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Glucuronidation of the Green Tea Catechins, (−)-epigallocatechin-3-gallate and (−)-epicatechin-3-gallate, by Rat Hepatic and Intestinal Microsomes

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The flavonoids (−)-epigallocatechin-3-gallate (EGCg) and (−)-epicatechin-3-gallate (ECg) are major components of green tea and show numerous biological effects. We investigated the glucuronidation of these compounds and of quercetin by microsomes. Quercetin was almost fully glucuronidated by liver microsomes after 3 h, whereas ECg and ECGg were conjugated to a lesser extent (12.2 ± 0.2 and 7.5 ± 0.2%, respectively). The intestinal microsomes also glucuronidated quercetin much more efficiently than ECg and EGCg. Although the rates were lower than quercetin, intestinal microsomes exhibited higher activity on the galloyl group of ECg and EGCg compared to the flavonoid ring, whereas hepatic glucuronidation was higher on the flavonoid ring of EGCg and ECg compared to the galloyl groups. The low glucuronidation rates could partially explain why these flavanols are present in plasma as unconjugated forms.

Keywords: Catechins; Glucuronidation; Liver; Intestine; Green tea; Rat

Abbreviations: (−)-epicatechin, EC; (−)-epicatechin-3-gallate, ECg; (−)-epigallocatechin-3-gallate, EGCg; (−)-epigallocatechin, EGC; UDP-glucuronosyltransferase, UGT

INTRODUCTION

After water, tea (*Camellia sinensis*) is the most consumed beverage in the world. There are three main commercial tea products: green tea, black tea and oolong tea. They differ mainly in the manufacturing process, with green tea subjected to the least amount of fermentation/oxidation. Green tea contains polyphenols, which includes flavonols and phenolic acids. Most of the polyphenols present in green tea are flavanols, commonly known as catechins. The major catechins present in green tea are (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECg), (−)-epigallocatechin-3-gallate (EGCg) and (−)-epigallocatechin (EGC), with EGCg being the most abundant constituent (See Fig. 1). Currently there is a great interest in the role of green tea catechins for the maintenance of health as preventive agents against cancer and coronary disease. Several studies have shown various antioxidant[1,2] and anticarcinogenic[3–5] biological and pharmacological activities. Understanding the metabolism of these compounds is fundamental and an urgent priority in determining their biological effects. The principal steps of green tea catechin bioavailability have been described in the literature and seem to be similar in animals and humans. Catechins reach the systemic circulation but their oral bioavailability is low;[6] only 2% of EGCg was available in the systemic blood after oral administration in rats,[7] and in humans the value was 1.6–14% depending on the dose.[8] Catechins are metabolised by conjugating enzymes in both the intestine and liver,[9] and conjugated forms with methyl, glucuronyl and sulfate groups were detected in plasma.[10] However, the percentage

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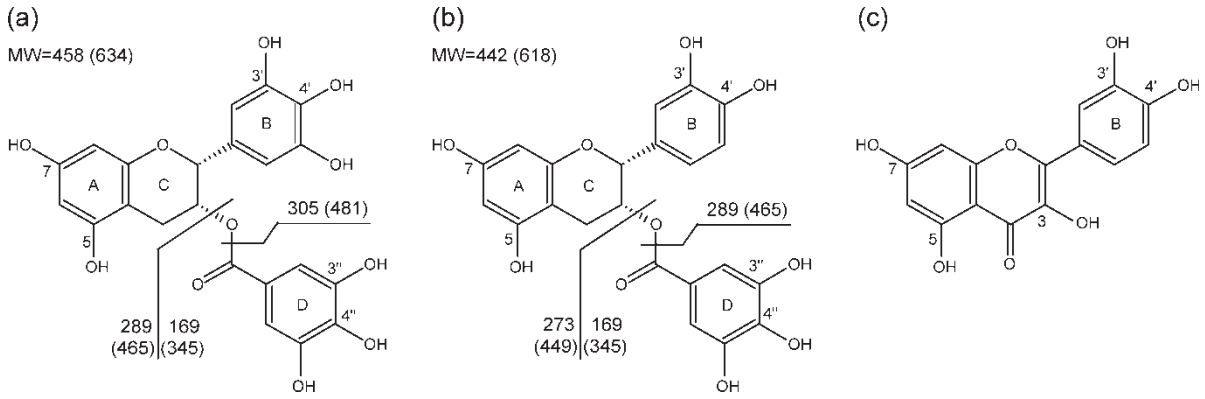

