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Absorption of 6-*O*-Caffeoylsophorose and Its Metabolites in Sprague−Dawley Rats Detected by Electrochemical Detector−High-Performance Liquid Chromatography and Electrospray Ionization−Time-of-Flight−Mass Spectrometry Methods

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ABSTRACT: Absorption and metabolism of a natural compound, 6-*O*-caffeoylsophorose (CS) from acylated anthocyanins in a red vinegar fermented with purple sweetpotato, were clarified. The absorption of CS and conjugated CS in blood from orally administrated Sprague−Dawley rats at a dose of 400 mg/kg was investigated by electrochemical detection−high performance liquid chromatography. As a result, CS was successfully detected in rat plasma (AUC_{0-6h}, 108.6 ± 8.1 nmol h/mL) and was found to be an intact absorbable polyphenol. In addition, half of the absorbed CS was detected as its conjugates (AUC_{0-6h}, 50.7 ± 5.7 nmol h/mL) as well as caffeic and ferulic acids from CS. By a time-of-flight-mass spectrometric analysis of CS-administered plasma sample, glucuronide and methylated conjugates of CS were identified, in addition to glucuronide, methylated, or sulfate conjugates of caffeic and ferulic acids. Consequently, CS was absorbed in intact form into rat blood and partly degraded to caffeic and ferulic acids or metabolized by glucuronidation, methylation, or sulfatation.

KEYWORDS: 6-*O*-Caffeoylsophorose, absorption, metabolite, antihyperglycemic effect

INTRODUCTION

Many have been paying more and more attention to physiological functions of dietary phenolic acids and flavonoids, including anti-inflammatory, anticancer, and antioxidative effects.[1−3] The physiological events of such bioactive compounds must be closely associated with their absorption, metabolism, or tissue distribution, and current issues seem to move to the clarification of bioavailability, along with the evaluation of physiological functionalities.[4−6]

A natural compound, 6-*O*-caffeoylsophorose (CS, Figure 1), with the structure of 6-*O*-(*E*)-caffeoyl-(2-*O*-*β*-D-glucopyranosyl)-D-glucopyranose, was newly identified from a red-colored vinegar fermented with the storage root paste of purple-fleshed sweet potato.[7] CS was predominantly produced from the acylated moiety of the mother of anthocyanin, peonidin 3-*O*-(2-*O*-(6-*O*-(*E*)-feruloyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside in purple sweet potato by acidic hydrolysis.[7,8] Because of the prevalence on some reported health promotion effects of anthocyanins,[9−13] our interest has been focused on investigating the physiological bioactivity of CS from the moiety of bioactive anthocyanins. In our previous studies, CS showed an antihyperglycemic effect on maltose-loaded Sprague−Dawley (SD) rats through the inhibition of maltase activity at the small intestine[7] as well as in vitro antioxidative activity.[8,9] In addition to the postprandial antihyperglycemic effect of CS, the results that caffeic acid or ferulic acid, composed of acylated anthocyanins, inhibited atherosclerosis onset or improved renal dysfunction after intact absorption[10,11,14] led us to investigate the possible physiological role of CS possessing a caffeoly group in our body after its absorption.

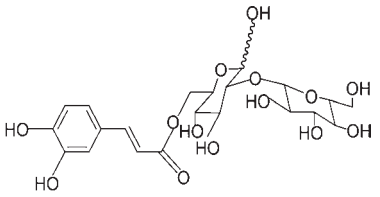

Figure 1. Structure of CS.

The absorption of glycosylated anthocyanins has been reported, such as cyanidin-3-*O*-glucoside,[10,15,16] which is absorbed intact in the blood system, while glycosylated phenolics such as chlorogenic acid[17] and rutin[18] were not or less absorbed as the intact form due to their high sensitivity to enzymatic hydrolysis during the absorption process. In contrast, a conflicting result on the absorption of chlorogenic acid was reported by Lafay et al.,[19] who demonstrated that it (or 5-*O*-caffeoylquinic acid) can be absorbed intact through

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rat stomach, together with intact absorption of caffeic acid cleaved from chlorogenic acid by mucosa esterase. According to the report that sugar substitution in anthocyanins had a significant impact on their absorption in rats,[20] it seems likely that the sugar moiety composed of CS may determine its digestive stability or intact absorption.

