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Sulfated Ferulic Acid Is the Main in Vivo Metabolite Found after Short-Term Ingestion of Free Ferulic Acid in Rats

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The bioavailability of ferulic acid (FA; 3-methoxy-4-hydroxycinnamic acid) and its metabolites was investigated in rat plasma and urine after an oral short-term ingestion of 5.15 mg/kg of FA. Free FA, glucuronoconjugates, and sulfoconjugates were quickly detected in plasma with a peak of concentration found 30 min after ingestion. Sulfoconjugates were the main derivates (∼50%). In urine, the cumulative excretion of total metabolites reached a plateau 1.5 h after ingestion, and ∼40% were excreted by this way. Free FA recovered in urine represented only 4.9 ±1.5% of the native FA consumed by rats. Glucuronoconjugates and sulfoconjugates represented 0.5 ± 0.3 and 32.7 ± 7.3%, respectively. These results suggested that a part of FA incorporated in the diet was quickly absorbed and largely metabolized in sulfoconjugates before excretion in urine.

KEYWORDS: Hydroxycinnamic acid; ferulic acid; bioavailability; absorption; metabolites; urine; plasma; rat; pharmacokinetic

INTRODUCTION

Phenolics are well-known secondary plant products showing a wide range of interest. They are reducing agents and thus exhibit an antioxidant effect, which depends on their structure and on the medium in which they act (*1*−*3*). Some epidemiological studies have demonstrated that the incidence of coronary heart disease was inversely proportional to flavonoid intake (*4*). Moreover, the Mediterranean diet, which consists on a high consumption of fruits, vegetables, and fish, associated with a low consumption of saturated fats, is related to a lower mortality by cardiovascular diseases. In France, the incidence of heart diseases is also lower than in other countries despite a high consumption of saturated fat (*5*). This phenomenon, also called the "French paradox", is attributed to a moderate but regular intake of red wine rich in polyphenols. Other studies involve the consumption of tea and apples in the decrease of breast and lung cancers (*6*, *7*).

However, the bioavailability of phenolics and their active forms in the human metabolism remains unclear. Moreover, most of the studies deal with flavonoids, and only a few results are available on phenolic acids (i.e., hydroxycinnamic acids) despite their large occurrence in cereals, vegetables, and beverages. Some data have been obtained either from the consumption of fruits (i.e., prunes and tomatoes) by a small number of human subjects (*8*, *9*) or by the intake of pure molecules by rats (*10*). Animal experiments gave more information on the metabolic forms because they deal with only a single compound. Unfortunately, the amounts administered were generally higher than the usual hydroxycinnamate uptake and may have modified the metabolic pathway.

Thus, the present work was performed to determine the pharmacokinetic profiles of ferulic acid (FA) and its metabolites in the plasma and urine of rats, after a single ingestion of a small quantity of FA incorporated in a complete standard food. The analysis was performed using a specific and sensitive detection: high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD).

MATERIALS AND METHODS

Chemicals. Ferulic acid, sulfatase (EC 3.1.6.1) type H-1 from *Helix pomatia*, *β*-glucuronidase (EC 3.2.1.31) type IX-A from *Escherichia coli*, sodium acetate trihydrate, and ethyl acetate (Chromasolv) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Acetone (Spectronorm grade), hydrochloric acid 37%, and anhydrous sodium sulfate were from Prolabo (Fontenay sous bois, France). Acetonitrile and methanol (HPLC grade) were purchased from Carlo Erba (Val de Reuil, France). Sodium dihydrogenophosphate dihydrate, disodium hydrogenophosphate dihydrate, glacial acetic acid (HPLC grade), and white wipers (Wypall L25) were from Fisher Scientific (Elancourt, France). Water was double distilled with an Elix 3 system (Millipore).

Animals and Diets. Sixty-six male Sprague-Dawley rats were housed in individual cages in a temperature-controlled room (22 ± 1 °C) with a 12 h inverse light/dark cycle: day was from 9:00 p.m. to 9:00 a.m. and night from 9:00 a.m. to 9:00 p.m.

