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In vitro and *in vivo* conjugation of dietary hydroxycinnamic acids by UDP-glucuronosyltransferases and sulfotransferases in humans☆

Chi Chun Wong[a], Walter Meinl[b], Hans-Rudolf Glatt[b], Denis Barron[c], Angélique Stalmach[d], Heike Steiling[c], Alan Crozier[d], Gary Williamson[a,c,*]

[a]*School of Food Science and Nutrition, University of Leeds, LS2 9JT, Leeds, UK*
[b]*German Institute of Human Nutrition (DIfE) Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany*
[c]*Nestle Research Center, Vers Chez les Blanc, 1000 Lausanne 26, Switzerland*
[d]*Plant Products and Human Nutrition Group, Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, G12 8QQ Glasgow, UK*

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Abstract

Hydroxycinnamic acids are a class of phenolic antioxidants found widely in dietary plants. Their biotransformation in the human organism primarily involves Phase II conjugation reactions. In this study, activities of UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) towards major dietary hydroxycinnamic acids (caffeic, dihydrocaffeic, dihydroferulic, ferulic and isoferulic acids) were investigated. Conjugate formation was evaluated using human liver and intestinal S9 homogenates, and *in vitro* characterization was carried out using recombinant human UGTs and SULTs. Analysis of the kinetics of hydroxycinnamic acid conjugation in human S9 homogenates revealed that intrinsic clearance (V_{max}/K_m) is much greater for sulfation than for glucuronidation. Assessment of activity using a panel of recombinant human SULTs showed that SULT1A1 is most active in the sulfation of caffeic, dihydrocaffeic and isoferulic acids, while SULT1E1 is most active in the sulfation of ferulic and dihydroferulic acids. Only isoferulic acid was significantly glucuronidated by human liver S9 homogenates, explained by the high activity of liver-specific UGT1A9. Studies on the kinetics of active SULTs and UGTs demonstrated a markedly lower K_m for SULTs. To further corroborate our findings, we carried out an intervention study in healthy humans to determine the hydroxycinnamic acid conjugates in urine after consumption of hydroxycinnamate-rich coffee (200 ml). Analysis showed that sulfates are the main conjugates in urine, with the exception of isoferulic acid, which is mainly glucuronidated. These data suggest that sulfates are the predominant hydroxycinnamic acid conjugates in humans, and that SULT mediated sulfation is a major factor determining the bioavailability of hydroxycinnamic acids *in vivo*.

Keywords: Hydroxycinnamic acid; Phase II metabolism; Sulfotransferase; UDP-glucuronosyltransferase; Conjugation; Coffee

1. Introduction

Various phenolic phytochemicals such as hydroxycinnamates are being increasingly recognized to be associated with the beneficial effects of plant foods against degenerative diseases such as cardiovascular diseases [1]. Hydroxycinnamic acids are among the most abundant phenolic compounds in the diet, occurring widely in fruits, vegetables and coffee [2]. Depending on the diet, intake can be very high (500–1000 mg/day), especially among coffee drinkers, which could be a major contributor to the intake of hydroxycinnamates [3]. In the majority of foods, hydroxycinnamates are esterified to organic acids and sugars, but they can also be found in the free form in products such as tomato and beer [4]. *In vitro* reports, together with a limited number of human studies, have suggested that hydroxycinnamates exert beneficial effects linked to their antioxidant capacity, such as enhancing the resistance of human low-density lipoprotein to *ex vivo* oxidation [5–8]. Nevertheless, similar to other xenobiotics, dietary hydroxycinnamates are regarded by the body as foreign compounds. As a result, after ingestion and absorption, they are highly metabolized, resulting in metabolic forms (that may possess biological properties markedly different from those present in foods) being present in the body [9].

Recent human studies indicated that esterified hydroxycinnamic acids found in foods are extensively metabolized following ingestion, and that, as a consequence, free hydroxycinnamic acids and their conjugates are detected in human plasma and urine [6,10–13]. In particular, glucuronides and/or sulfates of hydroxycinnamic acids are the predominant forms, while free acids are found at low levels. First-pass metabolism of hydroxycinnamic acids thus has a significant

Abbreviations: DTT, DL-dithiothreitol; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SULT, sulfotransferase; UDPGA, uridine-5′-diphospho-glucuronic acid; UGT, UDP-glucuronosyltransferase.

☆ In this article, K_m is used to refer to the measured Michaelis constant. In some cases, it is actually "apparent" K_m, as it is derived from an impure homogenate, or it refers to the production of one product out of two products formed.

* Corresponding author. School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK. Tel.: +44 113 3438380; fax: +44 113 343 2982.
E-mail address: g.williamson@leeds.ac.uk (G. Williamson).

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influence on the bioavailability, and hence bioefficacy, of these compounds. Moreover, the strong antioxidant capacity of hydroxycinnamic acids is endowed by the presence of free hydroxyl groups [14], and these are the major sites of glucuronidation and sulfation [15]. Given the significance of Phase II conjugation reactions in modulating bioavailability and biological activity, it is important to define the regioselectivity of Phase II metabolic enzymes for — and the relative activity of Phase II metabolic enzymes towards — dietary hydroxycinnamates. Up to now, most of the studies have determined the level of conjugates indirectly by measuring the hydroxycinnamic acids released by hydrolysis with β-glucuronidase or sulfatase. This loses all information regarding the regioselectivity of conjugation in humans [4,12]. Recent HPLC-MS studies using *in vitro* Caco-2 cell cultures and rat models have directly determined the nature and regioselectivity of Phase II metabolic transformation using ferulic and dihydrocaffeic acids as model hydroxycinnamates [16,17]; however, information on specific isozymes catalyzing these conjugation reactions remains unclear.

The glucuronidation and sulfation of xenobiotics in the intestine and liver are carried out by UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), respectively. UGTs from the UGT1A locus are active, in general, towards phenolic hydroxyl groups [18]. UGTs show tissue-specific expression. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B4 are expressed in the human liver, whereas UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8 and UGT1A10 are found in the intestine [19–21]. In humans, cytosolic SULTs consist of 11 members (SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C1, SULT1C2, SULT1C3, SULT1E1, SULT2A1, SULT2B1a and SULT2B1b) that catalyze the sulfation of low-molecular-weight endogenous compounds and xenobiotics [22]. For two other forms of this superfamily (SULT4A1 and SULT6B1), no substrate has been detected to date. SULT1A1 and SULT2A1 are the major forms found in the liver; SULT1A3, SUL1B1 and SULT1E1 have also been detected in this tissue [23]. SULT1A1, SULT1A3 and SULT1B1 were found at high levels in all sections of the intestinal tract investigated; SULT1A2, SULT1C1, SULT1E1 and SULT2A1 are expressed at lower levels and restricted to certain intestinal sections [23].

The regioselectivity and relative activity of these UGTs and SULTs have a major influence on the profile of conjugates found in the body, as well as on their bioactivity. In order to understand the role of these enzymes in the disposition of hydroxycinnamic acids, we analyzed the regioselectivities of individual UGTs and SULTs for — and the activities of individual UGTs and SULTs towards — five dietary hydroxycinnamic acids, including three cinnamic acids (caffeic, ferulic and isoferulic acids) and two dihydrocinnamic acids (dihydrocaffeic and dihydroferulic acids). For this purpose, we employed HPLC with diode array detection to determine individual glucuronidated and sulfated hydroxycinnamic acids formed in *in vitro* enzyme incubations. The overall position and the type of conjugation in human intestinal and liver S9 extracts of the five hydroxycinnamates were elucidated. To identify the specific UGT and SULT isoforms responsible for glucuronidation and sulfation, we assessed the activities and kinetics of conjugate formation using a panel of human recombinant UGTs and SULTs. These *in vitro* results were then further confirmed by analyses of human urine following ingestion of coffee, a rich source of hydroxycinnamates (5-*O*-caffeoylquinic acid and other caffeic acid esters).