FIGURE 1 Structures and characteristic fragments of green tea catechins: EGCg (a), ECg (b) and quercetin (c). The numbers in parentheses indicate the molecular mass with a glucuronic acid residue attached to the corresponding moiety of the molecule.

of catechin in the blood that is conjugated depends on the nature of the catechin. All (+)-catechin and (−)-epicatechin in the blood are conjugated,[11] whereas up to 90% of EGCg is unconjugated.[8] Since most flavonoids in blood such as quercetin are 100% conjugated, [12] we were interested in why the galloylated catechins were an exception. In addition, for the proportion of EGCg and ECg which is conjugated in blood, we determined the positional specificity of glucuronidating enzymes in the rat jejunum, ileum and liver.

MATERIALS AND METHODS

Reagents

Quercetin, EGCg and ECg were purchased from Extrasynthese (Genay, France). β-glucuronidase/sulfatase from *Helix Pomatia* (type H2) was purchased from Sigma (Sion, Switzerland).

Animals and Diets

Male Wistar rats, two per cage and weighing approximately 300 g, were housed in temperature-controlled rooms (22°C), with a dark period from 20:00 to 8:00 h and access to food from 8:00 to 16:00 h. They were fed a standard semi-purified diet (73% wheat starch, 15% casein, 6% mineral mixture, 1% vitamin mixture, 5% corn oil) for 2 weeks.

In Vitro Assay for Flavonoid Glucuronidation

Hepatic, jejunal and ileal microsomes from rat intestine were prepared by differential ultracentrifugation at 105,000*g* at 4°C for 1 h. To prepare intestinal microsomes, mucosal scrapings (50 cm for jejunal and 20 cm for ileal intestine) were homogenized in ice cold buffer of a similar composition to that used for liver homogenisation (50 mmol/lM Tris–HCl, pH 7.2, 100 mmol/l sucrose, 10 mmol/l EDTA, 2 mmol/l dithiothreitol and 1 μmol/l leupeptin), except that trypsin inhibitor (25 mg/100 ml) was added to the buffer to prevent UGT inactivation by pancreatic enzymes. The final microsomal pellet was suspended in a buffer containing HEPES (100 mmol/l), pH 7.2 and sucrose (100 mmol/l) (supplemented with trypsin inhibitor for the intestinal fraction) and kept in a frozen state at −20°C until use. The preparations were adjusted to have a final protein concentration of about 5 mg/ml for liver microsomes and 3 mg/ml for intestinal microsomes, measured according to the Pierce B.C.A. protein reagent kit (Socochim, Lausanne, Switzerland).

Incubations were carried out as follows: 540 μl of buffer (75 mmol/l Hepes, pH 7.3, 10 mmol/l $MgCl_2$), 50 μl of UDP-glucuronic acid (4.5 mmol/l final concentration), 2.5 μl of Vitamin C (0.150 mmol/l final concentration, only for catechin glucuronidation) and 100 μl of microsomal suspension (50 μg protein) activated by 60 μl of a 0.2% solution of Triton X-100. The reaction was started by the addition of 2 μl of quercetin or catechin (151 mmol/l in DMSO). Incubations were performed at 37°C for 3 h. Aliquots of the reaction mixture were taken at 30, 60, 120 and 180 min and treated (with or without β-glucuronidase/sulfatase) for HPLC analysis (see "HPLC analysis Section").

HPLC Analysis

Sample Treatment

A measure of 175 μl of sample was acidified with 0.1 volume of acetic acid (0.58 mol/l) and directly extracted or incubated for (i) 30 min for quercetin and (ii) 15 min for catechins, at 37°C with 50 units β-glucuronidase and 2.5 units sulfatase from *Helix pomatia*. In each case, the addition of 500 μl of acetone was used to stop the reaction. After centrifugation, the samples were taken to dryness under nitrogen. They were then diluted with 175 μl of (i) perchloric acid (0.25 mol/l) for catechins or (ii) 15% acetonitrile for quercetin. The concentrations of conjugated

derivatives present in samples were estimated as the difference between the concentrations of aglycone recorded before and after adequate enzymatic treatment.