In the present study, thus, we tried to evaluate whether CS can be absorbed in intact form and how it is metabolized after oral administration to SD rats. An electrochemical detector−high-performance liquid chromatography (ECD-HPLC) system was applied for CS, caffeic acid, and ferulic acid assays, because of low ionization efficiency for CS by an electrospray ionization−time-of-flight−mass spectrometer (ESI-TOF-MS), whereas overall metabolic analyses of CS in plasma were conducted with the ESI-TOF-MS.

MATERIALS AND METHODS

Materials. β-Glucuronidase type B-1 and sulfatase type H-1 were purchased from Sigma Chemical Co. (St. Louis, MO). Caffeic and ferulic acids were purchased from Wako Pure Chemical Industry (Osaka, Japan). Other reagents were of analytical grade and used without further purification.

Preparation of CS. CS was obtained from a red-colored vinegar that was developed via fermentation of the storage root paste of purple-fleshed sweet potato (*I. batatas* cv. Ayamurasaki).[9] Briefly, a red vinegar (3 L) was evaporated and freeze-dried under reduced pressure. The dried powder of vinegar (4.9 g) was dissolved in methanol and applied to an ODS-HPLC analysis (L-6200 intelligent pump system, Hitachi Co., Tokyo, Japan) on a column (Inertsil ODS 5, 20 mm × 250 mm, GL Sciences, Tokyo, Japan) with 30% acetonitrile containing 15% acetic acid at 7.0 mL/min. An L-4200 UV−vis detector (Hitachi, Tokyo, Japan) was used for monitoring at 310 nm. A peak corresponding to CS elution with a retention time of 12.9 min was collected and evaporated to dryness (purity, >95%; no caffeic acid[9]).

Animal Experiments. Experimental animals, 21 male SD rats (SPF/VAF Crj: SD, 7 weeks old, 294.4 ± 12.5 g) supplied by Charles River Japan (Kanagawa, Japan) were divided into seven groups according to fixed times (0, 0.25, 0.5, 1, 2, 4, and 6 h) after oral administration of CS (three rats in each group). The rats were acclimatized under laboratory conditions (21 ± 1 °C, 55.5 ± 5% humidity, 12 h dark/light cycle) for 1 week before experiments and fed on an MF diet (Oriental Yeast, Tokyo, Japan) and given water ad libitum. All rats were handled in accordance with the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government. The rats were fasted for 16 h prior to a single oral administration of 400 mg/kg CS in 1 mL of saline solution. Before and after administration at each fixed time, rats were killed to take blood (approximately 10 mL). Blood in a tube with 0.5 mg heparin was immediately centrifuged at 3500*g* for 15 min at 4 °C to obtain a plasma sample.

Determination of CS in Blood. CS was determined by an HPLC equipped with an ECD (Coulochem III, ESA, Bedford, MA). First of all, an aliquot (500 μL) of plasma was deproteinized by 500 μL of 4% trichloroacetic acid and centrifuged at 10000*g* for 20 min at 4 °C. One milliliter of the supernatant was applied to a Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA), followed by an elution with 3 mL of 40% acetonitrile containing 0.1% trifluoroacetic acid. The extract was then evaporated to dryness under vacuum. The dried extract dissolved in 20 μL of distilled water was injected into ECD-HPLC system. The column used for the system was a Cosmosil 5C_{18}-AR II (4.6 mm × 150 mm, Nacalai Tesque, Kyoto, Japan), and isocratic elution was performed by methanol: water (18:82, v/v) containing 10 mM lithium acetate (pH 3.8) at 0.8 mL/min at 37 °C. The potentials of ECD detector were set at 100 mV for reference electrode and 800 mV for working electrode, respectively.

