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Enzymatic synthesis of substituted epicatechins for bioactivity studies in neurological disorders

Jack W. Blount [a], Mario Ferruzzi [b], Dan Raftery [c], Giulio M. Pasinetti [d], Richard A. Dixon [a,*]

[a] Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA
[b] Department of Food Science, Purdue University, 745 Agriculture Mall Dr., West Lafayette, IN 47907, USA
[c] Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA
[d] Department of Psychiatry, The Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1230, New York, NY 10029, USA

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ABSTRACT

Glucuronidated and/or methylated metabolites of the proanthocyanidin (PA) monomer (−)-epicatechin are detected in both blood and brain 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 metabolites for future mechanistic studies, we 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. Incubation of UGT1A9 with 3′-*O*-methyl-epicatechin resulted in two major products, one of which was identified as 3′-*O*-methyl-epicatechin 5-*O*-glucuronide, a major metabolite found in blood plasma and brain tissue of the rodents following feeding with a grape seed extract. We also investigated in vitro methylation of epicatechin and epicatechin glucuronides by human catechol *O*-methyltransferase. Enzymatic production of 3′-*O*-methyl-epicatechin 5-*O*-glucuronide was optimized to 50% overall yield. These studies form a basis for generation of mg quantities of pure epicatechin (methyl) glucuronides of biological significance, and provide clarification of structure of previously identified epicatechin metabolites.

1. Introduction

The monomeric units of grape seed proanthocyanidins (PAs) inhibit β-amyloid (Aβ) oligomerization and improve cognitive function in the Tg2576 mouse model of Alzheimer's disease [1–3]. PA monomers and their metabolites reach the brain at a concentration of 300–400 nM following 10 days of repeated dosing, while PA polymers do not reach the brain [2]. Potential development of polyphenols such as PAs for preventing/treating neurological disorders is largely hindered by their complexity and our limited knowledge regarding their bioactivity, metabolism and bioavailability, especially in the brain.

Several studies have reported the presence of PA metabolites in blood plasma after feeding animals with PA preparations or foods rich in PAs [4–7]. The metabolites have been partially identified as sulfonated, glucuronidated and/or methylated derivatives of epicatechin (Fig. 1) and catechin [5,8–10], but definitive structures have yet to be assigned for many biologically relevant metabolites, with some notable exceptions. 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 [11], whereas rat plasma and urine contained 3′-*O*-methyl-epicatechin, epicatechin-5-*O*-glucuronide, epicatechin-7-*O*-glucuronide, 4′-*O*-methyl-epicatechin, 3′-*O*-methyl-epicatechin-5-*O*-glucuronide and 3′-*O*-methyl-epicatechin-7-*O*-glucuronide. Another study identified epicatechin glucuronide and 3′-*O*-methyl-epicatechin glucuronide in plasma and brain tissue following oral administration of epicatechin, suggesting that epicatechin is undergoing both methylation and glucuronidation in vivo, although the site(s) of glucuronidation was not identified [8].

To test the hypothesis that specific PA metabolites might improve cognitive function by binding to molecular targets in the brain, it is necessary to synthesize them in quantities suitable for mechanism of action studies. We here utilize mammalian enzymes to biochemically synthesize the epicatechin metabolites found in brain and blood plasma of rodents following chronic application of the monomer-enriched fraction of a grape seed polyphenolic extract (GSPE). The identities of the synthetic metabolites were unequivocally established, and the procedures optimized to allow for efficient production of mg quantities of the target molecules.

* Corresponding author. Fax: +1 580 224 6692.
E-mail address: radixon@noble.org (R.A. Dixon).

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2. Materials and methods

2.1. Biochemical synthesis of epicatechin derivatives

UDP-glucuronosyl transferase enzymes, buffers, and UDP-glucuronic acid were purchased from BD Biosciences (San Diego, USA). Standard glucuronosyltransferase assay conditions from BD Biosciences were used for initial studies on glucuronidation of epicatechin; 350 μl purified water, 100 μl UGT Reaction Mix Solution B (BD Biosciences), 20 μl UGT Reaction Mix Solution A (BD Biosciences), 10 μl 50 mM (−)-epicatechin (Sigma Aldrich), and 20 μl BD-Supersomes (5 mg protein per ml) were used in a 500 μl assay. Reactions were incubated at 37 °C for 0, 6, and 24 h, then stopped by addition of 94% acetonitrile/6% glacial acetic acid or methanol/3% phosphoric acid at a ratio of 2:1 (v:v). The samples were centrifuged at 10,000*g* for 3 min at room temperature and analyzed by HPLC, monitoring at 280 nm.

