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Uptake and Metabolism of Hydroxycinnamic Acids (Chlorogenic, Caffeic, and Ferulic Acids) by HepG2 Cells as a Model of the Human Liver

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Hydroxycinnamic acids are antioxidant polyphenols common in the human diet, although their potential health benefits depend on their bioavailability. To study the hepatic uptake and metabolism, human hepatoma HepG2 cells were incubated for 2 and 18 h with caffeic, ferulic, and chlorogenic acids. Moderate uptake of caffeic and ferulic acids was observed versus a low absorption of chlorogenic acid, where esterification of the caffeic acid moiety markedly reduced its absorption. Methylation was the preferential pathway for caffeic acid metabolism, along with glucuronidation and sulfation, while ferulic acid generated glucuronides as the only metabolites. Ferulic acid appeared to be more slowly taken up and metabolized by HepG2 cells than caffeic acid, with 73% and 64% of the free, nonmetabolized molecules detected in the culture medium after 18 h, respectively. In conclusion, hydroxycinnamic acids can be metabolized by the liver as suggested by the results obtained using HepG2 cells as a hepatic model system.

KEYWORDS: Hydroxycinnamic acids; metabolism; in vitro conjugation; HepG2 cells; LC-MS analysis

INTRODUCTION

Dietary polyphenols have gained increasing interest because of their numerous biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, inhibition of cellular proliferation, and altering signal transduction pathways (*1*). Epidemiological studies have also highlighted the association between the consumption of polyphenol-rich foods and the prevention of degenerative human diseases such as cardiovascular diseases, cancer, and other degenerative disorders (*2*). Polyphenols are abundant in our diet, widely distributed in foods and beverages of plant origin such as fruits, vegetables, cereals, cocoa, tea, coffee, and wine. Total polyphenol intake may reach 1 g/day in people who eat several servings of fruit and vegetables per day and is mainly constituted by hydroxycinnamates and flavonoids (about 1/3 and 2/3 of the total intake, respectively) (*3*).

Hydroxycinnamic acids are low-molecular-weight phenylpropanoids (C6−C3) ubiquitously found in fruits, vegetables, and cereals (*4*). The major hydroxycinnamic acids, *p*-coumaric, caffeic, ferulic, and sinapinic acids, are rarely found free except in processed foods, and they mainly exist esterified with organic acids, sugars, and lipids (*4*). Caffeic and quinic acids combine to form chlorogenic acid (5-caffeoylquinic acid), which is found in many fruits and in high concentrations in coffee (varying from 70 to up to 350 mg/cup). Ferulic acid is present in free form in tomatoes or beer, being the most abundant phenolic in cereal grains, mainly conjugated with arabinoxylans in plant cell walls. Consumers with regular intake of cereal products can have a daily intake of ferulic acid of up to 100 mg (*5*).

The interest of these compounds is related to their antioxidant activity (*6*), which may have some health beneficial effects in vivo. However, the extent of their protective effect in vivo depends on their bioavailability for intestinal absorption, metabolism, and subsequent interaction with target tissues.

Several human (*7*−*10*) and animal (*11*, *12*) studies have shown that the esterification of caffeic acid, as in chlorogenic acid, markedly reduced its absorption. In fact, absorption of chlorogenic acid occurs mainly after hydrolysis by esterases either in the small intestinal mucosa or by esterases of the colonic microflora. There are controversial results about the predominant form of chlorogenic acid in human plasma; while several researchers (*10*) have found only caffeic acid in plasma after the ingestion of coffee, intact chlorogenic acid has been detected at low concentrations in nonhydrolyzed urine samples (*7*, *8*, *12*). Recently, direct absorption of intact chlorogenic acid from the stomach in rats has been reported (*13*), although hydrolysis to caffeic acid prior to absorption seems to predominate.

Absorbed caffeic acid appears to be glucuronidated or sulfated as well as *O*-methylated to ferulic or isoferulic acids (*14*) (**Figure 1**). The colonic microflora may metabolize these compounds further to phenolics like *m*-coumaric acid and derivatives of phenylpropionic and hippuric acids (*12*, *15*, *16*). In contrast, most ingested chlorogenic acid would reach the

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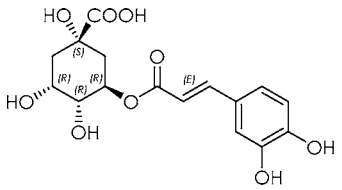
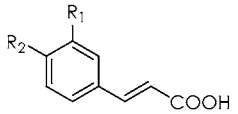


Chlorogenic acid (**1**)



R_1=R_2=OH	Caffeic acid (**2**)
R_1= OCH_3; R_2=OH	Ferulic acid (**3**)
R_1=OH; R_2=OCH_3	IsoFerulic acid (**M8**)
R_1=OH; R_2=OGlucAc	Monoglucuronide conjugate of **2**
R_1= OCH_3; R_2= OGlucAc	Monoglucuronide conjugate of **3**
R_1=OH; R_2= OSO_3H	Monosulfate conjugate of **2**

Figure 1. Chemical structures of chlorogenic, caffeic, and ferulic acids and their metabolites formed by HepG2 cells.

colon intact, where it would be cleaved into caffeic and quinic acids by the gut microflora and then absorbed and metabolized as described above for caffeic acid (*9*, *12*, *16*).

On the other hand, free ferulic acid is efficiently absorbed (*17*−*19*), but similarly to chlorogenic acid, esterification of ferulic acid to the arabinoxylans or lignins in the grain cell walls hampers its absorption in humans (*20*) and rats (*21*−*23*). Glucuronidated, sulfated, and sulfoglucuronidated are the main identified metabolites of ferulic acid and its esters (*22*).

Considering the scarce and contradictory results reported in the literature relative to the uptake and metabolism of chlorogenic acid and taking into account that the liver is the major organ for the metabolism of many drugs and xenobiotics, an established hepatic cell culture could be useful to study the metabolism of polyphenolic compounds. In a previous study we showed that human hepatocarcinoma HepG2 cells might be adequate for such metabolic studies as a model system of the human liver (*24*).

Thus, the aim of this work was to study the uptake and metabolism of caffeic and ferulic acids as representative of hydroxycinnamic acids and of chlorogenic acid as a hydroxycinnamate using HepG2 cells. Identification of metabolites was performed by HPLC-DAD and LC-MS analysis and by studying the behavior of the resulting metabolites after in vitro treatment with conjugating and deconjugating enzymes.