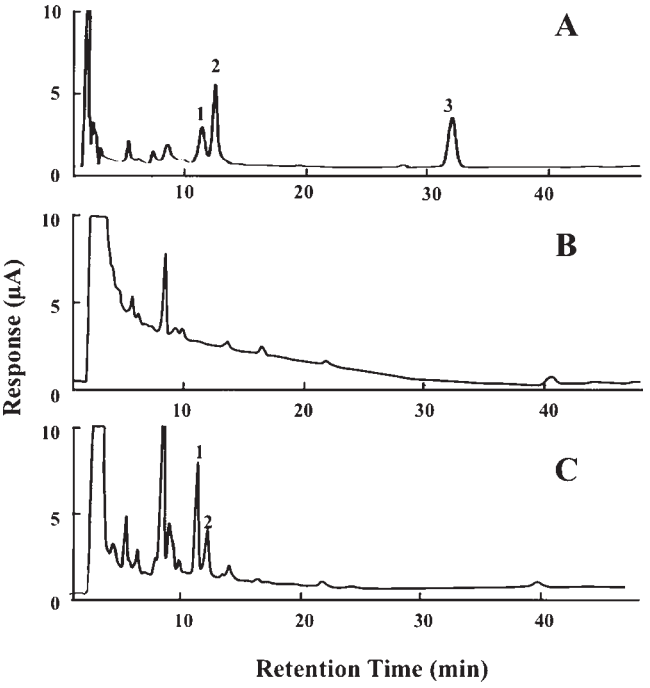

Figure 2. Typical ECD-HPLC chromatograms of standard (A) and rat plasma before (B) and 30 min after (C) a single oral administration of 400 mg/kg CS to SD rat. Peaks 1, 2, and 3 denote CS, caffeic acid, and ferulic acid, respectively. HPLC and ECD conditions are described in the Materials and Methods.

ESI-TOF-MS Analysis. The plasma (100 μL) collected after CS administration was subjected to an ESI-TOF-MS for analyzing CS metabolites. The dried extract from CS-administered blood mentioned above was dissolved in 0.1% aqueous formic acid and then separated using an Agilent 1200 series (Agilent, Germany) equipped with a microdegasser (G1379B), a binary pump (G1312A), a high-performance autosampler SL (G1367B), and a thermostatically controlled oven apartment (G1316A). Chromatographic separation was carried out at 40 °C on a Cosmosil 5C_{18}-MS-II column (2.0 mm × 150 mm, Nacalai Tesque). The mobile phase consisted of 0.1% aqueous formic acid (A) and methanol with 0.1% formic acid (B) using a 20 min linear gradient from 0 to 100% of solvent B. The flow rate was 0.2 mL/min, and the injection volume was 20 μL. MS analysis was performed by a microTOF II (Bruker Daltonics, Bremen, Germany). The ESI-TOF-MS analysis worked in negative mode, and the mass range was set at 100−1000 *m*/*z*. The conditions of ESI source were as follows: drying gas, N_2; flow rate, 8.0 L/min; drying gas temperature, 200 °C; nebulizing gas pressure, 1.6 bar; and capillary voltage, 3800 V. All of the acquisitions and analyses of data were controlled by a Bruker Data Analysis 3.2 Software. To tune the detector to optimal conditions, calibration was performed with sodium formate clusters [10 mM sodium hydroxide in water:acetonitrile (1:1, v/v)]. The calibration solution was injected at the beginning of the run, and all of the spectra were calibrated prior to identification.

Sulfatase/β-Glucuronidase Treatment of Plasma. The plasma (100 μL) collected after CS administration was also subjected to a CS conjugate assay. The obtained plasma was evaporated to dryness and dissolved in 100 μL of 0.1 M sodium acetate buffer (pH 5.0) followed by enzymatic treatment with 300 μL of enzyme solution containing 250 units of sulfatase and 500 units of β-glucuronidase at 37 °C for 4 h. The reactant was then extracted with a Sep-Pak Plus C_{18} cartridge, and the amounts of CS and caffeic acid in enzymatic treated plasma were assayed with ECD-HPLC system as mentioned above. The amount of conjugate from CS or