The optimized procedure for glucuronidation of 3′-*O*-methyl-epicatechin was to incubate 210 μl purified water, 100 μl UGT Reaction Mix Solution B, 40 μl UGT Reaction Mix Solution A, 10 μl of 20 mM 3′-*O*-methyl epicatechin, and 120 μl human UGT1A9 (5 mg protein per ml). After 6 h, a further 40 μl of UGT Reaction Mix Solution A, 40 μl of Reaction Mix Solution B, 120 μl UGT1A9, and 10 μl of 20 mM 3′-*O*-methyl epicatechin were added. Then at 12 h, an additional 40 μl UGT Reaction Mix Solution A and 120 μl UGT1A9 were added. The reactions were incubated at room temperature for 24 h and stopped by the addition of 400 μl of methanol containing 3% phosphoric acid.

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 [12]. 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).

2.2. Purification of glucuronidated compounds

Enzymatic products were partially purified by ethyl acetate extraction (1:1 v:v). 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 sample (5 ml) was loaded onto the cartridge, 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 resuspended in a small volume of methanol for HPLC purification.

2.3. HPLC analysis

HPLC was performed on either an Agilent HP1200 HPLC or a Beckman System Gold HPLC, monitoring at 280 nm. 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 system had a model 126 solvent module, 168NM diode array detector, and a manual injector. A Varian Metasil 5 Basic C18 250 × 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 × 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. 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.

2.4. LC-MS-TOF analysis of EC metabolites

Analysis of epicatechin metabolites was conducted on an Agilent 1100 system equipped with an Agilent MSD-TOF (Palo Alto, CA) using a Varian C18 amide column (3 μm, 150 × 2.1 mm i.d.) as previously published [13]. Briefly, a binary mobile phase consisting of solvent systems A [0.1% formic acid (v/v) in double distilled water] and B [0.1% formic acid (v/v) in acetonitrile] were used under gradient conditions. ESI capillary voltage was −3.5 kV, nebulizer gas pressure was set at 35 psig, gas temperature was 350 °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).

2.5. NMR-spectroscopy

Epicatechin glucuronides were dissolved in 0.6 ml methanol-*d4* (Cambridge Isotope Laboratories), evaporated to dryness under nitrogen, re-dissolved in 0.2 ml CD_3OD, and placed in an OD 3 mm NMR tube. 1H 1D NMR, 1H–1H TOCSY (total correlated spectroscopy), 1H–1H NOESY (nuclear Overhauser enhancement spectroscopy), gradient enhanced 1H–1H COSY (correlated spectroscopy), 1H–13C HSQC (heteronuclear single quantum coherence), and 1H-13C HMBC (heteronuclear multiple bond coherence) spectra were acquired on a Varian Inova-500 MHz spectrometer at 308 K (35 °C) at the Complex Carbohydrate Research Center, University of Georgia. Chemical shifts were measured relative to the methyl signal of CD_3OD (δH = 3.30 ppm, δC = 49.0 ppm). Additional experiments were conducted with a Bruker Avance-III 800 MHz spectrometer using an OD 5 mm shigemi tube, at the Purdue University's Chemistry department.