MATERIALS AND METHODS

Chemicals. Acetonitrile, methanol, sodium chloride, disodium hydrogen phosphate anhydrous, and potassium dihydrogen phosphate, as well as formic acid, were acquired from Panreac (Barcelona, Spain). Hydroxycinnamic acids (caffeic, ferulic, and chlorogenic acids), antibiotics (gentamicin, penicillin, and streptomycin), enzymes (catechol-*O*-methyltransferase, β-glucuronidase from *Helix pomatia*, sulfatase), *S*-adenosyl-L-methionine chloride, UDP-glucuronic acid, and adenosine-3′-phospho-5′-phosphosulfate were purchased from Sigma Chemical Co. (Madrid, Spain). DMEM F-12 medium and fetal bovine serum (FBS) were from Biowhitaker (Innogenetics, Madrid, Spain). All reagents were of analytical or chromatographic grade.

Cell Culture. Human hepatoma HepG2 cells were grown in DMEM F-12 medium supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and 50 mg/L of each gentamicin, penicillin, and streptomycin. Cells were grown in 100 mm diameter plates in a humidified incubator containing 5% CO_2 and 95% air at 37 °C, changing the culture medium every second day. Since HepG2 cells grew well in serum-free medium, as tested in the laboratory, the incubation with hydroxycinnamic acids was carried out in the same medium containing the antibiotic mixture but deprived of serum to prevent any potential interference from serum components.

The experiments were carried out when cells reached 80% confluence (concentration of 5×10^6 cells/plate, approximately), when plates were changed to the different conditions: control plates were treated only with FBS-free medium, and experimental plates contained 100 μmol/L of the different phenolic compounds, in duplicate. To all plates, ascorbic acid (150 μmol/L) was added to prevent potential oxidation of the hydroxycinnamic acids.

After incubation for 2 h (short term) or 18 h (long term), the culture medium was aspirated and frozen at −20 °C until analysis. Cells were washed twice with PBS (0.01 M phosphate buffered saline solution, pH 7.4) and then collected by scraping in PBS, combining cells from duplicate plates corresponding to a particular condition. After centrifugation at 1250 rpm for 5 min at 4 °C, the supernatant was removed and the cell pellet resuspended in 200 μL of PBS and then sonicated for 7 min at room temperature to break down the cell membrane and to release the total amount of metabolites, separating the supernatant from the cell debris by centrifugation at 5000 rpm for 10 min at 4 °C. Supernatants, containing the cytoplasmatic contents, were transferred into Eppendorf vials and kept frozen at −20 °C.

Analysis of Hydroxycinnamic Acids and Metabolites. *HPLC Procedure.* An Agilent 1100 liquid chromatographic system (Agilent Technologies, Waldrom, Germany) equipped with a diode array UV/vis detector and a Rheodyne injection valve (20 μL loop) was used. Separation was performed in a 250 mm × 4.6 mm i.d., 5-μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by an ODS precolumn, eluted with a mobile phase made of a mixture of 4% (v/v) formic acid in deionized water (solvent A) and acetonitrile (solvent B). The solvent gradient changed according to the following conditions: from 90% A to 80% A in 20 min, 75% A in 10 min, 65% A in 10 min, and then maintained isocratically for 10 min. Elution was performed at a flow rate of 1 mL/min at room temperature, and chromatograms were acquired at 324 nm. An Agilent Chemstation software system controlled the equipment and carried out the data processing.

Quantification of chlorogenic, ferulic, and caffeic acids and their metabolites was performed by comparison with standards of the three parent compounds, prepared in serum-free culture medium in a range of concentrations from 1 to 100 μmol/L. A linear response was obtained for all the standard curves, as checked by linear regression analysis. Percentage of recovery of standards added to the culture medium (varying from 94.1 to 100%), limits of detection (ranging from 0.25 to 0.45 μmol/L), limits of quantification (0.88−1.60 μmol/L), and precision of the assay (as the coefficient of variation, ranging from 2.1 to 7.2%) were considered acceptable and allowed quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecules).

LC-MS analysis was carried out on an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a diode array detector and a quadrupole (G1946D) mass spectrometer. Data acquisition and analysis were carried out with an Agilent ChemStation. Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above, only varying solvent A that consisted of 1% formic acid in deionized water (v/v). Eluent flow (1 mL/min) was split 8:1 between the diode array detector and the mass spectrometer ion source. Ionization was achieved by atmospheric pressure electrospray ionization (ESI) source, operated in negative ion mode, with the electrospray capillary voltage set to 3000 V, a nebulizing gas (N_2) flow

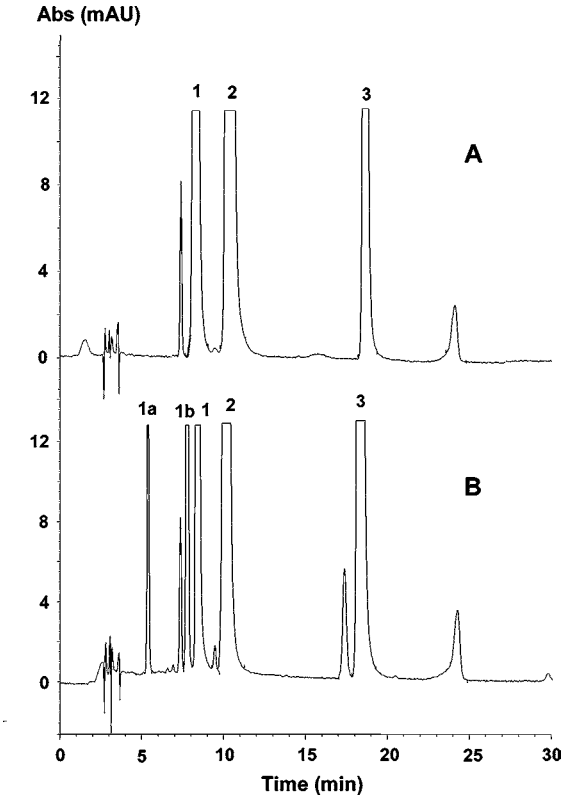


Figure 2. Chromatographic profile at 324 nm: (**A**) serum free culture medium spiked with 100 μmol/L chlorogenic acid (**1**), caffeic acid (**2**), and ferulic acid (**3**); (**B**) serum free culture medium spiked with phenols after 18 h of incubation.

rate of 12 L/h, and a drying gas temperature of 300 °C. Mass spectrometry data were acquired in the scan mode (mass range *m*/*z* 100−500) at a scan rate of 1.5 s. Diode array spectra were registered from 230 to 450 nm, with an interval of 2 nm.