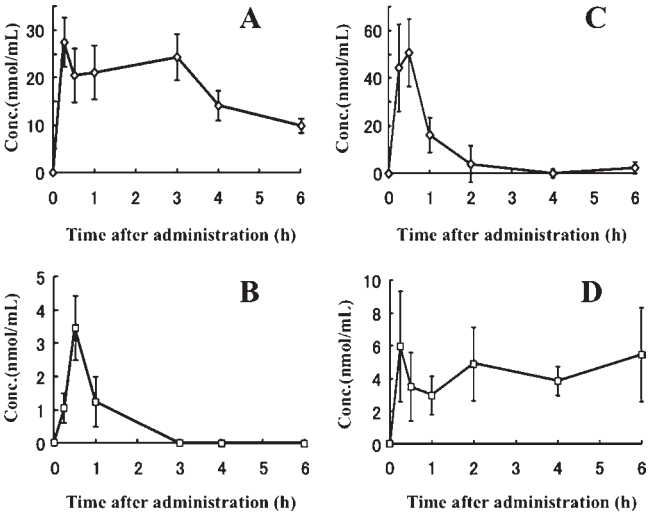

Figure 3. Time course of plasma concentrations of CS (A) and caffeic acid (B) in plasma without sulfatase/β-glucuronidase treatment after a single oral administration of 400 mg/kg CS to SD rat. Conjugated CS (C) and caffeic acid (D) in plasma obtained at fixed times were calculated from the difference in their amount between before and after enzymatic treatment. Each value is expressed as the mean ± SEM (n = 3).

caffeic acid in plasma was calculated from the difference in their amount between before and after enzymatic treatment.

Pharmacokinetic Analysis of CS and Its Metabolites in Plasma. The pharmacokinetic parameters, such as C_{max} and t_{max}, were analyzed from 0 to 6 h after CS administration to SD rats. The elimination of half-life ($t_{1/2}$) was calculated by plotting the plasma level against logarithmic time. The mean residence time (MRT) was calculated by dividing the area under the moment curve ($AUMC_{0-6h}$) by area under the curve (AUC_{0-6h}). The AUC_{0-6h} was calculated using the trapezoidal rule. All pharmacokinetic analyses were performed using a GraphPad Prism5 (GraphPad Software, La Jolla, CA).

RESULTS

Absorption of CS in SD Rats. Figure 2 shows the typical ECD-HPLC chromatograms of plasma samples before (Figure 2B) and 30 min after administration of CS (400 mg/kg) to SD rats (Figure 2C). Caffeic acid and ferulic acid were also assayed as targeted compounds of CS metabolites. Under the present ECD-HPLC conditions, the standards of CS, caffeic acid, and ferulic acid were separately detected with high sensitivities of >7.5, > 4.8, and >1.2 nmol/mL, respectively (Figure 2A). As compared with the chromatogram of the standards (Figure 2A), two peaks (peaks 1 and 2) corresponding to CS and caffeic acid were, respectively, detected at 11.7 and 12.7 min in CS-administered plasma (Figure 2C), while no corresponding peaks were detected in blank plasma (Figure 2B). This indicates that CS can be absorbed in the intact form into a SD rat's blood system even if it possesses a sugar (sophorose) moiety. A low concentration of caffeic acid shown in Figure 2C suggested that a small part of CS was hydrolyzed into caffeic acid and sophorose during the CS absorption process. No peak corresponding to ferulic acid was observed in the 30 min plasma sample.

The pharmacokinetic profiles of CS and caffeic acid are illustrated in Figure 3, and their pharmacokinetic parameters are summarized in Table 1. As shown in Figure 3A,B, the maximal absorption of CS (C_{max} at 0.25 h; 27.5 ± 5.2 nmol/mL) was much higher than that of its main metabolite, caffeic acid (C_{max} at 0.5 h; 3.5 ± 1.0 nmol/mL), together with a higher absorption amount of CS (AUC_{0-6h}, 108.6 ± 8.1 nmol h/mL) than that of caffeic acid (AUC_{0-6h}, 3.1 ± 0.9 nmol h/mL) (Table 1). In addition, CS kept the high plasma level to 3 h. The longer MRT value (2.6 h) of CS than that of caffeic acid (0.7 h) also demonstrated the high stability of CS in plasma (Table 1).

Table 1. Pharmacokinetic Parameters of Nonconjugated and Conjugated CS and Its Metabolite, Caffeic Acid, after a Single Oral Administration of 400 mg/kg CS to SD Rats[a]

	nonconjugated form		conjugate form	
	CS	caffeic acid	CS	caffeic acid
C_{max} (nmol/mL)	27.5 ± 5.2	3.5 ± 1.0	50.8 ± 14.3	
t_{max} (h)	(0.25)	0.5	0.5	
AUC_{0-6h} (nmol h/mL)	108.6 ± 8.1	3.1 ± 0.9	50.7 ± 5.7	25.6 ± 2.5
$t_{1/2}$ (h)	(4.7)	0.9	0.5	
MRT (h)	2.6	0.7		

[a] Data are the means ± SEMs (n = 3). t_{max}, time to reach maximum concentration; C_{max}, maximum concentration; $t_{1/2}$, half-life; AUC, area under the curve; and MRT, mean residence time. t_{max} and $t_{1/2}$ were calculated by plotting plasma levels at 0.25, 3, 4, and 6 h against logarithmic time (h). The value shown in parentheses is taken as "tentative" data. C_{max} was defined as a maximum concentration of compound among the levels obtained at each fixed time.

