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Enzymatic synthesis of substituted epicatechins for cognitive research

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Abstract

Bioavailability studies have indicated that several glucuronidated and/or methylated metabolites of the proanthocyanidin (PA) monomer (–)-epicatechin can be detected in both blood and brain tissues following feeding of rodents with a monomeric grape seed PA extract shown to reduce symptoms in a mouse model of Alzheimer's disease. To generate quantities of key metabolites suitable for future mechanistic studies, we have investigated the ability of recombinant human glucuronosyl transferases of the UGT1A and UGT2B families to glucuronidate epicatechin or 3'-*O*-methyl epicatechin in vitro. Of twelve enzymes tested, UGT1A9 was the most efficient, producing epicatechin 3'-*O*-glucuronide as the major product, a compound which is not, however, commonly observed in the biological fluids. Incubation of UGT1A9 with 3'-*O*-methyl-epicatechin resulted in two major products, one of which co-chromatographed with a major metabolite found in blood plasma and brain tissues; this was shown by LC-MS-TOF and NMR analysis to be 3'-*O*-methyl-epicatechin 5-*O*-glucuronide. Conditions were optimized to produce this compound at around 50% overall yield using UGT1A9, which was the most efficient enzyme of the twelve tested. We also investigated the in vitro methylation of epicatechin and epicatechin glucuronides by human catechol *O*-methyltransferase to devise strategies for the synthesis of radiolabeled epicatechin metabolites. These studies form a basis for generation of mg quantities of pure epicatechin (methyl) glucuronides of biological significance, and provide unequivocal assignment of the substitution positions of epicatechin metabolites that have been incompletely identified in previous studies.

Introduction

Plants collectively produce a staggering array of specialized metabolites, also known as natural products). Many of these compounds are polyphenolic, eg. flavonoids, proanthocyanidins, hydroxycinnamic acids, coumarins and stilbenes. Many polyphenolic compounds have been shown to confer health benefits, acting as general antioxidants and also likely functioning through binding to more specific molecular targets (Baba et al., 2000;

Hartman et al., 2006; Haza and Morales, 2010; Rezai-Zadeh et al., 2005; Spencer, 2003; Terao, 1999; Thomas et al., 2009; Wang et al., 2009). Potential development of polyphenols for preventing/treating neurological disorders is largely hindered by their complexity and our limited knowledge regarding the bioactivity, metabolism and bioavailability of these compounds, especially in the brain.

In a previous study, we have shown that a grape seed polyphenolic extract (GSPE) could inhibit β -amyloid (A β) oligomerization and improve cognitive function in the Tg2576 mouse model of Alzheimer's disease (Wang et al., 2008). We then developed procedures for fractionation of the GSPE into monomeric, oligomeric and polymeric fractions (Sharma et al., 2011), and showed that the monomeric units of proanthocyanidins (PAs) are the bioactive components in the GSPE (Sharma et al., 2011; Wang et al., 2011). PA monomers and their metabolites reach the brain at a concentration of 300–400 nM following 10 days of repeated dosing, while PA polymers are largely not bioavailable (Wang et al., 2011). Chronic oral application of the monomer-enriched fraction of the GSPE significantly reduced A β oligomer content in the brain and improved spatial memory in Tg2576 mice (Wang et al., 2011).

These studies point to the possibility that specific PA metabolites might improve cognitive function by binding to specific molecular targets in the brain. To test such hypotheses, it is necessary to firstly identify the metabolites, and secondly to synthesize them in quantities suitable for mechanism of action studies, preferably in a radiolabeled form. Several studies have reported the presence of PA metabolites in blood plasma after feeding animals with PA preparations or foods rich in PAs, such as cocoa (Baba et al., 2001; Baba et al., 2000; Feng, 2006; Spencer et al., 2001c). Although the metabolites have been partially identified as sulfonated, glucuronidated and/or methylated derivatives of the PA monomers epicatechin and catechin (Abd-el-Mohsen et al., 2002; Baba et al., 2000; Piskula and Terao, 1998; Spencer, 2003), assignment of definitive structures (ie with the positions of all substitutions proven unequivocally) have yet to be made for most biologically relevant metabolites, with some notable exceptions (Natsume et al., 2003; Okushio et al., 1999)

In the present study, we utilized mammalian enzymes to biochemically synthesize the epicatechin metabolites consistent with major forms found in brain tissues and blood plasma of rodents following chronic application of the monomer-enriched fraction of GSPE. The identities of the synthetic metabolites were unequivocally established, and the synthetic procedures optimized to allow for efficient production of mg quantities of the target molecules.