2. Materials and methods

2.1. Reagents

Caffeic, ferulic and isoferulic acids were obtained from Sigma-Aldrich (St. Louis, MO). Dihydrocaffeic and dihydroferulic acids were purchased from Alfa Aesar (Lancashire, UK). The conjugates caffeic-3-*O*-glucuronide, caffeic acid-4-*O*-glucuronide, caffeic acid-3-*O*-sulfate, caffeic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulfate, ferulic acid-4-*O*-glucuronide, ferulic acid-4-*O*-sulfate, isoferulic acid-3-*O*-glucuronide and isoferulic acid-3-*O*-sulfate were synthesized as will be described elsewhere (Fumeaux et al., unpublished data). Uridine-5′-diphospho-glucuronic acid (UDPGA) and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) were obtained from MP Biochemicals (London, UK). Pooled liver S9 homogenate was purchased from Sigma-Aldrich, while pooled intestinal (duodenum/jejunum) S9 homogenate was obtained from Xenotech (Lenexa, KS). Recombinant human UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17) expressed in baculovirus-infected insect cells (Supersomes) were obtained from BD Gentest (Woburn, MA). The total protein content (5 mg/ml) of Supersomes was specified on data sheets provided by the manufacturer. SULTs were expressed in *Salmonella typhimurium* and purified as previously described [24]. Crude bacterial cytosols were directly used for activity determinations, as SULTs are particularly stable in this matrix and as PAP (the product of the consumed cofactor, which is a potent inhibitor of various SULTs) is rapidly degraded by bacterial enzymes. All activities were adjusted to the expression levels of SULTs measured by immunoblot analysis [24]. All other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich. Nescafe Gold Blend soluble coffee powder was used for the human study.

2.2. Phase II metabolism of hydroxycinnamic acids by human liver and intestinal S9 homogenates

The incubation mixture, in a final volume of 50 μl, consisted of 100 mM potassium phosphate buffer (pH 7.4), with 100 μM vitamin C, 1 mM UDPGA, 100 μM PAPS, 1 mM DL-dithiothreitol (DTT), 5 mM saccharolactone (a β-glucuronidase inhibitor) and 0.025 mg/ml alamethicin (a UGT activator). Human liver S9 and intestinal S9 homogenates were used at 2 and 0.8 mg/ml, respectively [25]. Reaction was initiated by adding 50 μM hydroxycinnamic acid from an 8 mM stock solution dissolved in ethanol/water (50:50, vol/vol). After 1 h of incubation in a 37°C water bath, the reaction was stopped by addition of 10 μl of ice-cold acetonitrile containing 500 mM HCl. Controls were treated under the same conditions and consisted of samples without cofactor, S9 homogenate or substrate. The samples were stored at −70°C until analysis. Recoveries of the hydroxycinnamic acid conjugates from the homogenates were as follows: caffeic-3-*O*-glucuronide, 102%; caffeic acid-4-*O*-glucuronide, 100%; caffeic acid-3-*O*-sulfate, 98%; caffeic acid-4-*O*-sulfate, 97%; dihydroferulic acid-4-*O*-sulfate, 97%; ferulic acid-4-*O*-glucuronide, 99%; ferulic acid-4-*O*-sulfate, 103%; isoferulic acid-3-*O*-glucuronide, 102%; isoferulic acid-3-*O*-sulfate, 99%. Apparent enzyme kinetic parameters were estimated in terms of product formation, using 6–12 different concentrations of the substrate. For the analysis of the enzyme kinetics of glucuronidation, reaction conditions were optimized for each hydroxycinnamic acid; the reaction was linear over the time period indicated, and the percentage of conversion of the substrate was less than 15%. Reaction buffer consisted of 100 mM potassium phosphate buffer (pH 7.4), with 100 μM vitamin C, 1 mM UDPGA, 5 mM saccharolactone and 0.025 mg/ml alamethicin. The incubation conditions were as follows: 20–3200 μM caffeic acid, 2 mg/ml liver S9 homogenate or 0.8 mg/ml intestinal S9 homogenate for 1 h; 10–3200 μM ferulic acid, 2 mg/ml liver S9 homogenate for 30 min or 0.8 mg/ml intestinal S9 homogenate for 1 h; 10–3200 μM isoferulic acid, 2 mg/ml liver S9 homogenate for 30 min or 0.8 mg/ml intestinal S9 homogenate for 1 h. For the determination of sulfation kinetics, reactions were carried out in 100 mM potassium phosphate buffer (pH 7.4), with 100 μM vitamin C, 100 μM PAPS and 1 mM DTT. The substrate and S9 concentrations employed were as follows: 2.5–100 μM caffeic acid, 1 mg/ml liver S9 homogenate or 0.4 mg/ml intestinal S9 homogenate for 30 min; 2.5–250 μM ferulic acid, 1 mg/ml liver S9 homogenate or 0.4 mg/ml intestinal S9 homogenate for 30 min; 2.5–250 μM isoferulic acid, 1 mg/ml liver S9 homogenate or 0.4 mg/ml intestinal S9 homogenate for 30 min; 10–120 μM dihydrocaffeic acid, 3 mg/ml liver S9 homogenate or 0.8 mg/ml intestinal homogenate S9 for 1 h; 25–1000 μM dihydroferulic acid, 2 mg/ml liver S9 homogenate or 0.8 mg/ml intestinal S9 homogenate for 1 h.

2.3. Glucuronidation of hydroxycinnamic acids by recombinant human UGTs

The contribution of individual isozymes to the glucuronidation of hydroxycinnamic acids was assessed using human recombinant UGTs. The reaction was performed in a 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM vitamin C, 1 mM UDPGA, 5 mM saccharolactone and 0.025 mg/ml alamethicin (final volume, 50 μl). Control Supersomes and UGTs were added at a concentration of 1 mg/ml. Reaction was started by the addition of 50 μM hydroxycinnamic acid, allowed to proceed at 37°C for 1 h and finally stopped by addition of 10 μl of ice-cold acetonitrile containing 500 mM HCl. Recoveries of hydroxycinnamic acid conjugates from the control UGT Supersomes were as follows: caffeic-3-*O*-glucuronide, 104%; caffeic acid-4-*O*-glucuronide, 100%; ferulic acid-4-*O*-glucuronide, 100%; isoferulic acid-3-*O*-glucuronide, 99%. Apparent enzyme kinetic parameters were estimated in terms of product formation, using 10–12 different concentrations of the substrate. Glucuronidation kinetics of glucuronidation were carried out with UGT1A1 and UGT1A9 on caffeic acid, with UGT1A1 on ferulic acid and with UGT1A7, UGT1A8 and UGT1A9 on isoferulic acid. The respective substrate concentrations and the times of incubation were as follows: 10–3200 μM caffeic acid, 1 mg/ml UGT1A1 or UGT1A9 for 1 h; 2.5–1600 μM ferulic acid, 0.5 mg/ml UGT1A1 for 30 min; 2.5–3200 μM isoferulic acid, 0.2 mg/ml UGT1A9, 0.4 mg/ml UGT1A7 or UGT1A8 for 30 min.