Chromatographic Conditions

The HPLC system used consisted of an autosampler (Hewlett-Packard type 1050), a UV detector (HP type 1050, set at 370 nm for quercetin and 280 nm for catechins) and the HP ChemStation system for data recording and processing. Separation was performed on a 5 μm C-18 Nucleosil 100-5 analytical column (250 × 3 mm) (Macherey-Nagel, Switzerland). The mobile phase consisted of water–H_3PO_4 (99.5: 0.5, solvent A) and acetonitrile (solvent B).

To separate the conjugated metabolites of catechins, the following gradient was used at a flow rate of 0.7 ml/min: 0–5 min, isocratic at 4.5% B; increase to 17.25% B at 20 min and further increase to 30% B at 25 min, hold for 5 min, then back to initial conditions, followed by equilibration for 5 min. For the quercetin samples, the following gradient was used at a flow rate of 0.5 ml/min: 0–2 min, isocratic at 15% B, increase to 40% B at 22 min; hold conditions for 3 min, then return to initial conditions and equilibrate for 8 min.

HPLC–MS

HPLC–MS analyses were performed on a Waters "Alliance" 2695 system, coupled to a Micromass (Manchester, UK) "Quattro-LC" mass spectrometer. Forty μl of the sample was injected from a polyethylene vial onto a C-18 CC-250/3 Nucleosil 100-5 HPLC column (Macherey-Nagel, Switzerland). Separations were carried out at a temperature of 22°C. Solvent A consisted of water with 4.5% acetonitrile, solvent B was 30% acetonitrile in water, both adjusted to pH 2.65 with formic acid. A flow rate of 0.6 ml/min was used. After sample injection, solvent composition was held at 100% A for 5 min, then changed linearly to 50% B at 20 min and 100% B at 25 min. Solvent composition was then held constant until 30 min, returned to initial conditions at 35 min and equilibrated for another 5 min before the next injection. The solvent flow was split 1:4 after the UV detector so that a flow of approximately 150 μl/min entered the ion source of the mass spectrometer.

The MS was fitted with an "Z-spray" electrospray interface. The electrospray voltage was set to −3.5 kV, the temperature of the source block to 120°C and that of the desolvation gas to 200°C. Nitrogen was used for the nebuliser, the cone gas (50 l/h) and the desolvation gas (600 l/h). Mass spectra were acquired by scanning over the mass range m/z 100–1000 with a scan time of 1 s, with the cone voltage alternating between 25 and 50 V. MS/MS experiments were performed with Argon as collision gas and at varying gas pressures and collision energies. Instrument control, data acquisition and data evaluation were performed using the Micromass MassLynx software, v4.0. UV data were acquired using a Waters (Rupperswil, Switzerland) 996 diode array detector scanned from 210 to 400 nm.

RESULTS

In Vitro Quercetin Glucuronidation

The capacity of rat liver and intestinal fractions to transfer *in vitro* glucuronic acid from UDP-glucuronic acid to quercetin was demonstrated using microsomal fractions obtained from rats adapted to a semi-purified diet. After the hepatic microsomal glucuronidation of quercetin (316 ± 28 μmol/l as measured at the beginning of each incubation by HPLC), the reaction products were analyzed by HPLC. Four metabolites of quercetin, labelled P3, P7, P4′ and P3′, were present in the samples (Fig. 2a, trace 1); the numbering represents the position of glucuronidation as previously determined.[12] As expected, these peaks disappeared after hydrolysis by β-glucuronidase/sulfatase (Fig. 2a, trace 4). Microsomes isolated from jejunum and ileum also exhibited quercetin UDP-glucuronosyltransferase activity and the chromatographic profile (Fig. 2a, traces 2 and 3) showed the same four products (P3, P7, P4′ and P3′) as those obtained with liver microsomal fraction. Independent of the microsomal fraction, P3 was the dominant peak present in the HPLC profile (Fig. 2a, traces 1–3) and P7, P4′ and P3′ were much lower (Fig. 2a, traces 1–3). Nevertheless, peak P7 was more abundant after intestinal glucuronidation compared to hepatic glucuronidation and in parallel, peak P4′ was less pronounced in the intestinal fraction compared to the liver microsomes. The apparent initial rate for each glucuronidated form of quercetin was determined. The initial rate of glucuronidation was especially high for quercetin in the liver (Table I) and this was coupled with a high extent of glucuronidation (96.3 ± 0.1%) after 3 h. The initial rate and extent of glucuronidation with intestinal microsomes was lower (Table I), but still higher than for catechin derivatives (see "ECg glucuronidation Section").