Materials and Methods

Biochemical synthesis of epicatechin glucuronides, methyl-epicatechins, and methyl-epicatechin glucuronides

UDP-glucuronosyl transferase enzymes, buffers, and UDP-glucuronic acid were purchased from BD Biosciences (San Diego, USA). UGT insect cell control Supersomes (BD Biosciences) were used as the negative control and pooled male mouse liver microsomes, or human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9,

UGT1A10, UGT2B4, UGT2B7, UGT2B15, or UGT2B17 supersomes were used for the glucuronidation of epicatechin or 3'-*O*-methyl epicatechin.

Initially, the glucuronidation of epicatechin was performed under similar assay conditions as described by BD Biosciences for their BD-Supersomes cDNA-expressed UDP-glucuronosyl transferase enzymes. For a 500 μ l assay, 350 μ l purified water, 100 μ l UGT Reaction Mix Solution B (BD Biosciences Cat. No. 451320), 20 μ l UGT Reaction Mix Solution A (Cat. No. 451300), 10 μ l 50mM (-)-epicatechin (Sigma Aldrich), and 20 μ l BD-Supersomes (5 mg protein per ml) were used. Reactions were incubated at 37 °C for 0, 6, and 24 h, then stopped by the addition of 94% acetonitrile/6% glacial acetic acid or methanol/3% phosphoric acid at a ratio of 2:1 (v:v). The samples were then centrifuged at $10,000 \times g$ for three min at room temperature and analyzed by HPLC, monitoring at 280nm.

Catechol-*O*-methyltransferase (C-OMT) from porcine liver (Sigma Aldrich) and S-(5'-adenosyl)-L-methionine (SAM) (Sigma Aldrich) were used to methylate epicatechin under standard assay conditions (Ni et al., 1996). The sample was incubated at 37 °C for 6 h and analyzed by HPLC, monitoring at 280 nm. 3'- and 4'-*O*-methyl-epicatechin were purchased from Nacalai USA, Inc. (San Diego, CA).

Several attempts were made to increase the efficiency of the glucuronidation of 3'-*O*-methyl-epicatechin by modifying either incubation temperature, pH, reaction time, or substrate and enzyme concentration. The final assay procedure was to incubate 210 μ l purified water, 100 μ l UGT Reaction Mix Solution B, 40 μ l UGT Reaction Mix Solution A, 10 μ l of 20 mM 3'-*O*-methyl epicatechin, and 120 μ l human UGT1A9 (5 mg protein per ml). After 6 h, a further 40 μ l of UGT Reaction Mix Solution A, 40 μ l of Reaction Mix Solution B, 120 μ l UGT1A9, and 10 μ l of 20 mM 3'-*O*-methyl epicatechin were added. Then at 12 h, an additional 40 μ l UGT Reaction Mix Solution A and 120 μ l UGT1A9 were added. The reactions were incubated at room temperature for 24 h and stopped by the addition of 400 μ l of methanol containing 3% phosphoric acid.

Purification of glucuronidated compounds

Enzymatic products were first partially purified from the aglycone substrate by ethyl acetate extraction (1:1 v:v); the aglycone is extracted into the organic phase, while the majority of the epicatechin glucuronides remain in the aqueous fraction. This strategy was utilized in the scale-up prior to purification of the glucuronide by HPLC. Residual ethyl acetate in the aqueous phase was evaporated under nitrogen. The aqueous fractions were then acidified to 1% phosphoric acid final concentration, and desalted using a Waters Sep-Pak Plus C18 cartridge. The cartridge was first washed with 10 ml methanol, followed by 20 ml water. The sample (5 ml) was loaded onto the cartridge, then washed twice with 5 ml water, and finally eluted twice with 5 ml methanol. The majority of the compounds were in the first methanol wash. The methanol was dried under nitrogen and then resuspended in a small volume of methanol for HPLC purification.

HPLC analysis

HPLC analysis and purification were performed on either an Agilent HP1200 HPLC or a Beckman System Gold HPLC, monitoring at 280nm. The Agilent HPLC with Chemstation

software version B.02.01.SRI was equipped with a G1322A degasser, a G1311A quaternary pump, a G1367B autosampler, a G1316A thermostatic column compartment, and a G1315C diode array detector. The Beckman HPLC with the System Gold operating system had a model 126 solvent module, 168NM diode array detector, and a manual injector. A Varian Metasil 5 Basic C18 250 X 4.6 mm column was used for analytical and micro-preparative HPLC on the Agilent HPLC system, while an Alltech Econosil 10 μ C18 250 X 22 mm column was used for larger scale preparative purification on the Beckman HPLC.