2.4. Sulfation of hydroxycinnamic acids by recombinant human SULTs

Sulfation of hydroxycinnamic acids by SULTs was performed in 100 mM potassium phosphate buffer (pH 7.4) containing 100 μM vitamin C, 100 μM PAPS and 1 mM DTT (final volume, 50 μl). Control cytosolic fraction and cytosolic preparations from SULT-expressing bacteria were added to a final concentration of 0.25 mg/ml total protein. The reaction was initiated by the addition of 50 μM hydroxycinnamic acid. The reaction was allowed to proceed at 37°C for 1 h and stopped by adding 10 μl of ice-cold acetonitrile with 500 mM HCl. Sulfation kinetics were determined using the most active isozyme SULT1A1. Apparent enzyme kinetic parameters were estimated in terms of product formation, using at least six different concentrations of substrate. Recoveries of the hydroxycinnamic acid conjugates measured using the control cytosolic fraction were as follows: caffeic acid-3-*O*-sulfate, 98%; caffeic acid-4-*O*-sulfate, 96%; dihydroferulic acid-4-*O*-sulfate, 98%; ferulic acid-4-*O*-sulfate, 102%; isoferulic acid-3-*O*-sulfate, 99%. The substrates and SULT1A1 concentrations used were as follows: 1–40 μM caffeic acid, 3.5 μg/ml (0.05 mg/ml total cytosolic protein from recombinant bacteria) for 10 min; 2.5–100 μM ferulic acid, 3.5 μg/ml for 15 min; 2.5–100 μM isoferulic acid, 3.5 μg/ml for 30 min; 5–100 μM dihydrocaffeic acid, 7 μg/ml for 30 min; 5–100 μM dihydroferulic acid, 17.5 μg/ml for 1 h. The sulfation kinetics of SULT1E1 on ferulic and dihydroferulic acids were also determined. For ferulic acid, 0.5 μg/ml enzyme (0.1 mg/ml total cytosolic protein from recombinant bacteria) was incubated with 2.5–100 μM substrate for 30 min; for dihydroferulic acid, it was performed at 5–100 μM substrate, with 2.5 μg/ml SULT1E1 for 1 h.

2.5. HPLC methodology

HPLC analyses were carried out using the Agilent 1200 series liquid chromatography system. For the analysis of caffeic, ferulic, isoferulic and dihydroferulic acids and their conjugates, chromatography was performed with a Zorbax XDB-C18 column (4.6×150 mm^2, 5 μm). The mobile phase consisted of 20 mM ammonium formate (pH 2.8) (A) and methanol (B). For the analysis of caffeic acid and conjugates, samples were eluted at 1 ml/min with 5–25% (B) in 20 min, followed by 80% (B) in 2 min and back to 5% (B) for 3 min. For the analysis of ferulic and isoferulic acids and their conjugates, the gradient was from 10% to 20% (B) in 10 min, to 60% (B) in 15 min, then set at 80% (B) for 2 min and back to 10% (B) for 3 min, at 1 ml/min. Analysis of dihydroferulic acid and conjugate was carried out at 1 ml/min, from 10% to 20% (B) in 15 min, to 60% (B) in 10 min, up to 80% (B) for 2 min and, finally, to 10% (B) for 3 min. For dihydrocaffeic acid and conjugates, the analyses were performed with a Zorbax XDB-C18 column (4.6×50 mm^2, 1.8 μm), with 20 mM ammonium formate (pH 4.5) (A) and methanol (B) as mobile phase. The gradient started at 3% (B) kept for 15 min, followed by an increase to 40% (B) in 5 min, and then returned to 3% (B) for 5 min. Samples prepared were centrifuged, and 25 μl of the supernatant was injected into the column. Due to the coelution of dihydrocaffeic acid sulfates with unknown components in the S9 homogenates, an extraction procedure was performed only for samples from dihydrocaffeic acid. After cessation of the reaction, the buffer was evaporated *in vacuo*. Residue was then extracted first with 60 μl of methanol, and then with 60 μl of acetonitrile. The supernatants of the extracts were combined and evaporated to dryness. Finally, the extracted material was resuspended in 60 μl of the initial mobile phase and analyzed by HPLC. The extraction efficiency was 92±4% for dihydrocaffeic acid conjugates (n=3).

Table 1
Kinetics of the glucuronidation of hydroxycinnamic acids by liver S9 homogenate, intestinal S9 homogenate and individual UGTs

Substrate	Product(s)/enzyme	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (L/min/mg) ($\times 10^{-6}$)
Caffeic acid	Caffeic acid-3-*O*-glucuronide			
	Liver S9	2760±230	67.7±3.3	0.02
	Intestinal S9	1770±180	40.0±2.0	0.02
	UGT1A9	1900±140	206±8	0.11
	Caffeic acid-4-*O*-glucuronide			
	Liver S9	2480±400	26.0±2.4	0.01
	Intestinal S9	2620±180	66.5±5.5	0.03
	UGT1A1	4240±270	178±8	0.04
	UGT1A9	1910±130	325±12	0.17
Ferulic acid	Ferulic acid-4-*O*-glucuronide			
	Liver S9	456±69	40.4±2.5	0.09
	Intestinal S9	4610±100	809±12	0.18
	UGT1A1	158±21	108±4	0.68
Isoferulic acid	Isoferulic acid-3-*O*-glucuronide			
	Liver S9	686±116	452±36	0.66
	Intestinal S9	2210±100	217±6	0.10
	UGT1A7	605±134	952±76	1.57
	UGT1A8	1140±170	2630±170	2.31
	UGT1A9	596±69	6850±350	11.5

No glucuronide conjugate was detected with dihydrocaffeic acid or dihydroferulic acid.