Enzymatic Treatments. *In Vitro Conjugation of Hydroxycinnamic Acids.* By the treatment of standard solutions of the three hydroxycinnamic acids studied with pure enzyme (catechol-*O*-methyltransferase, COMT) or a rat liver microsomal fraction that contained both UDP-glucuronosyltransferase (UGT) and sulfotransferase (ST), the in vitro conjugation of hydroxycinnamic acids was carried out. Rat liver microsomes were obtained by successive centrifugation and ultracentrifugation of liver homogenates as described previously (*24*). In vitro glucuronidation of hydroxycinnamic acids was performed by incubation of the microsomes containing the enzyme activity UGT in the presence of UDP-glucuronic acid as the glucuronosyl donor. The precise protocol has been described elsewhere (*25*). In vitro methylation was carried out using commercial COMT and *S*-adenosyl-L-methionine (SAM) as the methyl group donor following the procedure described by Piskula and Terao (*26*). For in vitro sulfation, hydroxycinnamic acids were treated with cytosolic sulfotransferases from rat liver microsomes, using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfate donor (*27*).

Enzymatic Hydrolysis of Phenolic Conjugates from the Cell Culture. Metabolites obtained after incubation of HepG2 cells with phenolic compounds (100 μmol/L) during 18 h were hydrolyzed enzymatically by incubating the culture media with β-glucuronidase (*28*) or sulfatase (*26*), and analyzing the released compounds by HPLC to confirm conjugate identifications.

RESULTS

Figure 2A shows the chromatographic profile of fresh culture medium spiked with 100 μmol/L chlorogenic acid (**1**), caffeic acid (**2**), and ferulic acid (**3**). When the phenolic compounds were incubated in ascorbic acid-containing culture medium in the absence of HepG2 cells for 18 h, new peaks appeared as can be seen in **Figure 2B**. Two of these peaks appeared immediately before caffeic acid and ferulic acid, at 9.4 and 17.0 min, respectively. Although it might be suspected that these peaks originated from the hydroxycinnamic acids, their spectra (with absorption maxima at 300 and 310 nm, respectively) differed from those of caffeic and ferulic acids. Besides, recoveries of the standard acids were 100.6% and 100.2% for caffeic and ferulic, respectively, showing that the hydroxycinnamic acids were stable in the cell-free culture medium. On the contrary, chlorogenic acid suffered an extensive transformation when dissolved in FBS-free culture medium in the absence of cells. In this sense, two new chromatographic peaks at 5.2 (peak 1a), and 7.6 min (peak 1b) having spectra identical with that of the chlorogenic acid standard were obtained after 18 h of incubation (**Figure 2B**). After this time, recovery of chlorogenic acid was only 83% of the compound initially added. These two new peaks may correspond to isomers of chlorogenic acid (5-*O*-caffeoyl quinic acid), since isomerization to neochlorogenic (3-*O*-caffeoyl quinic) and cryptochlorogenic acids (4-*O*-caffeoyl quinic) has been shown to occur by treatment of chlorogenic acid for 30 min at pH 8, 100 °C (*7*). Another peak at 6.7 min, however, had a different UV−vis spectrum, suggesting it was not a chlorogenic acid isomer.

Metabolism of Hydroxycinnamic Acids by HepG2 Cells. Metabolism of chlorogenic, caffeic, and ferulic acids by the hepatoma cell line was monitored at short (2 h) and long (18 h) incubation times, analyzing the products formed as a consequence of cell metabolism in the extracellular culture medium as well as in the cytoplasmatic contents after cell lysis. Representative chromatograms for culture media after 18 h of incubation are shown in **Figure 3**. As can be seen, phenolic compounds and their metabolites were well-resolved from peaks corresponding to the culture medium, allowing quantitative and qualitative analysis of the studied phenols. When hydroxycinnamic acids were incubated for 18 h with HepG2 cells, extensive metabolism of these compounds took place, although appreciable amounts of the nonmetabolized parent phenolics could still be detected in the culture medium.

Caffeic acid (**2**) was metabolized by the hepatic cells with formation of four new peaks (M1−M4) (**Figure 3A**). Also, a peak with retention time and UV spectrum similar to ferulic acid (**3**) was detected. It has to be noted that ferulic acid is a methylated derivative of caffeic acid; therefore, this compound would have been formed in vitro by methylation of caffeic acid by hepatoma cells as confirmed by experiments described below. When pure ferulic acid (**3**) was incubated with HepG2 cells, three new peaks (M5−M7) were detected as potential conjugated metabolites (**Figure 3B**). Concerning chlorogenic acid (**1**), this hydroxycinnamate was not metabolized by HepG2 cells, and the only peaks detected were those observed when chlorogenic acid was maintained 18 h in culture medium in the absence of cells (peaks 1a,b) (**Figure 3C**).

At short incubation times (2 h), most of the phenolics were present in the culture medium as the parent molecules, suggesting slow cellular uptake and metabolism of these acids. Only traces of metabolites **M2**−**M4**, together with free ferulic were observed after 2 h of incubation of HepG2 cells with caffeic acid (data not shown). Ferulic acid metabolism was slightly more active, with formation of an intense peak corresponding to metabolite **M6** and smaller amounts of metabolites **M5** and **M7** (data not shown). When chlorogenic acid was incubated for 2 h with HepG2 cells, only peaks 1a,b were detected.