ECg Glucuronidation

As observed with quercetin, glucuronidation of ECg (315 ± 5 μmol/l) was catalysed by microsomes from the liver, jejunum and ileum (Fig. 2b). The HPLC profile shows that three peaks (P1, P2, P3) were detected irrespective of the microsomal fraction used and these disappeared after glucuronidase treatment

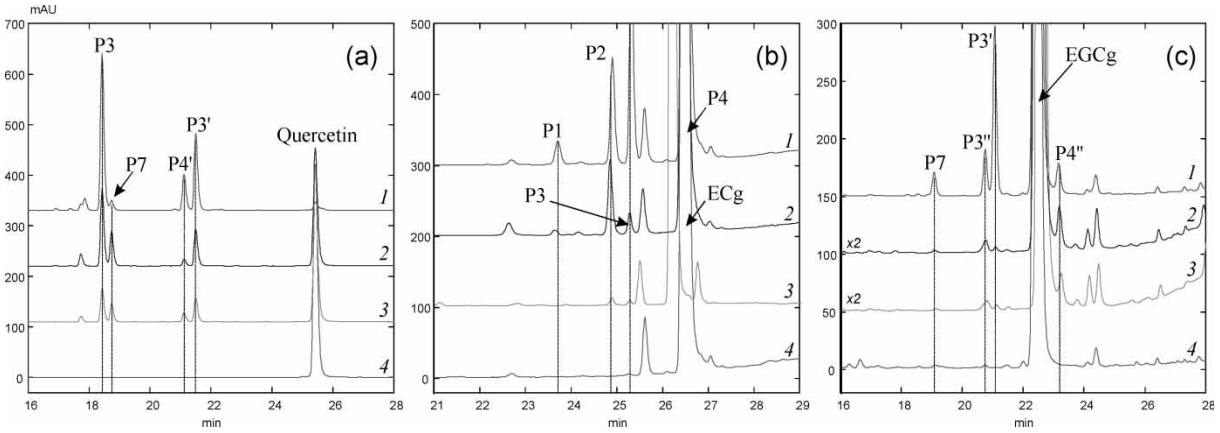

FIGURE 2 Representative HPLC–DAD chromatograms ($\lambda = 280$ nm) obtained after 3 h glucuronidation of (a) quercetin, (b) ECg and (c) EGCg with microsomal preparations from liver (1), from jejunum (2) and from ileum (3) and HPLC profile resulting from treatment of liver microsomes after glucuronidation by a β-glucuronidase/sulfatase (4). For quercetin, the peaks P3 corresponded to quercetin-3-*O*-glucuronide, P7 to quercetin-7-*O*-glucuronide, P4′ to quercetin-4′-*O*-glucuronide and P3′ to quercetin-3′-*O*-glucuronide. For EGCg, the P7 peak was EGCg-7-*O*-glucuronide, P3″ EGCg-3″-*O*-glucuronide, P3′ EGCg-3′-*O*-glucuronide and P4″ EGCg-4″-*O*-glucuronide. Traces are vertically offset for better visualization.