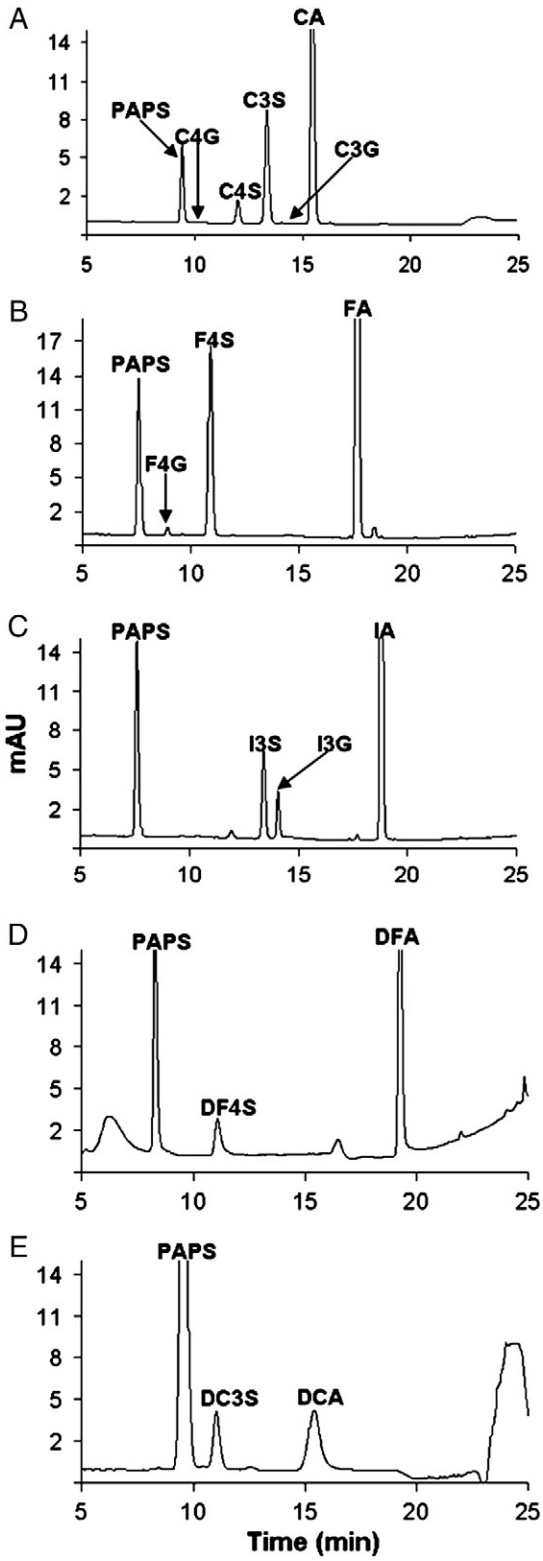

Fig. 1. HPLC chromatograms of hydroxycinnamic acids and conjugates identified by HPLC. Hydroxycinnamic acids (50 μM) were incubated with human liver S9 homogenate (2 mg/ml, for 1 h) and supernatant analyzed by HPLC-UV, each with different gradient elution conditions as described in Materials and Methods. CA: caffeic acid; C3S: caffeic acid-3-*O*-sulfate; C4S: caffeic acid-4-*O*-sulfate; DCA: dihydrocaffeic acid; DC3S: dihydrocaffeic acid-3-*O*-sulfate; DFA: dihydroferulic acid; DF4G: dihydroferulic acid-4-*O*-glucuronide; DF4S: dihydroferulic acid-4-*O*-sulfate; FA: ferulic acid; F4S: ferulic acid-4-*O*-sulfate; IA: isoferulic acid; I3G: isoferulic acid-3-*O*-glucuronide; I3S: isoferulic acid-3-*O*-sulfate. All peaks were identified and quantitated with authentic synthetic standards.

UV detection was carried out at 280 and 310 nm using a photodiode array detector. Caffeic, ferulic and isoferulic acids were quantified at 310 nm; dihydroferulic and dihydrocaffeic acids were quantified at 280 nm. The on-column limit of quantification of this HPLC method for the quantification of caffeic, ferulic and isoferulic acids was 1 pmol, and that for the quantification of dihydrocaffeic and dihydroferulic acids was 5 pmol. All conjugates were positively identified by comparing the retention times and UV absorption spectra with those of the authentic synthetic standards. Conjugates were quantified using calibration curves of the authentic standards of glucuronides and sulfates. Standard curves were linear over the range loaded (0.1–2 nmol, R^2>.99). The slopes of peak area (AU)/amount (nmol) are as follows: caffeic-3-*O*-glucuronide, 877; caffeic acid-4-*O*-glucuronide, 839; caffeic acid-3-*O*-sulfate, 1120; caffeic acid-4-*O*-sulfate, 512; dihydrocaffeic acid-3-*O*-sulfate, 113; dihydroferulic acid-4-*O*-sulfate, 157; ferulic acid-4-*O*-glucuronide, 833; ferulic acid-4-*O*-sulfate, 922; isoferulic acid-3-*O*-glucuronide, 626; isoferulic acid-3-*O*-sulfate, 872 (corrected for purity).

2.6. Human study

The research has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association—that the Ethical Committee of the institution in which the work was performed has approved it and that the subjects have given informed consent to the work.

Volunteers (eight male and three female) were in good health, nonsmoking, not pregnant and aged 19–35 years, with an average body mass index of 24.3±2.3 kg/m^2. The study and the protocol were reviewed by The Glasgow Royal Infirmary NHS Research Ethics Committee. Volunteers followed a low-polyphenol diet for 48 h before the study and fasted 12 h before the start of the study. Water was the only liquid allowed ad libitum. Coffee (200 ml) containing 3.4 g of powdered instant coffee was consumed within 5 min. Urine was collected over the period of 0–24 h. A standard lunch, containing low levels of phenolic and polyphenolic compounds, was provided after 3 h. After 8 h, the volunteers remained on the low-phenolic diet until the next morning, prior to collection of the 24-h time-point samples. The volumes of urine excreted were measured, and aliquots were stored at −80°C. The content of chlorogenic acids (μmol) in 200 ml of coffee was analyzed in triplicate by HPLC-PDA-MS^n, and the content of chlorogenic acids was as follows: 3-*O*-caffeoylquinic acid, 72±1.3; 4-*O*-caffeoylquinic acid, 78±1.5; 5-*O*-caffeoylquinic acid, 119±2.1; 3-*O*-feruloylquinic acid, 20±1.6; 4-*O*-feruloylquinic acid, 22±1.8; 5-*O*-feruloylquinic acid, 25±1.9; 3-*O*-caffeoylquinic acid lactone, 34±2.9; 4-*O*-caffeoylquinic acid lactone, 23±2.0; 3,4-*O*-dicaffeoylquinic acid, 5.8±0.6; 3,5-*O*-dicaffeoylquinic acid, 2.8±0.3; 4,5-*O*-dicaffeoylquinic acid, 4.0±0.4; 4-*O*-*p*-coumaroylquinic acid, 3.8±0.1; 5-*O*-*p*-coumaroylquinic acid, 3.1±0.1.

2.7. Analysis of urinary metabolites

Urine samples were defrosted and centrifuged at 16,110×*g* for 3 min at 4°C, prior to the triplicate analysis of 20-μl aliquots by HPLC-PDA-MS^n, using a Surveyor HPLC with a PDA detector and an LCQ Duo ion-trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). Separations were performed at 40°C with a SYNERGI 4-μm POLAR-RP 250×4.6 mm i.d. reverse-phase column (Phenomenex, Macclesfield, UK) using a 40-min 5–16% gradient of acetonitrile in 0.5% aqueous acetic acid. Sample was maintained at 4°C before HPLC analysis. The capillary temperature was 300°C; the sheath gas and auxiliary gas were 80 and 60 U/min, respectively; the source voltage was 3.0 kV; and collision energy was set at 35%. Following separation, hydroxycinnamates and conjugates were quantified using selective ion monitoring (SIM), based on the calibration curves of the corresponding standard. Data were expressed as mean±S.E. (n=11). The on-column limit of detection using HPLC-SIM was typically 3 pmol. Identification of quantified compounds was confirmed by selected reaction monitoring and co-chromatography with standards.

2.8. Data analysis

Data are shown as mean±S.D. (as mean±S.E. for enzyme kinetics data). Raw data from the enzyme kinetics studies were analyzed using OriginPro 7.5. K_m and V_{max} were derived from a nonlinear regression fit of the Michaelis–Menten model. The activity of SULTs was expressed in terms of K_{cat}, using estimated SULT concentrations by immunoblot analysis [24]. The specific contents of SULTs were as follows: SULT1A1, 0.512 mg/ml; SULT1A2, 0.244 mg/ml; SULT1A3, 0.080 mg/ml; SULT1B1, 0.182 mg/ml; SULT1C1, 0.144 mg/ml; SULT1C2, 0.039 mg/ml; SULT1C3, 0.088 mg/ml; SULT1E1, 0.042 mg/ml; SULT2A1, 0.09 mg/ml; SULT2B1a, 0.56 mg/ml; SULT2B1b, 0.093 mg/ml; SULT4A1, 0.048 mg/ml; SULT6B1, 0.037 mg/ml.