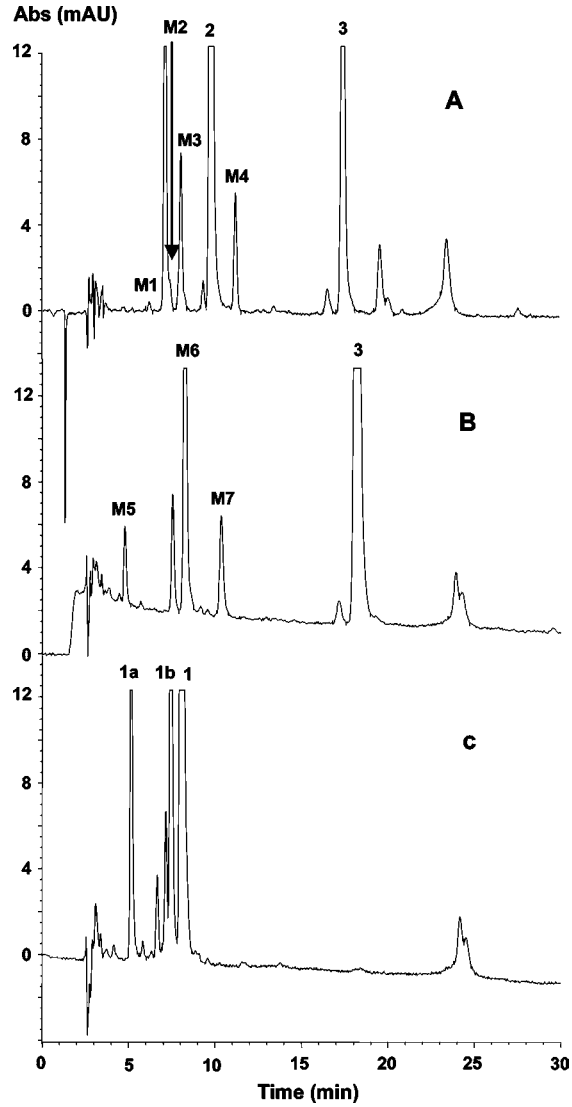


Figure 3. HPLC chromatograms of cell culture medium from HepG2 cells after incubation for 18 h with 100 μmol/L concentrations of the following compounds: (**A**) caffeic acid (**2**); (**B**) ferulic acid (**3**); (**C**) chlorogenic acid (**1**). Peaks M1–M8 correspond to different metabolites of the studied hydroxycinnamic acids (caffeic and ferulic acids) and hydroxycinnamate (chlorogenic acid) formed after incubation with the hepatic cells.

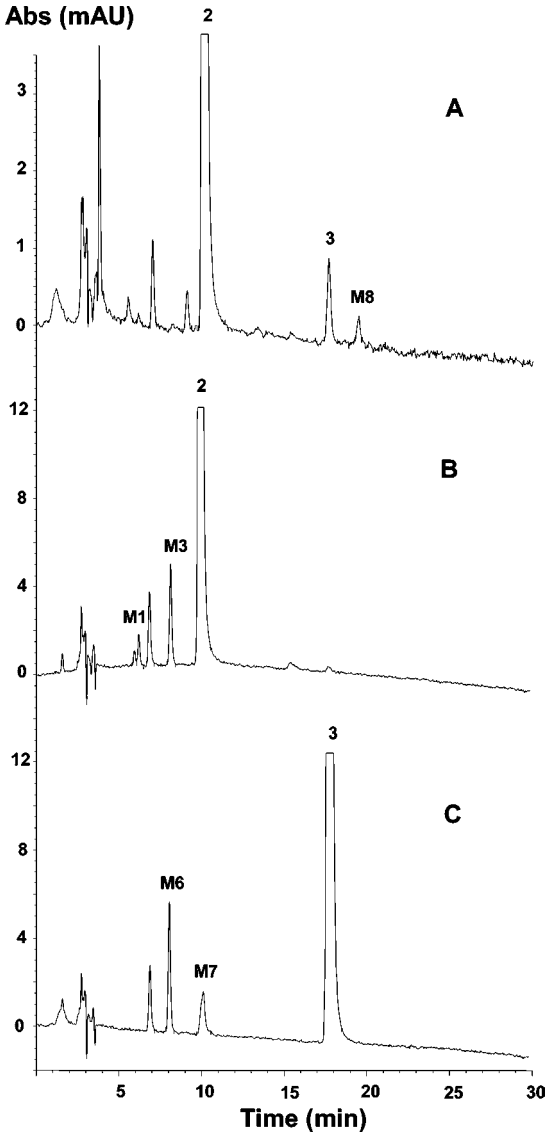


Figure 4. HPLC chromatograms of reaction media after in vitro methylation with catechol-*O*-methyl transferase and in vitro glucuronidation with rat liver microsomes. Shown are the medium after (**A**) in vitro methylation of caffeic acid and after in vitro glucuronidation of (**B**) caffeic acid (**2**) and (**C**) ferulic acid (**3**). Peaks 3 and M8 correspond to methylated caffeic acid, and peaks M1, M3, M6, and M7 correspond to glucuronidated metabolites.

Finally, analysis of the cytoplasmatic contents showed only trace amounts of caffeic and ferulic acids together with some of their potential metabolites in the cell lysates after 2 or 18 h of incubation, suggesting no intracellular accumulation of these compounds or their metabolites (data not shown). Chlorogenic acid could also be detected in the cytoplasmatic contents, confirming that the liver cells can absorb this phenolic compound although it does not undergo further intracellular metabolism.

Identification of Caffeic, Ferulic, and Chlorogenic Acids Metabolites. To identify the different metabolites formed after incubation of the selected phenolic compounds with human hepatoma cells, several steps were followed: in vitro conjugation of pure standards; enzymatic hydrolysis with β-glucuronidase and sulfatase of metabolites formed by HepG2 cells; confirmation of structures by LC-MS.

Two new peaks derived from caffeic acid were observed after in vitro conjugation with COMT (peaks 3 and M8, **Figure 4A**). The spectroscopic characteristics of these peaks (spectrum width and λ_{max} at 295 and 323 nm) were similar to those of ferulic acid; the peak at 17.7 min was coincident in retention time and spectral characteristics with the ferulic acid standard, while the peak at 19.5 min (M8) also had the same spectral characteristics, although it eluted at longer incubation times and thus it is proposed as isoferulic acid. No methyl conjugates of ferulic acid were observed (data not shown) as could be expected since methylation by COMT requires an *ortho*-diphenolic structure, absent in ferulic acid. Under the applied chromatographic conditions, methylation results in longer retention times of conjugated metabolites in comparison with parent compounds; since no peaks at longer retention times than chlorogenic acid were detected when this phenol was incubated with the liver cells (**Figure 3C**), in vitro methylation of this acid was not performed.

Concerning in vitro glucuronidation, besides the peak corresponding to glucuronic acid (at 6.9 min, **Figure 4B**,**C**), nonmetabolized caffeic and ferulic acids appeared after 4 h of treatment with liver microsomes. Of the new peaks observed