Analytical and small scale preparative HPLC was performed using the following gradient: isocratic at 5 % B for 5 min; 5 % B to 10 % B in 5 min; 10 % B to 17 % B in 15 min; 17 % B to 23 % B in 5 min; then 23 % B to 50 % B in 35 min. Alternatively, a shorter method was used which retained the same gradient except that the last step gradient was from 23 % B to 28 % B in 5 min, reducing the run time by 30 min. Solvent A was 1 % phosphoric acid in milliQ water, and solvent B was HPLC grade acetonitrile. Flow rate was 1 ml/min.

Preparative HPLC for purification on the Beckman system used the following gradient: isocratic at 5 % B for 7.5 min; 5 % B to 10 % B in 7.5 min; 10 % B to 17 % B in 22.5 min; then 17 % B to 23 % B in 7.5 min. Solvent A was milliQ water, and solvent B was HPLC grade acetonitrile. Flow rate was 15 ml/min.

LC-MS-TOF analysis of EC metabolites

Analysis of natural and biosynthetic catechin metabolites was conducted on an Agilent 1100 system equipped with an Agilent MSD-TOF (Palo Alto, CA) using a Varian C18 amide column (3 μ m, 150 $\times$ 2.1 mm i.d) as previously published (Ferruzzi et al., 2009). Briefly, a binary mobile phase consisting of solvent systems A [0.1% formic acid (v/v) in dd water] and B [0.1% formic acid (v/v) in acetonitrile] were used in a gradient elution. ESI capillary voltage was -3.5 kV, nebulizer gas pressure was set at 35 psig, gas temperature was 350 $^{\circ}$ C, drying gas flow rate was 9.0 l/min, fragmentor voltage was set to 165 V, skimmer 60 V and OCT RF V 250 V. Spectroscopic (UV at 280 nm) and mass data (from m/z 60–1000) were collected and analyzed using Analyst QS1.1 software (Applied Biosystems/MSD SCIEX).

NMR-spectroscopy

Epicatechin glucuronides were dissolved in 0.6 ml methanol-*d*₄ (Cambridge Isotope Laboratories), evaporated to dryness under a nitrogen stream, re-dissolved in 0.2 ml CD₃OD, and placed in an OD 3 mm NMR tube. 1-D Proton, TOCSY, and NOESY NMR spectra and gradient enhanced COSY, HSQC, and HMBC spectra were acquired on a Varian Inova-500MHz spectrometer at 308 K (35 $^{\circ}$ C). Chemical shifts were measured relative to the methyl signal of CD₃OD (δ _H = 3.30 ppm, δ _C = 49.0 ppm). Additional experiments were conducted with a Bruker 800 MHz spectrometer to determine the specific points of attachment of the glucuronide moiety; 1D NMR experiments used one-pulse and NOE (Nuclear Overhauser enhancement) sequences and 2D NMR experiments used TOCSY (total correlated spectroscopy).

Results

Epicatechin metabolites in blood plasma and brain of mice fed low molecular weight GSE fraction

In a previous study, we have shown that the monomeric units of PAs are the bioactive components in the GSPE (Sharma et al., 2011; Wang et al., 2011). Chronic oral application of the monomer-enriched fraction of the GSPE significantly reduced A β oligomer content in the brain and improved spatial memory in Tg2576 mice, while PA polymers are largely not bioavailable and are inactive in this assay (Wang et al., 2011). A number of metabolites of epicatechin and catechin were observed in both blood plasma and brain during pharmacokinetic experiments with the GSPE monomeric fraction. Typical HPLC traces of the plasma (epi)catechin and methyl-(epi)catechin metabolites are shown in Figures 1B and 1D. The metabolites marked **1**, **2** and **3** were all shown, by LC/MS analysis, to be glucuronidated derivatives of (epi)catechin while the metabolite marked **7** was shown to be a methyl-epicatechin glucuronide. These metabolites are also observed in brain tissues with the methyl-epicatechin glucuronide being the major peak. The peaks representing catechin derivatives were not addressed in the present study, since catechin does not appear to impact cognition in Tg2576 mice. It was not possible, based on previous data in the literature, to unequivocally assign positions to the glucuronosyl or methyl substituents on the epicatechin molecule, and there was an insufficient amount of metabolites from the plasma and brain tissues for NMR analysis.