3. Results

3.1. Glucuronidation and sulfation of hydroxycinnamic acids by human liver S9 homogenate

Comparative activities of glucuronidation and sulfation by human liver S9 homogenate and their kinetic parameters are shown in Tables 1 and 2. Liver S9 homogenate clearly showed a preference for the sulfation of hydroxycinnamic acids over glucuronidation (Fig. 1; Tables 1 and 2). Sulfates accounted for over 95% of the conjugates of caffeic and ferulic acids; in the case of dihydrocaffeic and dihydroferulic acids, sulfates were the sole conjugates found, and glucuronides could not be detected. Caffeic and dihydrocaffeic acids both possess a catechol group, and sulfation occurred preferentially at the 3-hydroxyl group. Analysis of the kinetics of conjugate formation showed that sulfate formation exhibited a much higher apparent

Table 2
Kinetics of the sulfation of hydroxycinnamic acids by liver S9 homogenate, intestinal S9 homogenate and individual SULTs

Substrate	Product(s)/enzyme	K_m (μM)	V_{max} (pmol/min/mg)	K_{cat} (s^{-1})	V_{max}/K_m (L/min/mg) ($\times10^{-6}$)	K_{cat}/K_m (mol/s) ($\times10^{-6}$)
Caffeic acid	Caffeic acid-3-*O*-sulfate					
	Liver S9	13.4±1.0	204±4		15.2	
	Intestinal S9	34.5±1.9	682±15		19.8	
	SULT1A1	11.6±2.4		62.1±6.5		5.35
	Caffeic acid-4-*O*-sulfate					
	Liver S9	11.9±1.6	52.7±2.0		4.43	
	Intestinal S9	41.3±7.3	297±22		7.20	
	SULT1A1	14.8±4.7		17.6±3.0		1.19
Ferulic acid	Ferulic acid-4-*O*-sulfate					
	Liver S9	59.0±2.7	162±4		2.75	
	Intestinal S9	62.9±7.2	572±26		9.10	
	SULT1A1	13.3±2.3		24.8±1.4		1.86
	SULT1E1	30.4±2.5		227±7		7.47
Isoferulic acid	Isoferulic acid-3-*O*-sulfate					
	Liver S9	152±17	147±9		0.97	
	Intestinal S9	152±12	466±19		3.07	
	SULT1A1	55.9±5.8		35.4±1.7		0.63
Dihydrocaffeic acid	Dihydrocaffeic acid-3-*O*-sulfate					
	Liver S9	106±17	165±16		1.56	
	Intestinal S9	15.8±5.8	592±65		37.4	
	SULT1A1	34.1±10.4		108±13		3.17
Dihydroferulic acid	Dihydroferulic acid-4-*O*-sulfate					
	Liver S9	356±61	42.2±3.0		0.12	
	Intestinal S9	567±46	810±33		1.43	
	SULT1A1	134±23		17.1±1.8		0.13
	SULT1E1	180±59		126±29		0.70

affinity (low K_m) and V_{max} compared to glucuronides (Table 2). Sulfation followed simple hyperbolic kinetics. Based on intrinsic clearance, caffeic acid was the most actively sulfated (primarily at the 3-position), followed by ferulic, dihydrocaffeic, isoferulic and dihydroferulic acids. Isoferulic acid was the only hydroxycinnamic acid that showed significant glucuronidation. Isoferulic acid-3-*O*-glucuronide formation showed the highest clearance (V_{max}/K_m) for glucuronidation, while ferulic and caffeic acids were conjugated with a much lower efficiency.

3.2. Glucuronidation and sulfation of hydroxycinnamic acids by human intestinal S9 homogenate

Profiles of conjugates obtained using human intestinal S9 homogenate also indicated that sulfation is the major pathway for the disposition of hydroxycinnamic acids (Tables 1 and 2). For all five hydroxycinnamic acids, the sulfates accounted for over 95% of all the conjugates formed. The V_{max}/K_m for sulfation was highest for dihydrocaffeic acid, again favoring 3-hydroxyl over 4-hydroxyl. Caffeic acid was also highly sulfated, followed by ferulic, isoferulic and dihydroferulic acids. The intrinsic clearance (V_{max}/K_m) of sulfation was, in general, higher in human intestinal S9 homogenate than in human liver S9 homogenate. In contrast, the rate of glucuronidation was comparatively lower. Ferulic acid was the most highly glucuronidated hydroxycinnamic acid; even then, the formation of ferulic acid-4-*O*-glucuronide only accounted for <5% of the total conjugates. Others, including isoferulic acid, were not significantly glucuronidated.

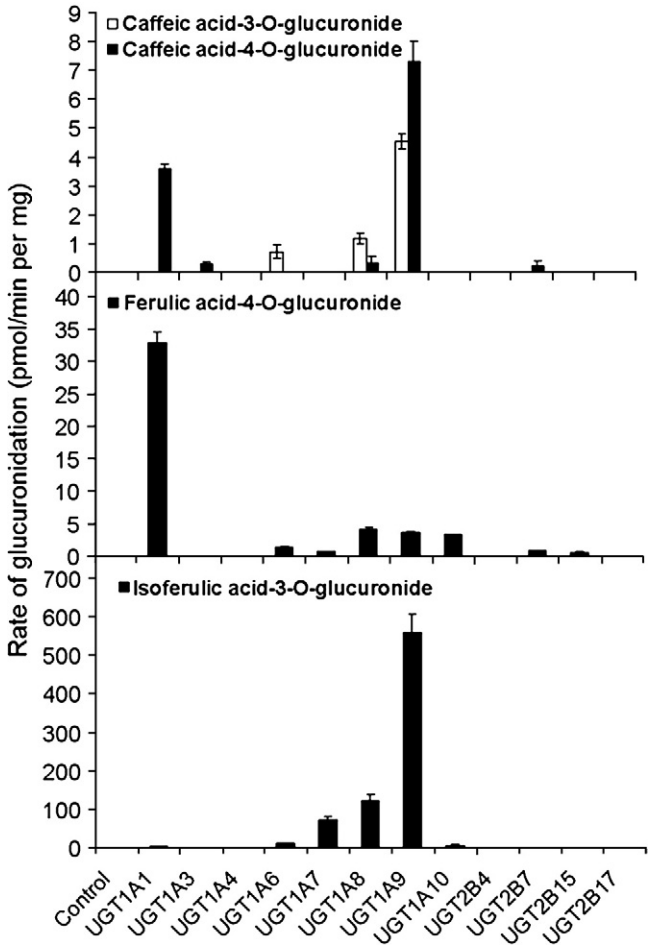

Fig. 2. The glucuronidation of caffeic, ferulic and isoferulic acids by recombinant human UGTs. Formation of conjugates was measured after incubation of 50 μM substrates with 1 mg/ml indicated UGT for 1 h.