TABLE I Apparent rate and extent of glucuronidation of quercetin, ECg and EGCg

		Glucuronidated forms	Apparent rate (nmol/min. mg)	Extent of glucuronidation (% ± SD) ($n = 3$)
Quercetin	Liver	P3	997	
		P7	31.2	96.3 ± 0.1
		P4′	129	
		P3′	339	
	Jejunum	P3	192	
		P7	97.9	56.6 ± 2.7
		P4′	21.6	
		P3′	136	
	Ileum	P3	102	
		P7	58.2	32.7 ± 4.0
		P4′	27.3	
		P3′	91.3	
ECg	Liver	P1	19.9	
		P2	65.6	12.2 ± 0.2
		P3	133	
	Jejunum	P1	12.8	
		P2	47.5	2.3 ± 0.1
		P3	23.9	
	Ileum	P1	3.20	
		P2	10.5	0.9 ± 0.02
		P3	4.36	
EGCg	Liver	P7	17.5	
		P3″	16.2	7.5 ± 0.2
		P3′	46.6	
		P4″	14.8	
	Jejunum	P7	2.91	
		P3″	9.06	1.2 ± 0.04
		P3′	5.06	
		P4″	25.9	
	Ileum	P7	2.66	
		P3″	8.46	1.0 ± 0.04
		P3′	4.69	
		P4″	24.2	

The glucuronidation rate was calculated after incubation for 3 h. P2 is glucuronidated on the galloyl group, P1 and P3 are glucuronidated on the flavonoid skeleton.

(Fig. 2b, traces 1–3). In the liver fraction, peak P3 was dominant, but its presence decreased in the jejunal and ileal fraction. In contrast to this, peak P2 was more abundant in the jejunal microsomes than in the hepatic microsomes. Compound P1 was synthesized in small amounts, whatever the microsomal fraction.

Unlike quercetin, the glucuronidation positions of ECg have not yet been reported in the literature. To identify the individual glucuronidation sites, LC–MS experiments were performed. All compounds show up as $[MH]^-$ ions when analyzed by electrospray ionization in negative ionization mode and upon in-source collision or MS/MS, the compounds yield characteristic fragment ions. Thus, the identity of the peaks of ECg glucuronidated forms observed with HPLC–DAD were confirmed by HPLCMS and HPLC–MS/MS and this technique revealed the presence of another peak (P4) which almost coeluted with ECg (Fig. 3; mass chromatograms not shown), indicating that indeed four major peaks of glucuronides were present.

Only one glucuronidated form was present in each peak; its mass of m/z 617 is characteristic for an ECg monoglucuronide (Fig. 3a). Fragmentation of this ion either by in-source fragmentation or by MS/MS yielded two peaks at m/z 169 and 289, corresponding to the gallic acid and epicatechin moiety, respectively (Fig. 3b). The P1 and P3 peaks showed a characteristic fragment at m/z 465 (Fig. 3b, spectra at 26.4 and 27.6 min), indicating that the glucuronidation site was located on the A or B ring (Fig. 1b). This fragment was not present in the mass spectra of the glucuronides detected for the P2 and P4 peaks, which indicates that in this case the glucuronidation was not on the epicatechin group. Instead, the presence of a fragment ion with m/z 345 suggested