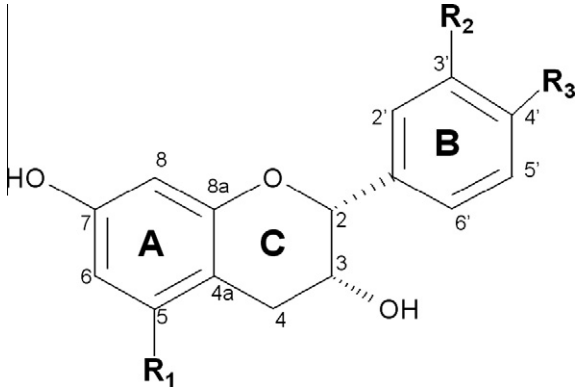

Fig. 1. The structure of (−)-epicatechin metabolites with substitutions (R groups) at the 3′, 4′, or 5 positions which may be hydroxyl, *O*-methyl, or *O*-glucuronide. (A) Epicatechin: $R_1 = R_2 = R_3 = OH$; (B) epicatechin-3′-*O*-glucuronide: $R_1 = R_3 = OH$, R_2 = *O*-GlA; and (C) 3′-*O*-methyl-epicatechin-5-*O*-glucuronide: R_1 = *O*-GlA, R_2 = *O*-Me, $R_3 = OH$.

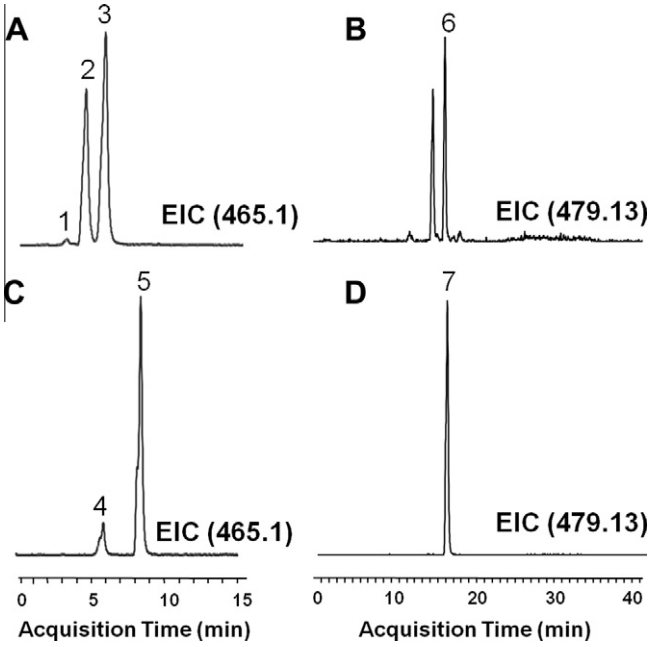

Fig. 2. LC/MS comparisons of epicatechin metabolites found in the plasma of mice fed GSPE or generated from epicatechin in vitro by the activity of human UGT1A9. (A) the glucuronidated (epi)catechin metabolites found in the plasma of mice that had been fed monomer enriched GSPE. (B) The glucuronidated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

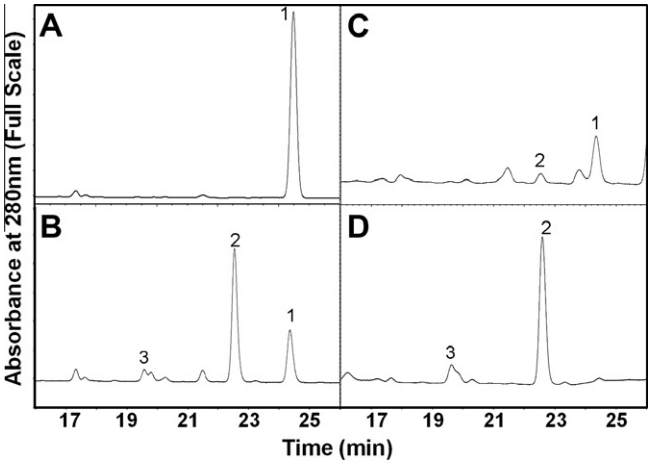

Fig. 3. Glucuronidation of epicatechin with UGT1A9 and partial purification of the glucuronidated products. HPLC chromatograms were monitored at 280 nm. (A) Control reaction stopped at zero time. (B) UGT1A9 reaction. (C) Organic phase of the ethyl acetate extraction of (B). (D) Aqueous phase of the ethyl acetate extraction of (B). Identified peaks are: **1**, (−)-epicatechin; **2**, (−)-epicatechin-3′-*O*-glucuronide; **3**, (−)-epicatechin glucuronide.