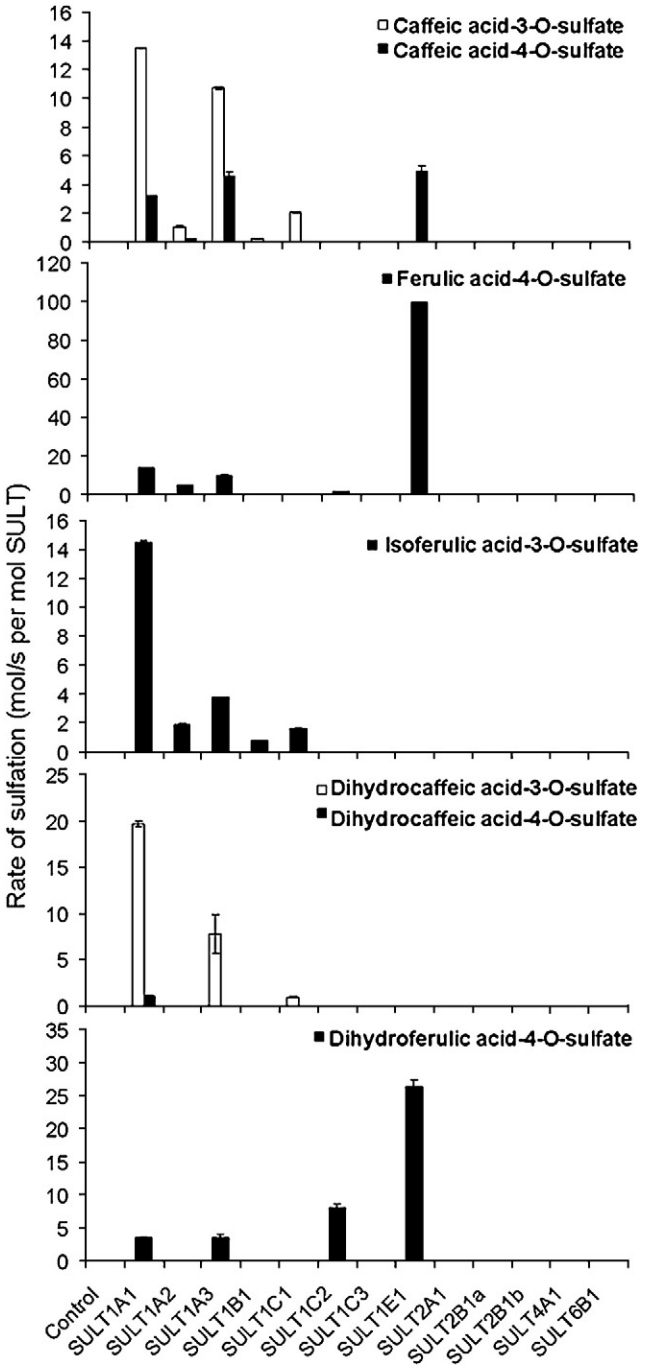

Fig. 3. The sulfation of caffeic, ferulic, isoferulic, dihydrocaffeic and dihydroferulic acids by recombinant human SULTs. Fifty micromolar of the substrates was incubated with cytosolic preparations from recombinant human SULT-expressing bacteria (0.25 mg/ml total protein) for 1 h.

3.3. Glucuronidation of hydroxycinnamic acids by recombinant UGTs

Some recombinant UGTs were found to generate relatively low but detectable levels of glucuronides with caffeic, ferulic and isoferulic acids (Fig. 2). Under the conditions employed, we could not identify

any glucuronide formation from dihydrocaffeic and dihydroferulic acids. It appeared that isozymes UGT1A1 and UGT1A9 were the enzymes most active for hydroxycinnamic acids. The glucuronidation of isoferulic acid was catalyzed at a high rate by UGT1A9, whereas UGT1A1 was the most active in the formation of ferulic acid-4-*O*-glucuronide. UGT1A1 and UGT1A9 were also active in the glucuronidation of caffeic acid, albeit at a much lower rate. UGT1A1 specifically catalyzes the formation of caffeic acid-4-*O*-glucuronide, whereas UGT1A9 conjugates both the 3-hydroxyl and the 4-hydroxyl groups of caffeic acid. UGT1B isozymes were considerably less active towards the hydroxycinnamic acids tested. The kinetic constants of glucuronide formation were also determined in UGTs demonstrating a high activity of conjugation. Glucuronidation kinetics followed a simple hyperbolic profile similar to that in human S9 homogenates. For isoferulic acid, UGT1A7 and UGT1A9 showed similar affinities, while UGT1A9 had a much greater V_{max}. The catalytic efficiency of UGT1A1 with ferulic acid was moderate. In agreement with the kinetic data in S9 homogenates, both UGT1A1 and UGT1A9 demonstrated low affinity and low activity for caffeic acid.

3.4. Sulfation of hydroxycinnamic acids by recombinant SULTs

The relative rates of sulfation of the five hydroxycinnamic acids were evaluated using 13 recombinant human SULTs. Significant sulfation activity was observed with SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C1, SULT1C2 and SULT1E1, while the rest were inactive (Fig. 3). In particular, SULT1A1 catalyzed the sulfation of caffeic, isoferulic and dihydrocaffeic acids at a much higher rate than other SULT isoforms, indicating its major role in the sulfation of these compounds *in vivo*. SULT1A1 catalyzed the sulfation of caffeic acid most effectively, followed by dihydrocaffeic, ferulic, isoferulic and dihydroferulic acids. For caffeic and dihydrocaffeic acids, SULT1A1 showed a clear preference for the conjugation of 3-hydroxyl over 4-hydroxyl. On the other hand, SULT1E1 is the most active isoform towards the methylated forms (i.e., ferulic and dihydroferulic acids). SULT1A3 was active at all the five hydroxycinnamic acids, but the level of activity was lower than that with SULT1A1. The kinetics of formation of sulfates were also examined for SULT1A1 for the five hydroxycinnamic acids and for SULT1E1 for ferulic and dihydroferulic acids. Sulfation of caffeic acid by SULT1A1 showed a significant substrate inhibition above 40 μM; for the rest of the hydroxycinnamic acids, it exhibited simple hyperbolic kinetics, and substrate inhibition was not observed over the concentrations tested. Intrinsic clearance (V_{max}/K_m) for SULT1A1 is in the order of caffeic>dihydrocaffeic>ferulic>isoferulic>dihydroferulic acid, in good agreement with the relative rate of sulfation in human intestinal and liver S9 homogenates. Caffeic acid-3-*O*-sulfate was the major conjugate formed from caffeic acid by SULT1A1. Ferulic acid-4-*O*-sulfate formation by SULT1E1 also exhibited a high clearance rate that was sixfold greater than that of SULT1A1, indicating that SULT1E1 is the major enzyme involved. The rate of sulfation of dihydroferulic acid is much greater for SULT1E1 than for SULT1A1.

Table 3
Urinary excretion of chlorogenic acid metabolites in 24-h urine samples of healthy (n=11) volunteers, following ingestion of 200 ml of coffee (composition in Materials and Methods)

Chlorogenic acid metabolite	Excretion (μmol)	Excretion (% intake)
Caffeic acid-3-*O*-sulfate	6.4±0.8	1.6±0.2
Caffeic acid-4-*O*-sulfate	0.6±0.1	0.1±0.0
Ferulic acid-4-*O*-sulfate	11±2	2.7±0.4
Isoferulic acid-3-*O*-glucuronide	4.8±0.5	1.2±0.1
Isoferulic acid-3-*O*-sulfate	0.4±0.1	0.1±0.1
Dihydrocaffeic acid-3-*O*-glucuronide	0.7±0.2	0.2±0.0
Dihydrocaffeic acid-3-*O*-sulfate	37±8	9.0±2.0
Dihydroferulic acid	10±2	2.4±0.5
Dihydroferulic acid-4-*O*-glucuronide	8.4±1.9	2.0±0.5
Dihydroferulic acid-4-*O*-sulfate	12±3	3.0±0.8

Data are expressed as mean±S.E. (μmol) and as percentage of chlorogenic acid intake.