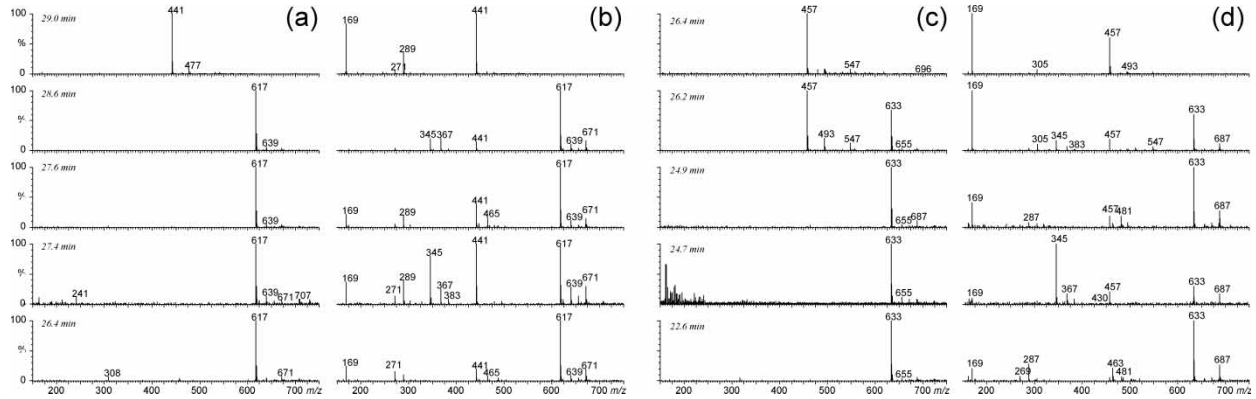

FIGURE 3 ESI(−) MS of (a) ECG and its conjugates at cone voltages of 25 V and (b) 50 V; from top to bottom: ECG, P4, P3, P2 and P1. (c) EGCg and its conjugates at cone voltages of 25 V and (b) 50 V; from top to bottom: EGCg, P4″, P3′, P3″ and P7.

that glucuronidation occurred on the galloyl group for P2 and P4 (Fig. 3b, spectra at 27.4 and 28.6 min).

This demonstrates that jejunal and ileal microsomes preferentially glucuronidate the galloyl group, whereas hepatic microsomes act preferentially on the flavonoid ring. Compared to quercetin, the rate and extent of ECg glucuronidation was low in all microsomal fractions (Table I). With liver microsomes, the rate for ECg was eight times less than that of quercetin (Table I). In the intestine, the extent of ECg glucuronidation rate was less than 3%, implying that glucuronidation of ECg in the gut does not play a major role in intestinal metabolism.

EGCg Glucuronidation

Figure 2c shows the HPLC profile obtained after incubation of ECGg (319 ± 2 μmol/l) with microsomes. Similar to the above, four peaks were detected (P7, P3″, P3′ and P4″; numbering corresponding to the position of glucuronidation as discussed below) and upon treatment with β-glucuronidase all four peaks disappeared as expected, with concomitant formation of the parent aglycone (Fig. 2c, traces 1 and 4). This demonstrates that each peak was a glucuronide of EGCg. The same peaks were observed independently of the source of the microsomes, suggesting that the same compounds were produced in all cases, although in different ratios (Fig. 2c). The analysis of the EGCg glucuronides by LC/MS corroborated the results found in HPLC–DAD, since four forms were detected again.

As was observed for ECg, only one EGCg monoglucuronide was present in each peak (m/z 633, Fig. 3c). The mass spectra of P7 and P3′ show a characteristic fragment with m/z 287 (Fig. 3d, spectra at 22.6 and 24.9 min), suggesting that the glucuronidation is on the A or B ring (Fig. 1a). In parallel, the mass spectra of P3″ and P4″ did not show this fragment with m/z 287 but a fragment with m/z 345 (Fig. 3d, 24.7 and 26.2 min) which, analogous to the results observed with ECg above, suggests that the glucuronidation occurred on the galloyl group (Fig. 1a).

The data from Lu *et al.*[13] allow further assignation of the position of glucuronidation based on elution position using very similar conditions. The glucuronidation kinetics of EGCg are shown in Table I. As observed for ECg, intestinal fractions preferentially glucuronidated the galloyl group, whereas hepatic fractions were higher on the flavonoid ring. Glucuronidation of position 7 was particularly low in the intestine. The initial rates were low compared to quercetin; only 7.5 ± 0.2% of EGCg was glucuronidated by liver microsomes. This rate was even decreased in the jejunum and ileum fractions, where it was 32 times lower than quercetin, again suggesting that the gut plays a minor role in ECg glucuronidation.

DISCUSSION

Glucuronidation *In Vivo*

Several pharmacokinetic studies have been carried out on green tea catechins and also on (+)-catechin and EC from other sources. (+)-catechin and EC are completely conjugated in plasma, as is quercetin.[11,14] However, EGCg and ECg are notable exceptions amongst flavonoids and are present mainly as the unconjugated parent compound in plasma.[8,15,16] The reasons for these differences have not been examined before. From our results, we suggest that one major reason is the low capacity of conjugation in the intestine and in the liver for EGCg and for ECg compared to quercetin.