Ability of human UGT1A isoforms to glucuronidate epicatechin

PA monomers and their metabolites reach the brain at a concentration of 300–400 nM following 10 days of repeated dosing in Tg2576 mice (Wang et al., 2011). The goal of the present study was to devise a biochemical synthesis for these compounds to facilitate subsequent mode of action studies, which would require mg quantities, in addition to providing standards for the identification of the in vivo metabolites.

We first tested the ability to generate epicatechin glucuronides using mammalian glucuronosyl transferase enzymes in vitro. Because the glucuronidation of epicatechin may theoretically occur at several positions (Figure 2), we tested a number of different isoforms of human UGT1A. The glucuronosyl transferase assays for epicatechin glucuronidation were performed under similar conditions as described by BD Biosciences for their BD-Supersomes cDNA-expressed UDP-glucuronosyl transferase enzymes. Four human UGT supersomes, UGT1A6, UGT1A7, UGT1A8, and UGT1A9, were initially tested as well as a mouse liver microsomal preparation. Based on this study, UGT1A9 was chosen as the enzyme of choice because it produced one major and one minor product with UV spectra similar to that of epicatechin when incubated with epicatechin and UDP-glucuronic acid (Figure 1A), whereas the mouse liver microsomes themselves contained and/or generated several products that did not give a UV spectrum similar to that of epicatechin. UGT1A8 produced a very small amount of product, and the other two UGTs did not produce any products from epicatechin.

Identification of the major product of UGT1A9 as epicatechin-3'-O-glucuronide

Incubation of epicatechin with UDP-glucuronic acid and UGT1A9 for 24 h under the conditions described above resulted in glucuronidation of about two thirds of the available substrate (Figure 3A,B). A partial purification of the products was first performed by extracting the reaction with ethyl acetate (1:1 v:v). All of the aglycone was found in the organic phase (Figure 3C) while most of the glucuronides remained in the aqueous phase (Figure 3D). Addition of phosphoric acid to a 1% final concentration then allowed the products to bind to a Waters C18 Sep-Pak cartridge, from which the products were eluted in 5ml methanol. They were dried under nitrogen, resuspended in a minimal volume of methanol, and then purified on a Beckman preparative HPLC. The products were collected in 1ml fractions, and these fractions were then analyzed on an Agilent HP1200 analytical HPLC.

The fractions eluting at 19, 20, 21, and 22 min, representing the major glucuronidated product, were combined; the acetonitrile was removed under a stream of nitrogen gas, and the aqueous fraction was frozen in liquid nitrogen and lyophilized. The purified product (600 µg) was analyzed by NMR at the Complex Carbohydrate Research Center, University of Georgia. The proton NMR spectrum of the sample exhibited five proton signals in the aromatic region (Table 1). Multiplicity and spin-spin coupling constant value (J) of signals at 7.08 (dd, $J_1=8\text{Hz}$, $J_2=1.5\text{Hz}$) and 6.85ppm (d, $J=8\text{Hz}$) indicated that they are ortho-coupled and therefore assigned to H6' and H5' of the aromatic B ring of the aglycone moiety. The doublet at 7.34 ($J=1.5\text{Hz}$) arises from the proton in the meta position to H6' and was assigned to H2' of the epicatechin. Broad singlets at 5.96 and 5.94 ppm, multiplicity and coupling constants of which were not determined due to fast exchange of these protons with deuterium, were assigned to H6 and H8 of the aglycone. Chemical shifts of the C ring protons (protons at C2, C3 and methylene protons at C4 positions) were assigned according to multiplicity and coupling constant values which were in agreement with those reported in the literature (Okushio et al., 1999). The broad signal around 4.85–4.87 ppm (br d, J not determined) had two proton intensities and the gHSQC spectrum confirmed that these were indeed 2 different protons (Table 1); the H2 of the aglycone and the anomeric proton of the β-glucuronosyl residue (H1''). All other protons of the β-glucuronosyl residue appeared in the carbohydrate ring proton region between 3.5–4 ppm.

The gHMBC spectrum allowed assignment of chemical shifts for C5 (158.1 ppm), C7 (157.4 ppm), C4a (99.9 ppm), C8a (157.4), C3' (146.2 ppm) and C4' (148.3 ppm) of epicatechin – carbons without directly linked protons. The cross peak in the gHMBC spectrum between C3' of the aglycone and the anomeric proton of the β-glucuronosyl residue indicated that β-glucuronic acid is glycosidically linked to the O-3' position of the epicatechin (Figure 2). This linkage position was further confirmed by a cross peak in the NOESY spectrum between H2' of epicatechin and the anomeric proton of the β-glucuronic acid residue), and therefore, the compound was identified as (–)-epicatechin-3'-O-glucuronide (Figure 2).

