (μA)
2.00
1.50
1.00
0.50
0.00
10.00
15.00
20.00
25.00
30.00
Retention time (minutes)

Figure 5

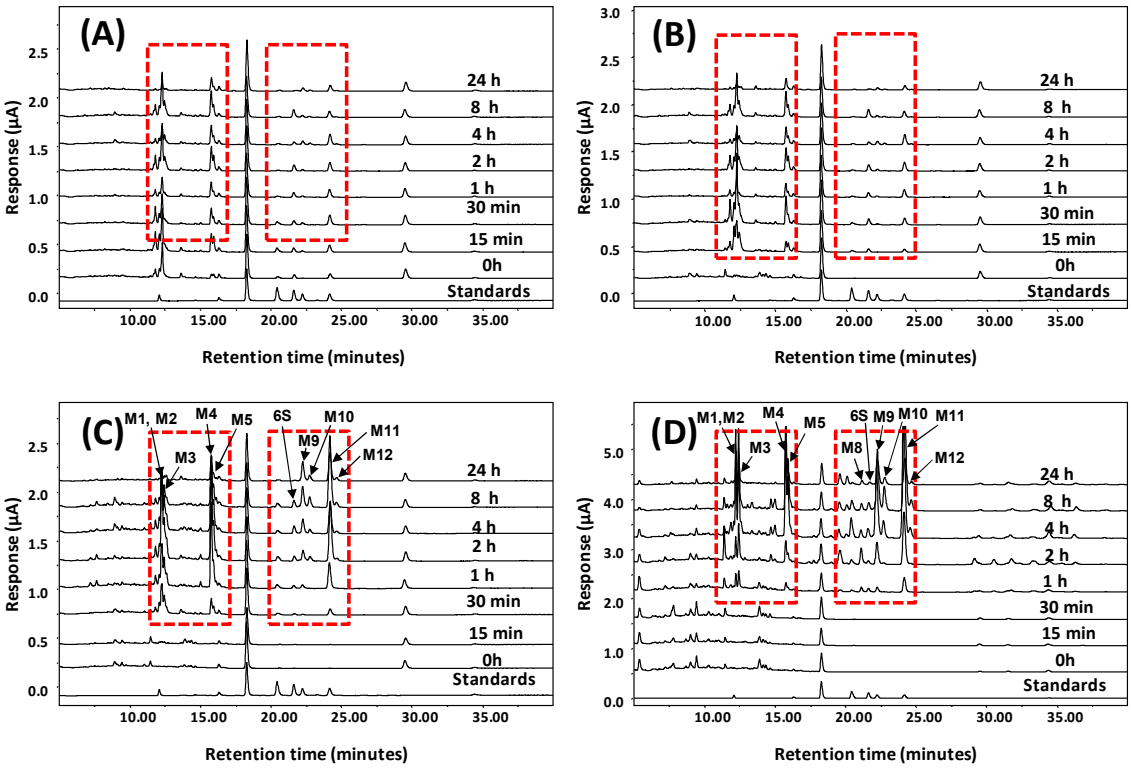
(A)
(B)
(C)
(D)
Response (μA)
Retention time (minutes)
24 h
8 h
4 h
2 h
1 h
30 min
15 min
0h
Standards
M1, M2
M3
M4
M5
6S
M8
M9
M10
M11
M12

Figure 6

GRAPHIC TOC