CS Conjugates in SD Rats. Figure 3C,D shows the absorption profiles of conjugated CS and conjugated caffeic acid in plasma treated by sulfatase and β-glucuronidase. The apparent increment of each compound indicates that some CS and caffeic acid were converted to conjugated forms after CS administration, and conjugated CS (AUC_{0-6h}, 50.7 ± 5.7 nmol h/mL) was calculated to be a half of nonconjugated (or intact) CS (AUC_{0-6h}, 108.6 ± 8.1 nmol h/mL).

ESI-TOF-MS Analysis of Plasma. To analyze the conjugates of CS and its metabolites, the plasma was subject to an ESI-TOF-MS, and their conjugates were identified on the basis of their exact mass. As shown in Figure 4 and Table 2, a total of 17 derivatives of CS and its metabolites were detected in plasma after the administration of CS. The conjugates of CS determined in plasma were glucuronide (CS-glcA, m/z 679.1727) and methylated (Me-CS, 517.1563; and 2Me-CS, 531.1719) compounds, which appeared at 30 or 60 min after administration, respectively. Glucuronide and sulfate conjugates of caffeic acid [CA-glcA, m/z 355.0671; CA-$(glcA)_2$, m/z 531.0992; and CA-S, m/z 258.9918] and ferulic or isoferulic acid (FA-glcA or isoFA-glcA, m/z 369. 0827; and FA-S or isoFA-S, m/z 273.0074) were detected in plasma at both 30 and 60 min after the administration of CS. Moreover, many dihydrides were found, including dihydrocaffeic acid-glucuronide (m/z 357.0827), dihydrocaffeic acid-sulfate (m/z 261.0074), and dihydroferulic acid-sulfate (m/z 275.0231). Interestingly, glucuronide of dihydrocaffeic acid existed in the plasma collected at 30 min rather than 60 min, while sulfates of dihydrocaffeic acid and dihydroferulic acid were detected at two time points.

DISCUSSION

So far, some researchers have reported that anthocyanins had an ability to show diverse functions including antihyperglycemia,[7]