LC/MS comparison of the epicatechin glucuronide products made with UGT1A9 and the metabolites in the plasma from mice which had been fed the GSPE monomer fraction revealed that the major UGT1A9 reaction product, epicatechin-3'-O-glucuronide, was not

found in the mouse plasma (Figure 1A, peak 5). However, the minor product, Peak 4, in the enzyme reaction mixture matched peak 3 in the mouse plasma (Figure 1B), and both peaks corresponded to epicatechin glucuronides (m/z 465). This minor product was tentatively assigned as epicatechin-5-*O*-glucuronide (see below).

Synthesis of 3'-*O*-methyl-epicatechin-5-*O*-glucuronide

Analysis of plasma and brain tissue had indicated that some of the epicatechin glucuronide derivatives were also methylated (Figure 1D, Supplementary Figure 1, peak 7), and this methylation would likely be on the 3'-*O* position, the preferred position for intestinal glucuronidation by UGT1A9. It was therefore important to determine how UGT1A9 would glucuronidate 3'-*O*-methyl-epicatechin, in which the 3'-*O* position was already substituted. The results of this experiment indicated that the major product (Figure 4, peak 2 and Figure 1C, peak 6) was a match, based on HPLC retention time and MS-TOF analysis, to the major product found in brain and plasma (Figure 1D, peak 7, Supplementary Figure 1, peaks 6 and 7). However, the overall in vitro conversion was only approximately 5% based on the optimal assay conditions previously established with epicatechin as substrate.

To increase the conversion rate to provide a cost-effective in vitro enzymatic synthesis of this product, we systematically investigated changes in temperature, pH, and reaction time with UGT1A8, UGT1A9, and UGT2B7. UGT1A9 consistently outperformed the other two UGTs in all of the assay modifications above and was therefore the enzyme of choice for the synthesis of the major product and further optimization of assay conditions. The standard pH for the UGT assays (7.0) was within the optimal range for the enzymatic reaction.

Interestingly, incubations at room temperature gave higher conversions than at 30°C or above. However, there was still a large amount of (costly) 3'-*O*-methyl-epicatechin left unconverted at the end of the reaction, with only 7.3% conversion after 24 h. Adding extra UDP-glucuronic acid every 4 h increased the overall conversion at 24 h to 8.5%, whereas adding extra UGT1A9 every 4 h doubled the 24 h conversion to 15.5%. The enzyme was therefore most probably the limiting factor. If extra enzyme and UDP-glucuronic acid were added together every 4 h, the product formation increased to ~20% (Figure 4, peak 2).

Since the assay appeared to be linear over a 12 h period and the addition of enzyme and UDP-glucuronic acid every 4 h provided an increase in product formation above the standard linear assay, we increased the amount of UGT1A9 and UDP-glucuronic acid in the initial reaction, and then added more of each at 6 and 12 h, along with additional buffer. The final assay conditions are described in Materials and Methods, and gave approximately 50% conversion after 24 h incubation. Eight UGT1A and four UGT2B family member enzymes were tested under the final optimized assay conditions (Table 2). UGT1A9 was much more efficient at synthesizing the desired product (Table 2, peak 2), giving an approximately 50% conversion rate. The second most productive enzyme was UGT1A8, producing only about 15% as much product as UGT1A9. Interestingly, both UGT1A8 and UGT1A10 produced a significant amount of a different 3'-*O*-methyl-epicatechin glucuronide (76% and 58% respectively compared to the amount of the desired product synthesized with UGT1A9) (Table 2, peak 5). The major product of UGT1A9 was purified by preparative HPLC as

previously described for the purification of (–)-epicatechin-3'-*O*-glucuronide and analyzed by LCMS (Figure 1C and D) and NMR (Table 3).

NMR analysis determined the position of glucuronide attachment to the 3'-*O*-methyl epicatechin. Using a series of NOE experiments which irradiated the signals from protons on both epicatechin and glucuronic acid moieties of the molecule, a positive correlation was observed between the anomeric proton (4.9ppm) and the proton on position 6 (Table 3). This NOE peak is consistent with glucuronidation at the 5-position on the A ring of the epicatechin structure (Harada et al., 1999). Considering the known configuration of 3'-*O*-methyl epicatechin and the evidence of glucuronidation at the 5 position, the compound was determined to be 3'-*O*-methyl-epicatechin-5-*O*-glucuronide (Figure 2), and was produced with a 24 h conversion rate of around 50% allowing for optimal purification for subsequent use in biochemical/biological assays (Table 2).