3. Results and discussion

3.1. Epicatechin metabolites found in blood plasma and brain of mice fed low molecular weight GSPE fraction

Typical HPLC traces of plasma (epi)catechin and methyl-(epi)catechin metabolites from mice fed the GSPE monomer fraction are shown in Figs. 2A and B. The metabolites marked **1**, **2** and **3** were shown by LC/MS analysis to be glucuronidated derivatives of (epi)catechin, while **6** 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. 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, since the amounts of metabolites from the plasma and brain tissues were insufficient for NMR analysis.

3.2. Glucuronidation of epicatechin by human UGT1A isoforms

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 [14–17]. In order to find the enzyme responsible for intestinal glucuronidation of epicatechin, and the best reagent for enzymatic synthesis of epicatechin glucuronides in vitro, we tested a number of different isoforms of human UGT1A as well as a mouse liver microsomal preparation for glucuronidation of epicatechin in vitro. Four human UGT supersomes, UGT1A6, UGT1A7, UGT1A8, and UGT1A9, were initially tested. 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 (Fig. 2C), 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.

Incubation of epicatechin with UDP-glucuronic acid and UGT1A9 for 24 h under the conditions described under Section 2 resulted in glucuronidation of about two thirds of the available substrate (Fig. 3A and B). On extraction with ethyl acetate (1:1 v:v), all of the aglycone was found in the organic phase (Fig. 3C) while most of the glucuronides remained in the aqueous phase (Fig. 3D). The products were subsequently purified using aC18 cartridge, fractionated using preparative HPLC, and further analyzed on an Agilent HP1200 analytical HPLC.

The fractions eluting at 19–22 min, representing the major glucuronidated product, were combined for NMR analysis. The proton NMR spectrum of the purified product (600 μg) exhibited five proton signals in the aromatic region (Supplementary Table 1). Multiplicity and spin–spin coupling constant value (J) of protons at 7.08 (dd, J_1 = 8 Hz, J_2 = 1.5 Hz) and 6.85 ppm (d, J = 8 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 Hz) arises from the proton in the meta position to H6′ and was assigned to H2′ of epicatechin. Broad singlets at 5.96 and 5.94 ppm 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 in the literature [18]. The broad signal around 4.85–4.87 ppm had two proton intensities and the HSQC spectrum confirmed that these were indeed two different protons (Supplementary 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 HMBC 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 HMBC 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. This linkage 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 (Fig. 1).

LC/MS comparison of the epicatechin glucuronide products made with UGT1A9 and the metabolites in the plasma from mice fed GSPE monomer fraction revealed that epicatechin-3′-*O*-glucuronide was not found in the mouse plasma (Fig. 2C, peak **5**). However, the minor product, Peak **4**, in the enzyme reaction mixture matched peak **3** in the mouse plasma (Fig. 2A), and both peaks corresponded to epicatechin glucuronides (*m/z* 465). This minor product was tentatively assigned as epicatechin-5-*O*-glucuronide (see below).

A previous study [16] did not detect any activity for UGT1A9 with epicatechin. It should be noted that this study used a 1 h total incubation time for glucuronidation as compared to our 24 h reactions [16].

3.3. Synthesis of 3′-O-methyl-epicatechin-5-O-glucuronide

Some epicatechin glucuronides in plasma and brain tissue are methylated (Fig. 2B, Supplementary Fig. 1, peak **6**), and this 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 major product of 3′-*O*-methyl-epicatechin glucuronidation (Fig. 4B, peak **2** and Fig. 2D, peak **7**) was a match, based on HPLC retention time and MS-TOF analysis, to the major product found in brain and plasma of GSE fed mice (Fig. 2B, peak **6**, Supplementary Fig. 1, peaks **6** and **7**). However, the overall in vitro conversion rate of 3′-*O*-methyl-epicatechin into glucuronidate 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 under all conditions and was therefore chosen for further optimization of assay conditions. The optimized conditions included incubations at room temperature, adding extra UDP-glucuronic acid every 4 h and adding extra UGT1A9 every 4 h. When extra enzyme and UDP-glucuronic acid were added together every 4 h, the product formation increased to ~20% (Fig. 4, peak **2**). We also increased the amount of UGT1A9 and UDP-glucuronic acid in the initial reaction. The final assay conditions are described in Section 2, 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. UGT1A9 was the most efficient at synthesizing the desired product (Supplementary Table 2, peak **2**). 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) (Supplementary 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 product as UGT1A9.