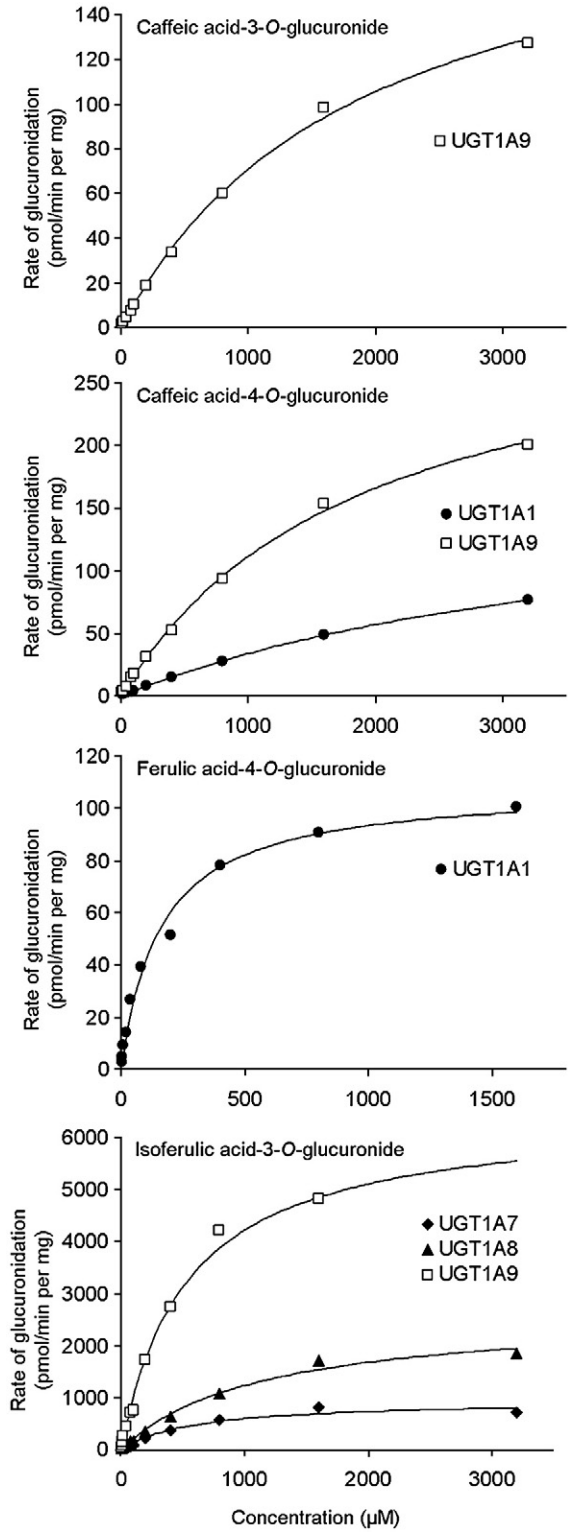

Fig. 4. The kinetics of glucuronidation of hydroxycinnamic acids by human UGTs.

3.5. Conjugates of hydroxycinnamic acids found in human urine after coffee consumption

Based on *in vitro* evidence, it was expected that, *in vivo*, hydroxycinnamic acids would exist mainly as sulfoconjugates. We analyzed the conjugates of hydroxycinnamic acids in urine from 11 volunteers for a 24-h period following consumption of coffee. As shown in Table 3, the hydroxycinnamic acids were extensively metabolized, with sulfates and glucuronides appearing in the urine. Among the hydroxycinnamic acids, only dihydroferulic acid could be detected in the free acid form. As expected, sulfates were the major conjugates. Only sulfates — but not the glucuronides — of caffeic and ferulic acids were detected. The levels of dihydrocaffeic acid-3-sulfate were, on average, 45-fold greater than the levels of the corresponding glucuronide. Similarly, the amount of dihydroferulic acid-4-*O*-sulfate in the urine was also much greater than the amount of dihydroferulic acid-4-*O*-glucuronide. Meanwhile, isoferulic acid is unique in that its glucuronide was the major conjugate observed.

4. Discussion

In the present study, we examined the disposition of hydroxycinnamic acids via glucuronidation (Fig. 4) and sulfation (Fig. 5), and identified the major UGTs and SULTs involved. *In vitro* results were then verified by measuring hydroxycinnamic acid conjugates (Fig. 6) in human urine following consumption of coffee, which is rich in hydroxycinnamate esters such as 5-*O*-caffeoylquinic acid. Previous investigations showed that hydroxycinnamic acids were absorbed and metabolized in humans to give sulfoconjugates and/or glucuronic acid conjugates in plasma, although the position of conjugation was not determined [12,26–28]. The site of absorption of hydroxycinnamic acids depends on their chemical forms [29]. Generally, esterified forms are only absorbed in the colon, where the "absorbable form" is released by bacterial esterases [30], while free acids are absorbed from the stomach and small intestine and metabolized in the liver [27]. Using human liver and intestinal S9 homogenates, we showed that hydroxycinnamates are extensively metabolized, with sulfates as their predominant conjugates (over 95%). Apart from isoferulic acid-3-*O*-glucuronide, glucuronide conjugates are not formed to a significant extent in *in vitro* incubations. Caffeic, ferulic and dihydrocaffeic acids are the most actively sulfated, whereas dihydroferulic acid is the least efficiently metabolized. Our *in vitro* data suggested that, with the probable exception of isoferulic acid, hydroxycinnamates exist *in vivo* predominantly as sulfoconjugates (Tables 1 and 2). Analysis of urinary conjugates revealed a good agreement with the *in vitro* data. Sulfates accounted for virtually all conjugate forms of caffeic, ferulic and dihydrocaffeic acids found in human urine (Table 3). Moreover, the regioselectivity preference for sulfation of the 3-hydroxyl of caffeic and dihydrocaffeic acids was consistent with the results from S9 homogenates. In agreement with its relatively slow rate of metabolism in S9 homogenates, dihydroferulic acid was the only free acid to be detected in urine, with dihydroferulic acid-4-*O*-sulfate as the major conjugate. As predicted from the *in vitro* data, isoferulic acid was significantly glucuronidated in humans, and the major conjugate was isoferulic acid-3-*O*-glucuronide. The ratio of glucuronidation to sulfation appeared to be greater than expected from experiments on conjugation with S9 homogenates. It cannot be excluded that factors other than SULT and UGT enzyme protein levels (e.g., availability of the cofactors *in vivo*) could affect the disposition of conjugates. As the conjugates are hydrophilic, uptake and efflux transporters are also important determinants of the major route of elimination and urinary conjugate profiles [31,32].

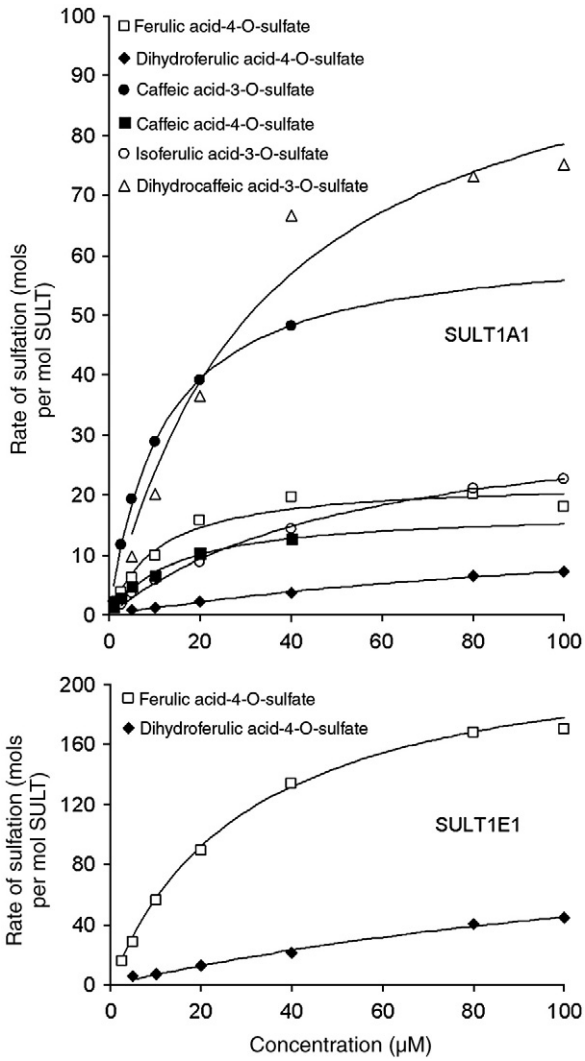

Fig. 5. The kinetics of sulfation of hydroxycinnamic acids by human SULTs.