Enzymatic substitution of epicatechin by methylation, glucuronidation followed by methylation, and methylation followed by glucuronidation

Mechanism of action studies will require radiolabeled (methyl)-epicatechin glucuronides. The approaches described above can readily provide epicatechin 3'-*O*-glucuronide or 3'-*O*-methyl-epicatechin-5-*O*-glucuronide labeled in either the epicatechin moiety (most easily with ³H through custom synthesis) or the glucuronide moiety (from UDP-¹⁴C-glucuronic acid). To provide a route for generation of ¹⁴C labeled methyl-epicatechin glucuronides with the label on the non-hydrolyzable methyl group, we investigated the in vitro methylation of epicatechin or epicatechin glucuronide using catechol-*O*-methyltransferase from porcine liver. Standards of 3'- and 4'-*O*-methylated epicatechins were used to positively identify the two major products of the reaction with epicatechin based on retention time and UV spectra (Figure 5E, peaks **3** and **4**). Glucuronidation of the methylated epicatechin mixture with UGT1A9 produced at least two glucuronidated methyl epicatechins which were 3'-*O*-methyl-epicatechin-5-*O*-glucuronide and a 4'-*O*-methyl-epicatechin-glucuronide (Figure 5F, peaks **5** and **6**).

The converse approach of glucuronidating the epicatechin prior to methylation did not work (Figure 5A–C), since allowing the glucuronidation to proceed to completion resulted in formation of (–)-epicatechin-3'-*O*-glucuronide which is the preferred methylation site of catechol-*O*-methyltransferase. Glucuronidation to completion was accomplished by using the optimized enzymatic assay conditions established for the glucuronidation of 3'-*O*-methylated epicatechin. The minor glucuronidated peaks did not appear to be methylated in this reaction either, perhaps because their concentrations were too far below the *K_m* value of the enzyme.

Discussion

In vivo metabolism of (epi)catechins in mammals

Tea, cocoa, chocolate, apples, and grape seed all contain relatively high levels of flavonoids, in particular catechin, epicatechin and their derivatives. These compounds and their metabolites are known to have antioxidative properties, aiding in the prevention of oxidative-

stress-induced cell death (Abd-el-Mohsen et al., 2002; Baba et al., 2000; Harada et al., 1999; Spencer et al., 2001a; Spencer et al., 2001b; Spencer et al., 2001c; Terao, 1999). Catechin and epicatechin both prevent heterocyclic amine-induced oxidative DNA damage (Haza and Morales, 2010), and in vivo glucuronidation and methylation of epicatechin may increase its bioactivity against oxidative stress-induced cell death in fibroblasts and neurons (Spencer et al., 2001a).

Because (epi)-catechin metabolites are beneficial for such a variety of health reasons, it is important to understand their metabolic fates and final disposition throughout the body. One study identified epicatechin as having higher in vivo bioavailability in rats than catechin (Baba et al., 2001), and several studies have examined the metabolic fates of (epi)catechins in rodents and humans. Some differences and similarities in metabolites were observed after feeding epicatechin orally to humans and rats (Feng, 2006; Natsume et al., 2003). In general, humans produced more sulfated conjugates than rats (Vaidyanathan and Walle, 2002). Both humans and rats had a variety of methylated and/or conjugated epicatechins in the urine and plasma following oral administration of epicatechin. Specifically, the metabolites identified in human urine and plasma were epicatechin-3'-O-glucuronide, 4'-O-methyl-epicatechin-3'-O-glucuronide, and 4'-O-methyl-epicatechin-5 or 7-O-glucuronide (Natsume et al., 2003), whereas rat plasma and urine contained 3'-O-methyl-epicatechin, epicatechin-7-O-glucuronide, and 3'-O-methyl-epicatechin-7-O-glucuronide. Only 8% of the orally administered epicatechin appeared in the urine in all metabolic forms 24 hours later in rats (Okushio et al., 1999); this publication identified epicatechin, 3'-O-methyl-epicatechin, 4'-O-methyl-epicatechin, epicatechin-5-O-glucuronide, and 3'-O-methyl-epicatechin-5-O-glucuronide (Okushio et al., 1999). Another study identified epicatechin glucuronide and 3'-O-methyl-epicatechin glucuronide in plasma and brain tissue following oral administration of epicatechin, although the site(s) of glucuronidation was not identified (Abd-el-Mohsen et al., 2002).