The major product of UGT1A9 was purified by preparative HPLC, and its structure was analyzed by LCMS (Fig. 2B and D) and NMR (Supplementary Fig. 2). The five aromatic protons 2′ (7.17 ppm; $J = 1.3$ Hz), 5′ (6.82 ppm; $J = 8.0$ Hz), 6′ (6.94 ppm; $J = 8.0$ Hz and 1.3 Hz), 6 (6.39 ppm; 7.1; $J = 2.0$ Hz) and 8 (6.11; $J = 2.0$ Hz) as shown (Supplementary Fig. 2) were monitored for NOE enhancement to identify the site 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 NOE was observed between the anomeric proton (4.9 ppm) and the proton on position 6. This NOE peak is consistent with glucuronidation at the 5-position on the A ring of the epicatechin structure [19]. 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 (Fig. 1). The 24 h conversion rate of around 50% achieved under optimal conditions will allow obtaining quantities of this product for use in biochemical/biological assays (Supplementary Table 2).

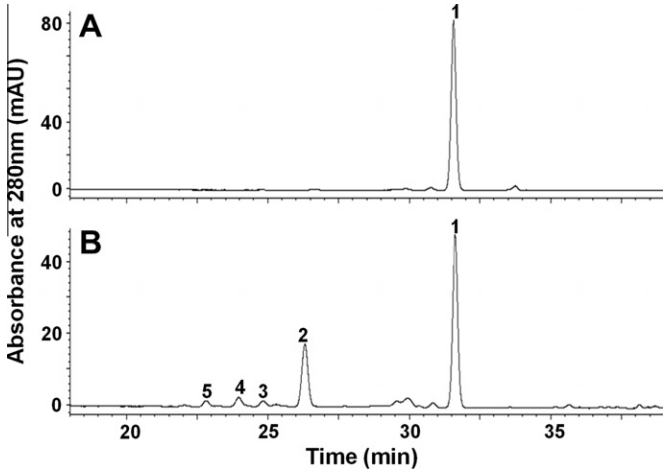

Fig. 4. Glucuronidation of 3′-*O*-methyl-epicatechin by UGT1A9. HPLC chromatograms were monitored at 280 nm. (A) Control reaction stopped at zero time. (B) UGT1A9 reaction. Identified peaks are: **1**, 3′-*O*-methyl-epicatechin; **2**, 3′-*O*-methyl-epicatechin-5-*O*-glucuronide; peaks **3**–**5** are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

3.4. Enzymatic substitution of epicatechin by methylation and glucuronidation followed by methylation, and by 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 ^{3}H epicatechin generated through custom synthesis) or the glucuronide moiety (from UDP-^{14}C-glucuronic acid). To provide a route for generation of ^{14}C labeled methyl-epicatechin glucuronides with the label on the non-hydrolyzable methyl group, we methylated epicatechin or epicatechin glucuronide in vitro using catechol-*O*-methyltransferase from porcine liver. Standards of 3′- and 4′-*O*-methylated epicatechins were used to positively identify the two major methylated epicatechin products based on retention time and UV spectra (Supplementary Fig. 3B, peaks **3** and **4**). Subsequent glucuronidation of the methylated epicatechin mixture with UGT1A9 produced at least two glucuronidated methyl epicatechins which were identified as 3′-*O*-methyl-epicat-

echin-5-*O*-glucuronide and a 4′-*O*-methyl-epicatechin-glucuronide (Supplementary Fig. 3C, peaks **5** and **6**).

The converse approach of glucuronidating the epicatechin prior to methylation did not produce glucuronidated methyl epicatechins (Supplementary Fig. 3D–F), since the glucuronidation resulted in formation of (−)-epicatechin-3′-*O*-glucuronide in which the preferred 3′-*O* methylation site of catechol-*O*-methyltransferase was already substituted. The minor glucuronidated products did not appear to be methylated in this reaction either, perhaps because their concentrations were too far below the km value of the enzyme.

Enzymatically methylating and subsequently glucuronidating epicatechin is a much quicker and safer method of synthesizing these metabolites than the standard chemical synthesis [20].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.139.

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