Rate of Glucuronidation of Different Flavonoids

As expected, liver microsomes exhibited a higher rate of glucuronidation compared to microsomes from the ileum or jejunum, in agreement with a low glucuronidation rate in the rat small intestine for

substrate.[13] However, glucuronidation rates of EGCg and ECg were low in all tissues compared to quercetin, even though quercetin and EGCg are substrates for the same UGT.[13,17,18] The limiting factor for glucuronidation does not appear to be the UDP-glucuronic acid cofactor (present in more than 10-fold molar excess compared to the tested flavonoids), but the nature of the flavonoid substrate itself. Unlike quercetin, EGCg and ECg have a galloyl group on the C ring and EGCg has a hydroxyl group in the 5′ position. Possibly this group could decrease the access to the active site of UGT, since ECGg (galloyl group and hydroxyl in 5′position) shows a glucuronidation rate even lower than that of ECg.

UGT Specificity

Recently, the 4 major EGCg glucuronides have been identified by MS/MS and NMR as EGCg-7-*O*-, EGCg-3″-*O*-, EGCg-3′-*O*- and EGCg-4″-*O*-glucuronides.[13] As observed for quercetin, the hydroxyls in 5, 5′ and 5″ positions were not glucuronidated. Microsomes from human, rat or mouse gave the same peaks, although the distribution between peaks was different.[13] UGT1A1, 1A3, 1A8 and 1A9 have a high activity towards quercetin,[17,18] as well as against EGCg and EGC,[13] but no glucuronidation occurred with UGT1A4 and 1A6 for green tea catechins.[13] In contrast to this, the flavonoids naringenin, galangin, chrysin and apigenin are substrates for UGT2B15, since quercetin and baicalein were weakly glucuronidated by this enzyme. The expression of UGT enzymes is different in each tissue;[19–21] for example, UGT1A9 was expressed in liver and in colon, UGT1A1 and 1A3 in various organs including liver, and jejunum colon and 1A8 in jejunum, ileum, colon and but not in kidney and liver. The observed difference in proportion of glucuronides could be due to differences in UGT expression in tissues. Indeed, UGT1A1 and 1A9, mainly present in liver, could be responsible for glucuronidation of EGCg-3′-*O*-glucuronide since this product was low in the intestine corresponding with a low level of UGT1A9 expression.[13,15]

Role of Sulfation

It has been shown *in vivo* that at low concentrations, sulfation prevails over glucuronidation,[22] but when aglycone concentration is high, glucuronidation predominates.[23] With quercetin, the observed glucuronidation rate was very high independent of the tissue, suggesting that quercetin was a "good" substrate for UGT. Indeed quercetin is found in plasma both as sulfates and especially as glucuronides.[14,24] In addition, no unconjugated quercetin was detected in rat plasma taken from the mesenteric vein during perfusion suggesting that the intestine was able to glucuronidate all absorbed quercetin during absorption.[25] Compared to quercetin, the glucuronidation rates of EGCg and of ECg were low, especially in the intestine. EGCg and ECg are present in human plasma partly or mainly in the unconjugated form.[15,16] The presence of unconjugated forms in plasma could be largely explained by the fact that the glucuronidation in the intestine is low and, in consequence, these two compounds could escape conjugation. Of the conjugated proportion of EGCg, some is found as sulfates.[15] As the absorption rate for EGCg was low,[26] and sulfation occurs at low concentrations,[22] this could contribute to catechin metabolism. However, based on the high percentage of free EGCg in the plasma, we would predict that the rate of sulfation of EGCg is also low compared to other flavonoids.

Effect of Conjugation on Biological Activity

Biological effects of green tea catechin aglycones are widely described in the literature, but very little information is available on the biological activity of the conjugates. It has been shown that, for quercetin, the substitution position of the glucuronide group has a influence on xanthine oxidase inhibition.[12] In contrast to the 3-glucuronide, the 3′-and 4′-glucuronides inhibit this enzyme. In the same way, EGCg-3′- and 3″-glucuronides have a potent free radical scavenging activity (similar to EGCg) when compared to EGCg-4″-glucuronides.[13] Clearly the conjugation is a major issue in flavonoid metabolism. For EGCg, the low absorption is compensated for by the low rate of conjugation, which may maintain a high biological activity of the aglycone *in vivo*. Our results provide at least a partial explanation of why the galloylated catechins are found as the aglycone form in plasma after consumption of green tea.

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