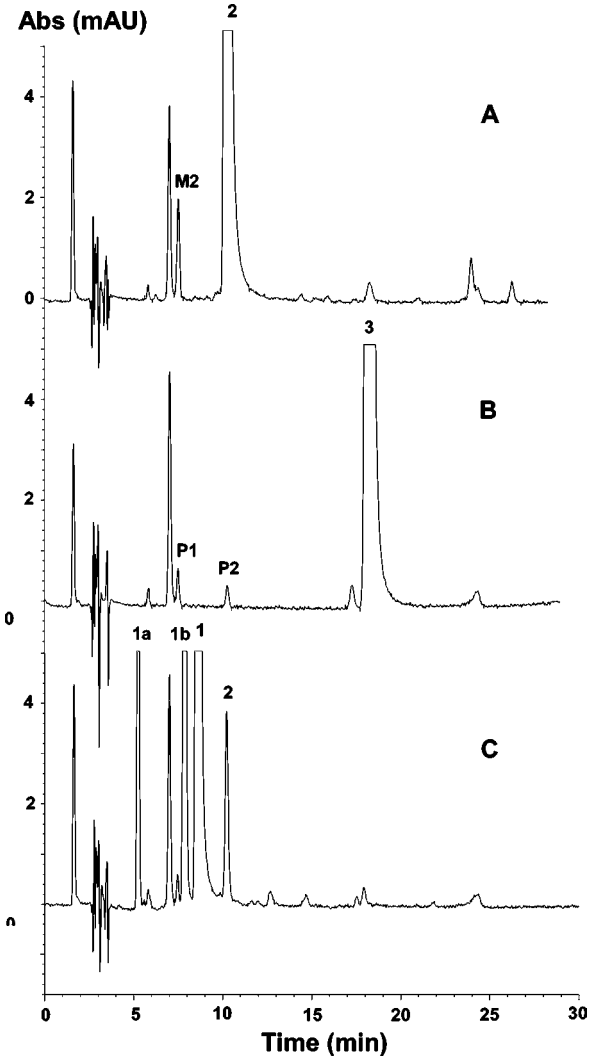


Figure 5. HPLC chromatograms of reaction media after in vitro sulfation with rat liver microsomes. Shown are the medium after in vitro sulfation of (**A**) caffeic acid (**2**), (**B**) ferulic acid (**3**), and (**C**) chlorogenic acid (**1**). Peak M2 corresponds to sulfated caffeic acid.

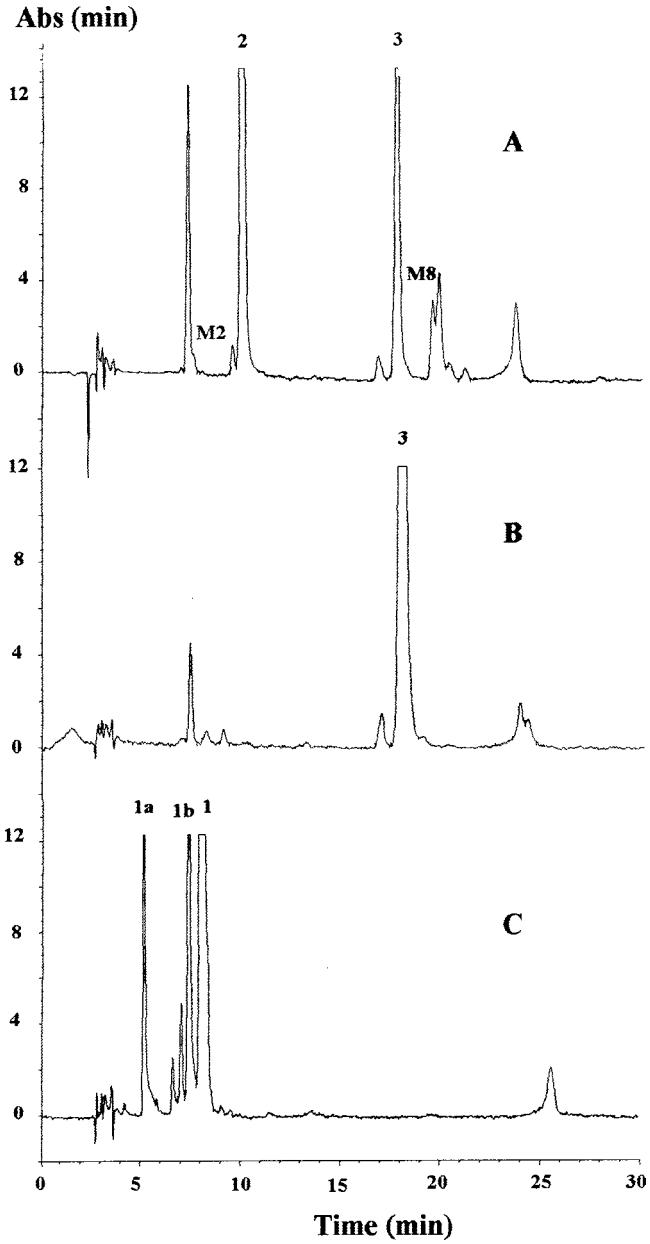


Figure 6. Chromatograms of culture media obtained after 18 h of incubation in the presence of 100 μM concentrations of the three hydroxycinnamic acids and subjected to in vitro hydrolysis with β-glucuronidase: (**A**) culture medium of cells treated with caffeic acid (**2**); (**B**) culture medium of cells treated with ferulic acid (**3**); (**C**) culture medium of cells treated with chlorogenic acid (**1**). Peaks M2 and M8 correspond to sulfated and methylated metabolites of caffeic acid, respectively.

when caffeic acid was subjected to in vitro glucuronidation (**Figure 4B**), two of them (M1 and M3) showed retention times and spectral characteristics similar to those of the peaks observed after incubation of caffeic acid with HepG2 cells, at 6.2 and 8.1 min, respectively (**Figure 3A**). Similarly, the two new peaks found after in vitro glucuronidation of ferulic acid, M6 at 8.25 min and M7 at 10.35 min (**Figure 4C**) were coincident with the metabolites formed after incubation of ferulic acid with the hepatoma cells (**Figure 3B**). In all cases, the yield of in vitro conjugation was very low, as it occurred in the methylation assay. When chlorogenic acid was subjected to in vitro glucuronidation, it suffered a partial hydrolysis to caffeic acid, but no further peaks suggestive of glucuronide derivatives of chlorogenic acid were detected (data not shown).

In vitro sulfation of caffeic acid resulted in an intense peak at 7.5 min (**Figure 5A**). The spectroscopic characteristics of this peak were identical with those of chromatographic peak M2 in **Figure 3A**. Sulfation of ferulic acid yielded two chromatographic peaks at 7.5 (P1) and 10.3 (P2) min but with spectral characteristics very different from those of any of the described metabolites (**M5**−**M7**), or those of peak M2, although retention times were similar (**Figure 5B**). No sulfate derivative of chlorogenic acid was detected (**Figure 5C**), although partial hydrolysis to caffeic acid was observed as it occurred during in vitro glucuronidation, probably due to the hydrolytical activity of microsomal esterases.