To further understand which specific isozymes are responsible for the metabolism of hydroxycinnamic acids, we tested the conjugating activities of a panel of UGTs and SULTs. UGT1A, in particular UGT1A1 and UGT1A9, is effective in the glucuronidation of hydroxycinnamic acids. A similar UGT activity profile is observed with polyphenols such as flavonoids [33,34]. UGT1A9 is highly active in catalyzing the formation of isoferulic acid-3-glucuronide. UGT1A9 is uniquely expressed in hepatic tissue [35], explaining the markedly higher level of glucuronidation of isoferulic acid in liver S9 homogenate compared to that in intestinal S9 homogenate. Thus, the liver would be the major site of glucuronidation of isoferulic acid. Evaluation of the relative activities of human SULTs expressed in *Salmonella* showed that the phenol SULT1A1 is highly active towards all five hydroxycinnamic acid substrates, indicating a major role in the disposition and bioavailability of hydroxycinnamic acids. Similar to the liver and intestinal S9 homogenates, SULT1A1 is most highly active towards caffeic and dihydrocaffeic acids, with regioselectivity for the 3-hydroxyl of the catechol moiety. SULT1A3 is also active in the sulfation of all hydroxycinnamic acids. In humans, SULT1A1 is highly expressed in the liver and intestine, whereas SULT1A3 is highly expressed in the intestine (and some other extrahepatic tissues) but virtually absent in adult liver [23,36]. Thus, human intestine can be expected to play a major role in the conjugation and disposition of hydroxycinnamic acids. On the other hand, SULT1E1 appeared to possess a unique regioselectivity for hydroxycinnamic acids, only

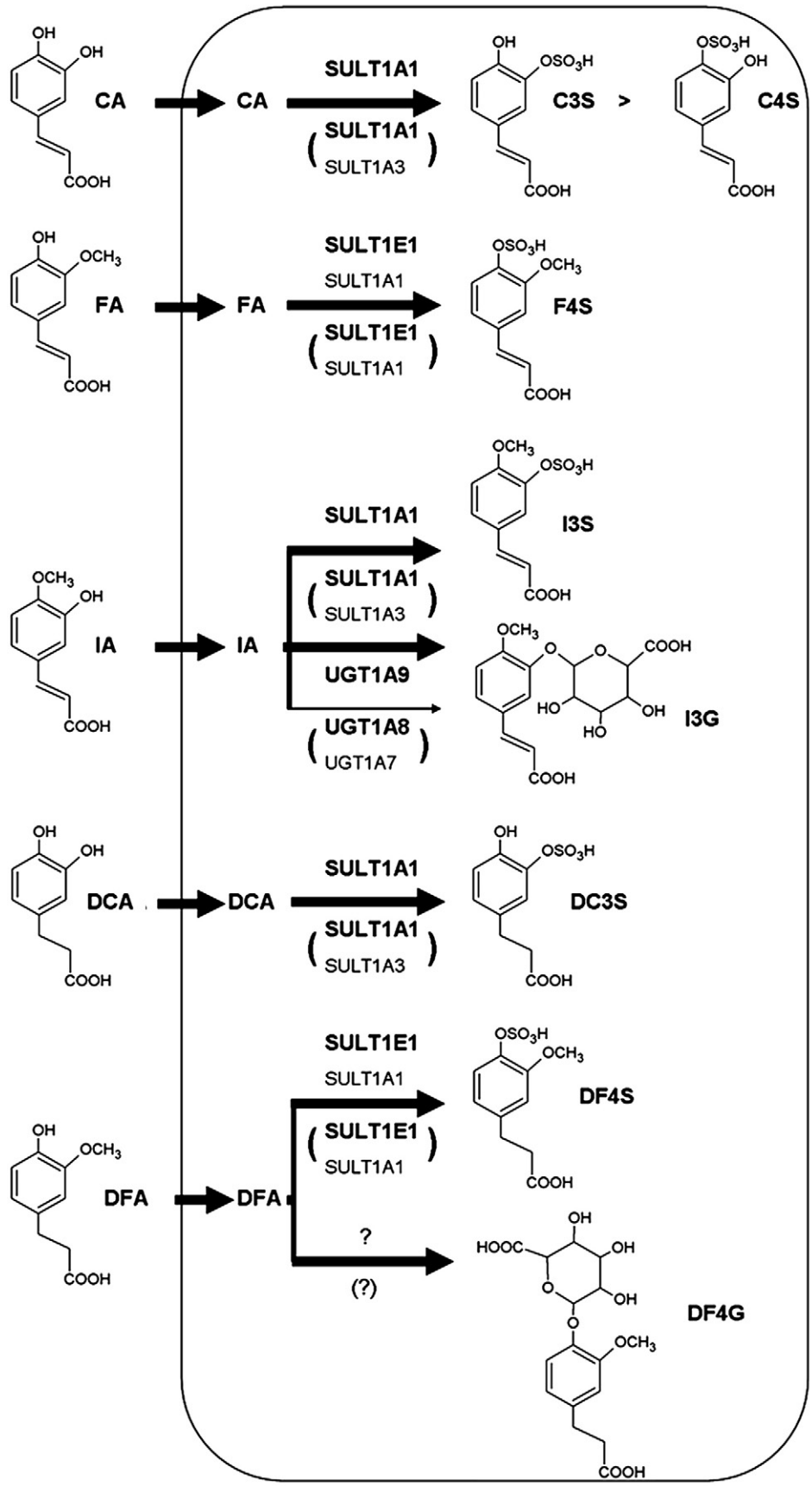

Fig. 6. Summary of the metabolic transformation of hydroxycinnamic acids in the human liver and intestine. Free hydroxycinnamic acids are absorbed and metabolized, with sulfates as major conjugates, with the exception of isoferulic acid, which is significantly glucuronidated in the liver. The predominant isozymes involved in metabolism in the human liver and intestine (in parentheses) are highlighted in boldface. Only conjugates found in human urine are considered in the figure.

catalyzing sulfation of the 4-hydroxyl group of ferulic acid and dihydroferulic acid with high efficiency. SULT1E1, also known as estrogen SULT, is expressed in both the liver and the intestine and catalyzes the conversion of endogenous estrogens into inactive storage forms [37]. Recent studies have shown that SULT1E1 is also active towards certain polyphenolic substrates such as flavonols and isoflavones [38,39]. The ability of ferulic and dihydroferulic acids to interact with SULT1E1 may lead to potential effects on the metabolism of estrogens.

Conjugation of hydroxycinnamic acids *in vivo* has important implications on the bioactivity of these compounds. For example, the catechol moiety of caffeic and dihydrocaffeic acids is critical to their high antioxidant activity, which could be drastically reduced by conjugation of catechol hydroxyl groups [40]. Thus, research should focus on the characterization of the bioactivity of conjugates, in particular hydroxycinnamate-*O*-sulfates, which are the predominant conjugates *in vivo*. In addition, prominent sulfation of hydroxycinnamates implies the potential for food–food and food–drug interactions. Sulfation is an important pathway for deactivation of drugs and toxins, and inhibition of SULT activity by hydroxycinnamic acids could lead to modified drug metabolism [41,42]. Conversely, inhibition of SULTs could protect the tissue from promutagens and procarcinogens bioactivated by SULTs [43].

In summary, the results indicate that hydroxycinnamic acids are metabolized *in vitro* and *in vivo* by glucuronidation and sulfation, with the latter being by far the predominant disposition pathway. Sulfation is a high-affinity and efficient pathway for the disposition of hydroxycinnamic acids. Our data also suggest that SULT1A1 and UGT1A9 are the most active isoforms in hydroxycinnamic acid sulfation and glucuronidation, respectively.

Acknowledgments

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