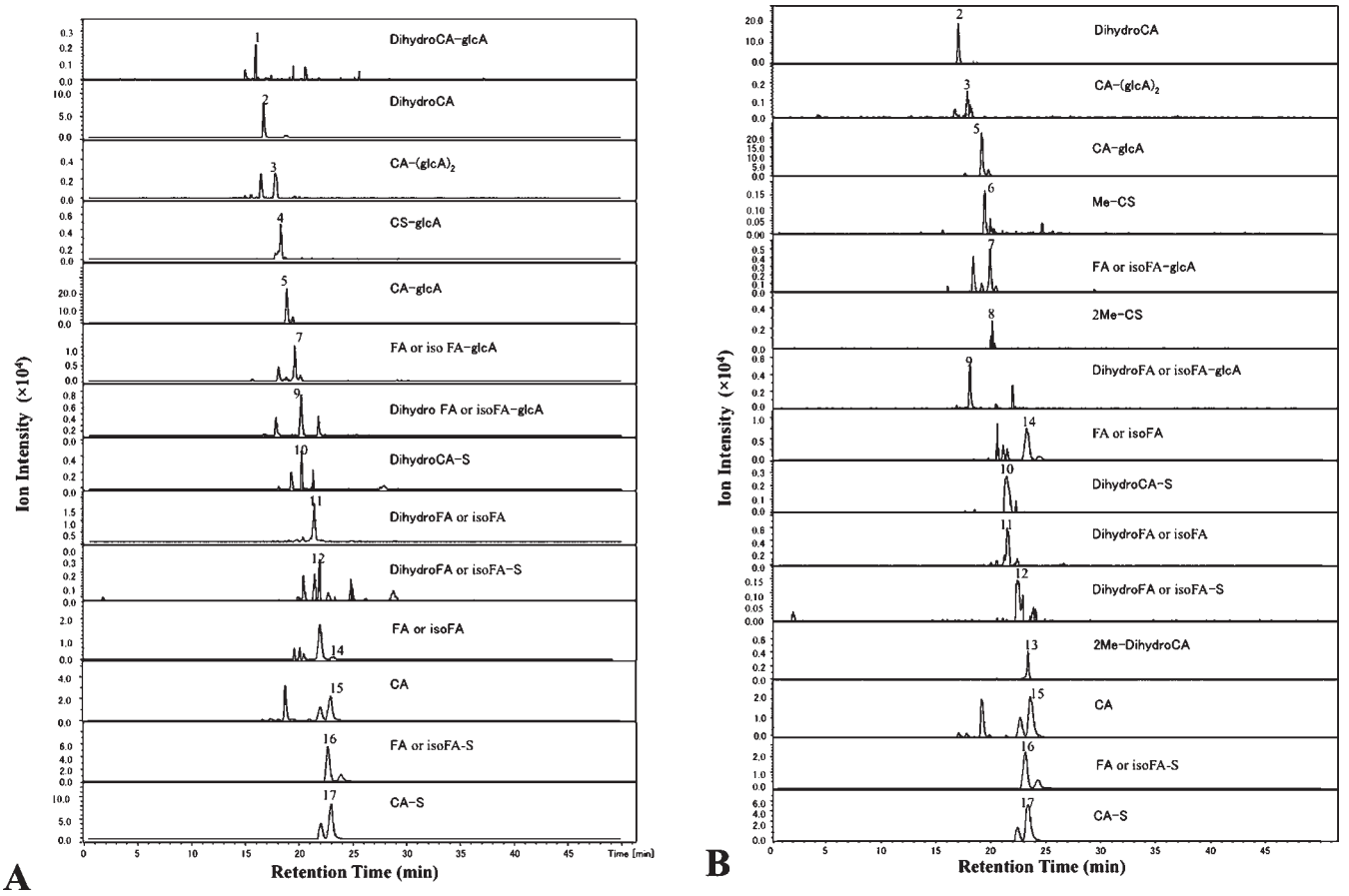

Figure 4. Extracted ion chromatograms of CS metabolites by LC-ESI-TOF-MS analysis in rat plasma collected at 30 (A) or 60 min (B) after a single oral administration of 400 mg/kg CS to SD rat. The condition of LC-ESI-TOF-MS analysis is described in the Materials and Methods.

Table 2. Derivatives of CS Identified by ESI-TOF-MS in CS-Administered Plasma

				compound		
peak	retention time (min)	molecular formula	$[M-H]^- m/z$	full name	abbreviation	collection time (min)
1	16.1	$C_{15}H_{18}O_{10}$	357.0827	dihydrocaffeic acid-glucuronide	dihydroCA-glcA	30
2	16.9	$C_9H_{10}O_4$	181.0506	dihydrocaffeic acid	dihydroCA	30, 60
3	17.7	$C_{21}H_{24}O_{18}$	531.0992	caffeic acid-(glucuronide)$_2$	CA-(glcA)$_2$	30, 60
4	18.4	$C_{27}H_{36}O_{20}$	679.1727	CS-glucuronide	CS-glcA	30
5	19.0	$C_{15}H_{16}O_{10}$	355.0671	caffeic acid-glucuronide	CA-glcA	30, 60
6	19.3	$C_{22}H_{30}O_{14}$	517.1563	methyl-caffeoylsophorse	Me-CS	60
7	19.7	$C_{16}H_{18}O_{10}$	369.0827	(iso)ferulic acid-glucuronide	FA or isoFA-glcA	30, 60
8	20.0	$C_{23}H_{32}O_{14}$	531.1719	dimethyl-CS	2Me-CS	60
9	20.3	$C_{16}H_{20}O_{10}$	371.0984	dihydro(iso)ferulic acid-glucuronide	dihydroFA or isoFA-glcA	30, 60
10	21.3	$C_9H_{10}O_7$	261.0074	dihydrocaffeic acid-sulfate	dihydroCA-S	30, 60
11	21.4	$C_{10}H_{12}O_4$	195.0663	methyl-dihydrocaffeic acid	dihydroFA or isoFA	30, 60
12	22.3	$C_{10}H_{12}O_7S$	275.0231	dihydro(iso)ferulic acid-sulfate	dihydroFA or isoFA-S	30, 60
13	23.1	$C_{11}H_{14}O_4$	209.0819	dimethyl-dihydrocaffeic acid	2Me-dihydroCA	30, 60
14	23.1	$C_{10}H_{10}O_4$	193.0506	(iso)ferulic acid	FA or isoFA	30, 60
15	23.1	$C_9H_8O_4$	179.0350	caffeic acid	CA	30, 60
16	23.1	$C_{10}H_{10}O_7S$	273.0074	(iso)ferulic acid-sulfate	FA or isoFA-S	30, 60
17	23.4	$C_9H_8O_7S$	258.9918	caffeic acid-sulfate	CA-S	30, 60