Figure 6 shows the chromatograms obtained after treatment with β-glucuronidase of culture media after 18 h incubation with caffeic acid (**Figure 6A**), ferulic acid (**Figure 6B**), or chlorogenic acid (**Figure 6C**). Metabolites **M1** and **M3**, formed after in vitro glucuronidation (**Figure 4B**) and present in the culture medium of cells incubated with caffeic acid (**Figure 3A**), together with metabolite **M4** disappeared after treatment with β-glucuronidase. In turn, the area corresponding to ferulic and caffeic acids increased and emerged a new chromatographic peak at 19.5 min of spectral characteristics identical with that of the peak obtained after in vitro methylation of caffeic acid

Table 1. Chromatographic and Spectroscopic Characteristics of Chlorogenic Acid (**1**), Caffeic Acid (**2**), and Ferulic Acid (**3**) and the Metabolites Formed after Incubation with HepG2 Cells

compd	MW	RT (min)	λ_{max} (nm)	$[M - H]^-$ (*m*/*z*)	fragment ions (*m*/*z*)	proposed structure
1	354	8.1	296–326	352.9	191.0–179	chlorogenic acid
2	180	9.8	294–324	179.1	135.1	caffeic acid
3	194	17.5	294–322	193.0	179.0–149.0	ferulic acid
M1	356	6.2	292–316	355.0	179.0	monoglucuronide caffeic acid
M2	260	7.3	306–328	259.1		sulfate caffeic acid
M3	356	8.1	294–314	355.1	179.0	monoglucuronide caffeic acid
M4	370	11.2	292–314	369.0	193.1	methylglucuronide caffeic acid
M8	194	19.5	296–324	193	179.0–149.0	isoferulic acid
M5	370	4.8	298–322			tentative glucuronide ferulic acid
M6	370	8.2	292–314	369.0	193.0	monoglucuronide ferulic acid
M7	370	10.3	300–330	368.9	193.0	monoglucuronide ferulic acid
1a	354	5.2	298–326	353.0	191.0–178.9	isomer chlorogenic acid
1b	354	7.5	298–326	353.0	191.0–178.9	isomer chlorogenic acid

(**Figure 4A**) corresponding to isoferulic acid (**M8**). This indicates that the peaks that disappeared were glucuronides and methylglucuronides of caffeic acid, since removal of the glucuronic acid moiety resulted in the formation of the parent caffeic (**2**), ferulic (**3**), and isoferulic (**M8**) acids. The only metabolite observed after enzymatic hydrolysis with β-glucuronidase was **M2**, which had been the only peak formed after in vitro sulfation of caffeic acid, suggesting that it was a sulfated conjugate. Hydrolysis with sulfatase yielded no conclusive results as to the putative nature of peak M2 (data not shown). Similarly, although only peaks M6 and M7 were formed after in vitro glucuronidation of ferulic acid (**Figure 4C**), these two peaks along with metabolite **M5** in the culture medium of cells treated with ferulic (**Figure 3B**) disappeared after hydrolysis with β-glucuronidase (**Figure 6B**), suggesting that these three metabolites corresponded to glucuronide derivatives of ferulic acid. None of these peaks were modified after hydrolysis with sulfatase (data not shown), indicating that no sulfated metabolites of ferulic acid were formed by HepG2 cells. Finally, when culture media from cells incubated with chlorogenic acid were hydrolyzed with β-glucuronidase, peaks 1a,b remained unaltered indicating the absence of glucuronide groups in those metabolites (**Figure 6C**).

Culture media from incubations of HepG2 cells for 18 h with hydroxycinnamic acids were analyzed by LC-MS to confirm the identity of different metabolites on the basis of *m*/*z* values of molecular ions and fragment ions. These results together with the UV/vis spectroscopic data are given in **Table 1**.

Metabolites **M1** and **M3** showed a $[M - H]^-$ ion with *m*/*z* 355, characteristic of a monoglucuronidated metabolite of caffeic acid. A fragment ion with *m*/*z* 179 corresponding to the deprotonated parent molecule (caffeic acid) after loss of the glucuronic acid moiety (176 amu) was also observed. These data, along with those from the in vitro conjugation and enzymatic hydrolysis experiments, confirmed that these metabolites corresponded to the monoglucuronides of caffeic acid, although we could not distinguish between structural isomers (4-hydroxy-3-glucuronide cinnamic acid or 4-glucuronide-3-hydroxy cinnamic acid). Peak M4, that disappeared from the culture medium when hydrolyzed with β-glucuronidase, showed the molecular ion $[M - H]^-$ with *m*/*z* 369 and fragment ions of *m*/*z* 193 and 179. These ions corresponded to the methylglucuronide, the monomethyl derivative (after the loss of glucuronic acid), and the deprotonated parent molecule caffeic acid (after a further loss of the methyl group), respectively. Metabolite **M2** had a $[M - H]^-$ ion of *m*/*z* 259, characteristic of a sulfated metabolite of caffeic acid, and a small fragment with *m*/*z* 179 (corresponding to caffeic acid after the loss of sulfate). Ferulic acid (**3**) and metabolite **M8** only had molecular and fragment ions with *m*/*z* 193 and 179, corresponding to methyl caffeic and free caffeic, respectively. Thus, metabolite **M8** was confirmed as isoferulic acid, which together with ferulic acid resulted from the methylation of caffeic acid by HepG2 cells.

Concerning the ferulic acid metabolites **M6** and **M7**, they were confirmed as the glucuronidated conjugates on the basis of their molecular ions of *m*/*z* 369 and fragment ions with *m*/*z* 193, corresponding to deprotonated ferulic acid after the loss of dehydrated glucuronic acid (**Table 1**). This is in agreement with the results obtained from the in vitro conjugation and enzymatic hydrolysis experiments. Although metabolite **M5** disappeared after enzymatic hydrolysis with β-glucuronidase (**Figure 6B**), it did not show any characteristic fragment corresponding to a glucuronidated derivative of ferulic acid when the sample was analyzed by LC-MS, and thus, it could only be suggested as a glucuronide of ferulic acid. Recently, Zhang et al. (*29*) identified two monoglucuronide conjugates of ferulic acid, together with a diglucuronide conjugate, in urine from rats administered ferulic acid.

Finally, chromatographic peaks 1a,b, with spectral characteristics identical with those of chlorogenic acid, presented a molecular ion $[M - H]^-$ with *m*/*z* 353. Therefore, these compounds may correspond to the 3-*O*-caffeoyl quinic acid and 4-*O*-caffeoyl quinic acid isomers of chlorogenic acid, although no further attempts to confirm their identity were done since they did not appear to be formed as a consequence of chlorogenic acid cell metabolism.

Quantification of Metabolites. Free and conjugated metabolites were quantified in the extracellular culture medium and in the cell lysates after 2 and 18 h of incubation with HepG2 cells (**Table 2**). Differences occurred in the extent of metabolism of hydroxycinnamic acids (caffeic and ferulic acids) with regard to the hydroxycinnamate assayed (chlorogenic acid).