In vitro formation of substituted (epi)catechins

Although several publications address the metabolic fate of epicatechin in rats and humans, only a few have considered the specific enzymes that carry out the various methylation, glucuronidation, and sulfation reactions (Sugatani et al., 2004; Taskinen et al., 2003; Vaidyanathan and Walle, 2002; Webb et al., 2005). One of these demonstrated that UGT1A9 glucuronidated a variety of catechols at a high rate (Taskinen et al., 2003). Contrary to our current data, this study did not detect any activity for UGT1A9 with epicatechin, and did not test activity with any methylated epicatechins. It should be noted that this study used a one hour total incubation time for glucuronidation as compared to our 24 hour reactions (Taskinen et al., 2003).

In the present study, four UGT1A family members were tested for activity with epicatechin while eight UGT1A and four UGT2B family members were tested for activity with 3'-O-methyl-epicatechin. UGT1A9 was the most active enzyme with both substrates. UGT1A9 glucuronidated epicatechin at the 3'-position (and also most probably at the 5-O, but as a minor product) and 3'-O-methyl-epicatechin at the 5-O position as well as minor amounts of the three other possible methyl-epicatechin monoglucuronides (3, 7, and 4'). UGT1A8

produced very small amounts of epicatechin-3'-*O*-glucuronide and 3'-*O*-methyl-epicatechin-5-*O*-glucuronide, but 1A8 and 1A10 also produced a significant amount of another 3'-*O*-methyl-epicatechin glucuronide while 1A9 only produced a small amount of the same 3'-*O*-methyl-epicatechin glucuronide (Table 2, peak 5). The UGT2B family of human supersomes essentially had no to negligible activity with 3'-*O*-methyl-epicatechin; the most active 2B enzyme, UGT2B7, produced only 7 percent as much of the 3'-*O*-methyl-epicatechin-5-*O*-glucuronide as UGT1A9.

A biosynthetic procedure for synthesis of substituted epicatechins

Enzymatically methylating and glucuronidating epicatechin is a much quicker and safer method of synthesizing these metabolites than the standard chemical synthesis described in an earlier publication (González-Manzano et al., 2009). Although the length of procedures appear to be about the same, in order to chemically methylate epicatechin the procedure requires the use of methyl iodide (CAS #74-88-4) which has a health risk of three and may be fatal if inhaled causing acute depression of the central nervous system; it is a Class 6.1 hazard. Likewise, to chemically glucuronidate epicatechin requires the use of sodium methylate (CAS #124-41-4) which also has a health risk of three and requires the use of a vapor and dust respirator. Sodium methylate is also a Class 4.2 spontaneously combustible compound and a Class 8 corrosive. This procedure also requires several extra handling steps which more than doubles the work effort including the removal of acetyl moieties bound to the glucuronide residue after product formation (González-Manzano et al., 2009). Given the added health risks in both procedures and extra work load in the glucuronidation method, the enzymatic approach described here appears to be the safest and most effective method available. Also by using enzymes, one can theoretically custom synthesize the glucuronide of choice as demonstrated in Table 2. UGT1A9 makes predominately the 3'-*O*-methyl-epicatechin-5-*O*-glucuronide (77% of its total product formation), while UGT1A8 and UGT1A10 make predominately peak 5 at 82% and 98% of their total product formation, respectively (Table 2), whereas the chemical synthesis method described will always give a mixture of products (González-Manzano et al., 2009).

The optimized UGT assay involves incubating donor and acceptor substrates with human UGT1A9. After 6 h, more donor and acceptor substrates are added as well as more enzyme. Then at 12 h, more donor substrate and enzyme are added. The reactions are incubated at room temperature for 24 h and stopped by the addition of acidified methanol. The OMT assay is very straightforward, with a 6h incubation at 37°C and needing only the donor and acceptor substrates combined with the catechol-OMT enzyme. Products are partially purified and concentrated using a C18 Sep-Pak cartridge, then purified on preparative HPLC. The ability to methylate and/or glucuronidate epicatechin at specific positions allows us to synthesize a variety of compounds which may be radiolabeled on the methyl group as well as the epicatechin or glucuronide moieties. These compounds will be very useful for future mechanism of action studies which should allow us to determine specific compound/cell component interactions in the brain or other organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

LC/MS comparisons of epicatechin metabolites found in the plasma of mice fed GSPE or generated from epicatechin in vitro by the activity of human UGT1A9. (A) the glucuronidated epicatechin products formed in vitro with UGT1A9. (B) the glucuronidated (epi)catechin metabolites found in the plasma of mice that had been fed monomer enriched GSPE. (C) the purified major product from the glucuronidation of 3'-O-methyl-epicatechin with UGT1A9. (D) the glucuronidated methyl-(epi)catechin compounds found in the plasma of rats that had been fed monomer enriched GSPE. Peak 5 is (–)-epicatechin-3'-O-glucuronide. Peak 4 is an epicatechin glucuronide and an exact match based on retention time and mass spectral data to peak 3 from the rat plasma. Peak 6 has been identified as 3'-O-methyl-epicatechin-5-O-glucuronide by microcoil NMR (Table 2) and is an exact match to peak 7 from the rat plasma based on retention time and mass spectral data. Mass spectral data for peaks 3,4,6, and 7 are shown in Supplemental Figure 1.