antioxidant functions,[8,9] anti-inflammatory functions, and antiatherosclerosis.[10,11] In addition, the moiety, such as caffeic acid and ferulic acid, also acted as a player to relax contracted vessels and inhibit proliferative inflammatory cells.[12,13] Therefore,

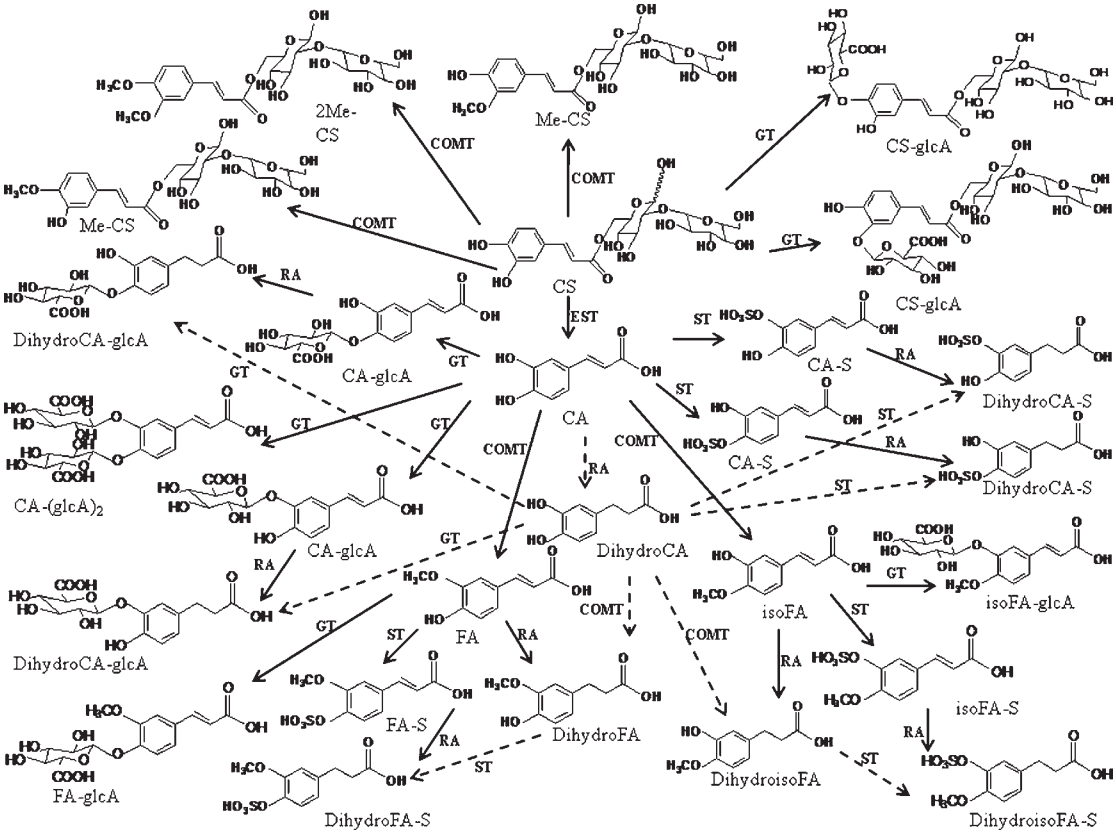

Figure 5. Proposed metabolism of CS during absorption on the basis of LC-TOF-MS analyses. CA, caffeic acid; FA, ferulic acid; COMT, catechol-*O*-methyltransferase; RA, reductase; GT, UDP-glucuronyltransferase; ST, sulfuryl-*O*-transferase; and EST, esterase. Bold arrows indicate major routes, and dotted arrows indicate minor ones.