Caffeic and ferulic acids showed a similar behavior, with 97% of the free molecules remaining intact in the culture medium after 2 h. Methyl conjugates (2.3% of the total phenols in the extracellular medium) were the main metabolites obtained from caffeic acid along with trace amounts of monoglucuronides, methylglucuronides, and sulfated derivatives. Glucuronidated conjugates were the only metabolites identified (3.0%) when ferulic acid was assayed. In contrast, no metabolism of chlorogenic acid would have taken place after 2 h, since only the isomers could be detected in the culture medium.

At 18 h of incubation, caffeic acid metabolites exceeded 35% of the analyzed phenols, with about 64% of nonmetabolized caffeic acid detected in the culture medium. Methylation was

Table 2. Concentrations of Free Molecules and Their Metabolites in the Extracellular Culture Media and in Cell Lysates after 2 and 18 h of Incubation of HepG2 Cells in the Presence of 100 μmol/L Chlorogenic (**1**), Caffeic (**2**), and Ferulic (**3**) Acids[a]

extracellular culture media		2 h		18 h	
acid	type	μmol/L	%	μmol/L	%
caffeic acid	free	94.2 ± 0.4	97.7	61.0 ± 0.3	63.8
	glucuronidated	traces		4.64 ± 0.03	4.8
	methylglucuronidated	traces		3.1 ± 0.1	3.2
	methylated	2.2 ± 0.3	2.3	19.6 ± 0.3	20.5
	sulfated	traces		7.3 ± 0.1	7.6
	total	96.4 ± 0.2	100.0	95.6 ± 0.2	100.0
ferulic acid	free	95.8 ± 0.3	97.0	68.9 ± 0.1	73.0
	glucuronidated	2.95 ± 0.01	3.0	25.5 ± 0.1	27.0
	total	98.8 ± 0.1	100.0	94.4 ± 0.1	100.0
chlorogenic acid	free	93.4 ± 0.1	94.4	73.5 ± 0.7	75.5
	isomers	5.5 ± 0.1	5.6	23.9 ± 0.3	24.5
	total	98.9 ± 0.2	100.0	97.4 ± 0.2	100.0

cells lysates		2 h		18 h	
acid	type	μmol/L	%	μmol/L	%
caffeic acid	free	0.015	15.4	traces	
	glucuronidated	0.067	66.9	0.059	60.8
	methylglucuronidated	traces		traces	
	methylated	0.018	17.7	0.038	39.2
	sulfated	traces		traces	
	total	0.100	100.0	0.097	100.0
ferulic acid	free	0.415	96.4	0.221	82.9
	glucuronidated	0.016	3.6	0.045	17.1
	total	0.431	100.0	0.266	100.0
chlorogenic acid	free	0.152	100.0	0.091	83.5
	isomers	traces		0.018	16.5
	total	0.152	100.0	0.109	100.0

[a] Values are the mean ± SEM, $n = 3$.

the main metabolic transformation undergone by this compound, doubling the extent of sulfation plus glucuronidation. Ferulic acid appeared to be more slowly taken up and metabolized (only to glucuronidated conjugates) than caffeic acid, since 73% of the free molecule was detected in the medium after 18 h. Finally, isomerization of chlorogenic acid was more pronounced after 18 h, since only 74% of the acid remained after 18 h in comparison with 94% after 2 h. On the other hand, considering that isomerization of chlorogenic acid in cell-free culture medium accounted for 17% of the initial amount of the acid, the higher extent of isomerization (24%) in the presence of HepG2 would suggest further contribution of the hepatoma cells to isomerization of chlorogenic acid in vitro.

The content of hydroxycinnamic acids and their metabolites was also measured in cell lysates. Total intracellular accumulation of compounds and their metabolites was minimal after 2 h of incubation, with 0.10, 0.15, and 0.43% of the amount of caffeic, chlorogenic, and ferulic acids initially added, respectively (**Table 2**). The intracellular concentrations of ferulic acid (0.27%) and chlorogenic acid (0.11%) were even lower after 18 h. Contrary to what was observed in the culture medium, the main intracellular metabolites of caffeic acid were glucuronide conjugates, especially at short incubation times (2 h), although at longer times intracellular levels of methylated derivatives increased. Since the methyl conjugates prevailed in the culture medium at both incubation times, these results might suggest a faster efflux of methylated derivatives into the extracellular medium. Free caffeic acid concentrations in the cell lysates were always low, with only trace amounts after 18 h of incubation, indicating rapid metabolism of the free molecule. On the contrary, free nonmetabolized ferulic acid was the compound predominantly accumulated within the cells after incubation with this phenol. Concentrations of ferulic acid and its glucuronides were always higher than the total amount of caffeic acid and its metabolites (0.43 vs 0.10 μmol/L, respectively, after 2 h), suggesting a more extensive uptake of this methylated derivative of caffeic acid as compared to the free molecule (**Table 2**). Although chlorogenic acid showed a negligible metabolism by HepG2, intracellular accumulation was observed with concentrations of 0.15 and 0.11 μmol/L after 2 and 18 h of incubation, respectively, showing that chlorogenic acid can be absorbed intact by the cells. Isomers of chlorogenic acid were also detected in the cell lysates, which might have been formed intracellularly or taken up by the cells from the culture medium.

DISCUSSION

The results from this study on the uptake and metabolism by human hepatoma cells of common dietary hydroxycinnamic acids showed that structural differences among the three studied compounds brought about important variations in the type of metabolites formed by HepG2 cells as a model system of the human liver. Caffeic acid gave rise to a wide range of metabolites including methylated, methylglucuronidated, glucuronidated, and sulfated conjugates, while its methyl derivative ferulic acid was only glucuronidated by hepatoma cells. This suggests that phase II metabolism of hydroxycinnamic acids like caffeic and ferulic acids takes place in the liver. In contrast, esterification of caffeic with quinic acid to form chlorogenic acid completely prevented its conjugation, although transformation into structural isomers took place. Also, differences in intracellular concentrations of the three compounds were observed, with higher values for ferulic than for caffeic and chlorogenic acids, which may reflect either higher cellular uptake of ferulic acid or lower efflux to the culture medium of the absorbed phenol.

To our better knowledge, this is the first work reporting on the metabolism of hydroxycinnamic acids by human hepatic cells. Moridani et al. (*30*) studied the metabolism of caffeic, chlorogenic, and dihydrocaffeic acids by rat hepatocytes and isolated microsomal fractions, showing that caffeic and dihydrocaffeic acids (but not chlorogenic acid) underwent *O*-methylation and glutathione conjugation, as well as hydrogenation and dehydrogenation catalyzed by phase I and phase II metabolizing enzymes. Using Caco-2 cells, Kern et al. (*31*) showed the capacity of these intestinal cells to deesterify and conjugate (through *O*-methylation, glucuronidation, and sulfation) different hydroxycinnamic acids in reactions involving also phase I (intracellular and extracellular esterases) and phase II enzymes.