Figure 2.

The structure of (-)-epicatechin with R groups at the 3', 4', or 5 positions which may be hydroxylated, *O*-methylated, or *O*-glucuronidated. 3'-OH, 4'-OH, 5-OH = epicatechin.

Figure 3.

Glucuronidation of epicatechin with UGT1A9 and partial purification of the glucuronidated products. HPLC chromatograms were monitored at 280nm. (A) control reaction. (B) UGT1A9 reaction. (C) organic phase of the ethyl acetate extraction of (B). (D) aqueous phase of the ethyl acetate extraction of (B). Identified peaks are: **1**, (–)-epicatechin; **2**, (–)-epicatechin-3'-O-glucuronide; **3**, (–)-epicatechin glucuronide.

Figure 4. Glucuronidation of 3'-O-methyl-epicatechin by UGT1A9. HPLC chromatograms were monitored at 280nm. (A) control reaction. (B) UGT1A9 reaction. Identified peaks are: **1**, 3'-O-methyl-epicatechin; **2**, 3'-O-methyl-epicatechin-5-O-glucuronide; peaks 3–5 are 3'-O-methyl-epicatechin glucuronides.

Figure 5.

Modification of epicatechin by glucuronidation followed by methylation or methylation followed by glucuronidation. HPLC chromatograms were monitored at 280nm. (A) control glucuronidation reaction. (B) 22 h glucuronidation reaction with UGT1A9. (C) 22 h glucuronidation reaction followed by 6 h methylation reaction with COMT. (D) control COMT reaction. (E) 6 h COMT reaction. (F) 6 h COMT reaction followed by 22 h glucuronidation reaction with UGT1A9. Identified peaks are: **1**, (–)-epicatechin; **2**, (–)-epicatechin-3'-O-glucuronide; **3**, 3'-O-methyl-epicatechin; **4**, 4'-O-methyl-epicatechin; **5**, 3'-O-methyl-epicatechin-5-O-glucuronide; **6**, 4'-O-methyl-epicatechin glucuronide.

Table 1Chemical shift assignments of Epicatechin-3-O- β -Glucuronide

Ring	Position	^1H	^{13}C	^1H - ^1H coupling
C	2	4.85	79.4	nd
	3	4.21	67.3	multiplet
	4	2.71(α)/2.85(β)	29.5	3.3 (α), 4.7 (β), 16.8 (gem)
	4a	-	99.4	-
A	5	-	156	-
	6	5.96	96	nd
	7	-	157.6	-
	8	5.94	95.5	nd
B	8a	-	157	-
	1'	-	132.4	-
	2'	7.34	117.6	1.5
	3'	-	146.2	-
GlcA	4'	-	148.3	-
	5'	6.85	116	8.0
	6'	7.08	122.9	1.5, 8.0
	1''	4.87	104.4	nd
	2''	3.53	74.2	nd
	3''	3.5	76.5	nd
	4''	3.59	72.6	nd
	5''	3.93	76.2	nd
	6''	-	172.5	-

nd – not determined

Table 2

Glucuronosyltransferase activity of human UGT1A and UGT2B supersomes

UGT	Peak 5	Peak 4	Peak 3	Peak 2
1A1	6	neg.	0	neg.
1A3	neg.	neg.	0	neg.
1A4	0	0	0	neg.
1A6	0	neg.	0	0
1A7	neg.	neg.	0	11
1A8	76	neg.	0	15
1A9	10	12	neg.	100
1A10	58	neg.	0	neg.
2B4	0	neg.	neg.	0
2B7	0	0	0	7
2B15	0	neg.	neg.	neg.
2B17	neg.	0	neg.	neg.

Data are expressed as percent of UGT1A9 product, peak 2 (3'-*O*-methyl-epicatechin-5-*O*-glucuronide) being 100 percent; actual amount of product formation is 2.2 µg/mg protein/h. If product formation is less than or equal to 5%, it is listed as negligible (neg.).