it seems likely that anthocyanins or their metabolites have a potential in promoting our health. Intact absorption of anthocyanins, caffeic acid, and ferulic acid into the human blood system[10,14] strongly supported their potential in our body. In this study, we investigated absorption behavior of CS from acylated anthocyanins[9] showing acute antihyperglycemic action in rats[7] and successfully clarified for the first time that CS was an intact absorbable natural compound into rat blood system. Along with intact CS absorption, it was also found that administered CS to some extent received metabolic degradation such as conjugation reaction and enzymatic hydrolysis to caffeic acid during absorption process.

The pharmacokinetic analysis in the present study revealed that CS was rapidly absorbed to blood system and kept stably for 6 h after single oral administration of 400 mg/kg CS (Table 1). The C_{max} and AUC_{0-6h} of CS were 27.5 ± 5.2 nmol/mL and 108.6 ± 8.1 nmol h/mL, respectively, which were much higher than those of cyanidin-3-*O*-glucoside (C_{max}, 3.49 nmol/mL; AUC_{0-4h}, 3.62 nmol h/mL; 320 mg/kg single oral administration)[10] and peonidin 3-*O*-caffeoylsophoroside-5-*O*-glucoside (C_{max}, 0.05 nmol/mL; AUC_{0-2h}, 0.047 nmol h/mL; 146.3 mg/kg single oral administration).[14] In addition to the preferable absorption of CS, it kept the high plasma level up to 3 h. This profile may be because CS entered into enterohepatic circulation,[21] like chrologenic acid and caffeic acid.[19] According to the AUC_{0-6h} of CS (108.6 ± 8.1 nmol h/mL) and its conjugate (50.7 ± 5.7 nmol h/mL), a half of absorbed CS was converted to conjugates. The lower percentage of CS conjugation (ca. 50%) as compared with other phenolics (cyanidin-3-*O*-glucoside, > 90% conjugates;[10] epigallocatechin gallate, 50−90% conjugates[22]) also support the preferable intact absorption and high digestive stability of CS.

ESI-TOF-MS analyses revealed that except for intact CS and caffeic acid, CS-administered plasma contained some glucuronide and methylated conjugates of CS, as well as glucuronide, methylated or sulfate conjugates of caffeic acid and ferulic acid (Figure 4 and Table 2). The current data provide a number of indications serving as the basis for a working model of CS metabolic pathways involved (Figure 5). Considering that the glucuronide conjugate of CS was observed in the plasma collected at 30 min, while the methylated conjugates appeared at 60 min, and taking into account that liver is a major site of methylation of flavonoids,[17,23] glucuronide and methylated conjugates of CS may be formed in the small intestine and liver, respectively. The dihydro derivatives of caffeic acid and ferulic acid and their subsequent conjugates with glucuronides and sulfates would involve the metabolism in colon or liver depending on the food source and delivery.[24,25] According to the report that rat intestinal epithelium favors the glucuronidation of dihydrocaffeic acid, whereas rat liver favors sulfation,[26] the caffeic and ferulic acid-related derivative reactions would occur in both organs during the absorption process.

The transporting mechanism of CS across the intestinal membrane is one of the attractive interests in this absorption study. In previous reports, glycosylated compounds including chlorogenic acid (5-*O*-caffeoylquinic acid)[27] and hesperidin[28] were absorbed mainly via paracellular diffusion through a tight junction. The transport of phenolic acids such as caffeic acid has

also been reported to be involved in the intestinal monocarboxylic acid transporter.[27] Together, CS has the possibility to transport via either or both of the pathways, or other underlying mechanisms, and the study on a caco-2 cell transported experiment is now in progress.

In conclusion, it was demonstrated for the first time that administered CS to SD rats can be absorbed in intact form with a high stability in plasma, and a part of CS was metabolized by methylation and glucuronidation. The CS metabolites including glucuronide and methylated or sulfate conjugates of caffeic and ferulic acids were also observed to some extent in plasma in the process of metabolism.

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