The HepG2 cell line is widely used to study the biotransformation of different drugs as a model system of the human liver (*24*). Genotyping of phase I and phase II enzymes and drug transporter polymorfisms in these cells confirmed HepG2 as a suitable model for metabolic studies (*32*). Only low levels of sulfotransferase and *N*-acetyltransferase were reported in this cell line, yet they were still high enough to allow metabolic assays by these enzymes.

In our study, sulfated metabolites were only detected after incubation of caffeic acid with HepG2 for 18 h and no sulfation of ferulic acid was detected, in spite of sulfated and sulfoglucuronidated conjugates being the major metabolites identified in rat urine and plasma after intake of ferulic acid (*19*, *33*−*35*). The limited sulfation by HepG2 cells in comparison with the in vivo animal data might question the suitability of this cell line for the metabolic studies aimed at this work. However, in

vitro treatment with rat liver microsomes also failed to sulfate ferulic acid and only sulfated caffeic acid was observed, reproducing the sulfation pattern after incubation with HepG2 cells. Moreover, in human studies ferulic acid was found only conjugated with glucuronic acid or glycine (*20*), confirming limited sulfation of ferulic acid in humans. On the other hand, the main metabolites formed after incubation of Caco-2 cell monolayers with different hydroxycinnamates were sulfoconjugates, suggesting that metabolic transformation of these compounds was predominant in the intestinal epithelium, with excretion of the conjugated forms to the apical (luminal) side of the cells (*31*).

Methylation was the preferential pathway for caffeic acid metabolism, while ferulic acid, lacking an *ortho*-diphenolic structure, could not by methylated by COMT. In consequence, only glucuronidated conjugates of ferulic acid were formed by this model system of the human liver. Glucuronidation of caffeic acid was also an important metabolic transformation of this phenol by hepatoma cells. This is in agreement with in vivo studies in humans, where glucuronide derivatives of both caffeic and ferulic acids were detected in plasma or urine, probably as products from hepatic metabolism (*10*, *14*, *16*, *18*, *36*). However, the major caffeic acid metabolites in humans and animals were ferulic and isoferulic acids, which agrees with our in vitro results showing methylated conjugates as the main caffeic acid metabolites.

These results show effective hepatic metabolism of ferulic and caffeic acids, yet with high amounts of nonmetabolized hydroxycinnamic acids in the culture medium, probably due to limited uptake of these compounds by the cell. Cellular uptake of cinnamic acids by intestinal mucosa has been shown to take place by Na^+-dependent saturable transport and also by nonsaturable diffusive mechanisms (*37*, *38*). Caffeic and ferulic acids have been suggested to enter the cell via active transport by the monocarboxylic acid transporter of epithelial cells (*39*−*41*), which would also mediate their efflux out of the cell, although paracellular diffusive mechanisms also operate for absorption (*39*, *40*). Gastric absorption of caffeic acid would take place by passive diffusion, while active transport would predominate in the small intestine (*7*). Free ferulic acid would be quickly absorbed from the stomach and then conjugated mainly in the liver (*19*). For passive diffusion mechanisms, a lower degree of hydroxylation of ferulic acid in comparison to caffeic acid (**Figure 1**) should facilitate diffusion through the lipid bilayer in cell membranes, minimizing interactions between the phenol hydroxyl groups and polar groups of membrane lipids (*42*). Actually, higher intracellular concentrations of ferulic acid were observed in the cell lysates (**Table 2**). However, the high concentration of nonmetabolized ferulic acid in the culture medium in comparison to caffeic acid seems to indicate lesser metabolism of ferulic acid by HepG2.

As for chlorogenic acid, no conjugation or hydrolysis to caffeic acid took place even after 18 h of incubation, indicating negligible metabolism of this caffeoyl quinic acid. Only isomeric forms were detected after treatment of hepatoma cells; even though these isomers were also formed spontaneously in cell-free culture medium, a higher extent of isomerization of chlorogenic acid in the presence of HepG2 cells would not rule out some influence of the cells on its isomerization. More importantly, chlorogenic acid was unequivocally detected in cell lysates. Although the extent of intracellular accumulation was very low (about 0.1−0.15% of the added compound), this indicates uptake of intact chlorogenic acid by the cells. Isomers were also detected in the cell lysates, yet it cannot be established whether these compounds were formed intracellularly or were taken up by the cells from isomers in the culture medium.

Several authors failed to detect chlorogenic acid in either plasma or urine of rats and humans fed pure chlorogenic acid or chlorogenic acid containing foods (*10*, *11*, *14*, *17*). However, in agreement with our results, other authors detected traces of chlorogenic acid in urine (*7*, *12*). Low absorption of chlorogenic acid in comparison with caffeic acid was demonstrated in a perfusion experiment with rat jejunum (*43*), while in an ileostomy study a gastrointestinal absorption of only 33% of this acid was calculated (*7*). More recently, Lafay et al. (*13*) clearly showed a fast absorption of native chlorogenic acid from the rat stomach. Therefore, the low urinary recovery of chlorogenic acid reported by different authors could be explained by its hydrolysis in the body. The rapid detection of caffeic and ferulic acid after the ingestion of chlorogenic acid by rats or humans (*10*, *11*), and the absence of degradation of chlorogenic acid in the upper intestinal tract (*7*, *11*) also support the hypothesis of hydrolysis by inner tissues after absorption. Mucosal esterases (intra- or extracellular) together with microbial esterases from the colonic bacteria would be responsible for hydrolysis of chlorogenic acid to caffeic and quinic acids (*31*, *39*, *40*, *44*). Thus, chlorogenic acid that is not absorbed in the upper gastrointestinal tract, together with the absorbed acid excreted in the bile, would reach the large intestine where it may be hydrolyzed by the microflora. Caffeic and quinic acids may be further metabolized to 3,4-dihydroxyphenylpropionic, 3-hydroxyphenylpropionic, 3-hydroxybenzoic, *m*-coumaric, hippuric, and 3-hydroxyhippuric acids (*8*, *12*, *39*, *40*).

In summary, moderate uptake and metabolism of caffeic and ferulic acids were observed using HepG2 cells. Caffeic acid underwent methylation, glucuronidation, and sulfation, while ferulic acid was only glucuronidated. Conversely, chlorogenic acid showed null metabolism but effective, although limited, uptake by this cell line as a model system of the human liver.

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