Animals were fed with a complete laboratory diet (P14) prepared at INRA (Jouy-en-Josas, France) (**Table 1**). Three grams of P14 powder mixed with water (1:1, w/w) was supplied between 9:00 a.m. and 9:30 a.m., and the mixture was available ad libitum between 11:00 a.m. and 6:00 p.m. for 10 days before experiments. On the day of sacrifice, rats were divided into two groups. The control group (average weight = 338 ± 5 g) received the standard diet, whereas the second group

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Table 1. Composition of the Standard Complete P14 Powder

ingredient	wt (g/kg)	ingredient	wt (g/kg)
milk proteins	140	vitamins	10
starch	623	cellulose	50
saccharose	100	choline	2
soy oil	40		
mineral salts	35	total	1000

(average weight = 329 ± 5 g) was fed with the same diet supplemented with FA, to give to each rat 5.15 mg/kg of body weight of ferulic acid. This second diet was prepared by dissolving FA in water (1.121 g/L) and by mixing this solution with P14 powder (1:1, w/w). Six rats were sacrificed before the meal, and six rats of each group were sacrificed 30 min and 1, 1.5, 2.5, and 4.5 h after the meal. The rats were deeply anesthetized with an overdose of sodium pentobarbital (48 mg/kg) and exsanguinated by section of the abdominal aorta and vena cava. Blood was collected into heparinized tubes and centrifuged at 3000*g* for 20 min at 4 °C. Plasma was then aliquoted and frozen at −30 °C until analysis. To collect urine, the bladder was clamped, pierced, and rinsed with distilled water. The urine was directly collected into tubes and frozen at −30 °C. To recover the urine excreted during the time between the meal and sacrifice, cages were underlaid with two sheets of wipers, which were frozen at −30 °C after the experiment.

Sample Preparation. Samples were enzymatically hydrolyzed to identify glucuronidated, sulfated, and sulfoglucuronidated metabolites of ferulic acid in plasma and urine according to procedures described in many papers (*8*, *10*−*12*).

Plasma. Free FA and conjugated FA were titrated in 100 μL of plasma incubated at 37 °C in a water bath for 2 h with 20 μL of sulfatase type H-1 solution in 1 M acetate buffer, pH 4.9, containing 2.5×10^5 units/L of sulfatase and 7.5×10^6 units/L of β-glucuronidase. After incubation, the plasma was precipitated with 250 μL of acetone and centrifuged at 800*g* during 10 min at 4 °C with a Jouan CT 422 centrifuge. The supernatant was filtered on a 0.2 μm filter (Gelman) and analyzed by HPLC. For the determination of free FA, a set of samples was incubated without enzyme and treated as previously described. Sulfates without glucuronidation were determined on a sample treated with 20 μL of sulfatase type H-1 solution in 1 M acetate buffer, pH 4.9, in the presence of 26 mM D-saccharic acid 1,4-lactone (*13*) and treated as previously described.

The determination of ferulic acid glucuronides was realized by incubating at 37°C for 2 h 100 μL of plasma with 20 μL of 0.2 M phosphate buffer, pH 6.8, containing 7.5×10^6 units/L β-glucuronidase type IX-A. If β-glucuronidase type IX-A is a pure enzyme, it is not the case of sulfatase type H-1 from Sigma, which also contains β-glucuronidase activity. It was, however, preferred to a pure sulfatase due to its high cost and the large number of experiments. Moreover, according to Hackett and Griffiths (*13*), the activity of β-glucuronidase can be totally inhibited by performing the enzymatic reaction in the presence of D-saccharic acid 1,4-lactone, in the conditions previously described. Such an experiment allows one to quantify specifically the sulfated metabolites. Calculations gave the proportion of each metabolite.

Urine. Ninety microliters of bladder urine was incubated for 2 h at 37 °C, either with 20 μL of 250 mM acetate buffer, pH 4.9, containing or not sulfatase type H-1 (2.5×10^5 units/L sulfatase and 7.5×10^6 units/L β-glucuronidase) or with 20 μL of 250 mM acetate buffer, pH 6.8, containing or not 7.5×10^6 units/L β-glucuronidase type IX-A. After incubation, the urine was acidified with 10 μL of hydrochloric acid 3.7% and extracted twice with 1 mL of ethyl acetate. The organic phases were combined, evaporated to dryness under nitrogen, and then redissolved in 400 μL of methanol and filtered on a 0.2 μm filter for HPLC analysis.

The excreted urine, absorbed on paper sheets, was treated as follows: sheets were cut in pieces of 1 cm^2 and mixed with 250 mL of water in a stoppered conical flask under orbital shaking at 140 rpm in a shaking water bath (Grant model OLS 200) for 30 min at 37 °C. The extract was then filtered through a glass frit, and the conical flask was rinsed with 20 mL of water. A 50 mL aliquot was incubated for 2 h at 37°C in the shaker water bath with 50 mL of 250 mM acetate buffer, pH 4.9, containing or not sulfatase type H-1 (2.5×10^5 units/L sulfatase and 7.5×10^6 units/L β-glucuronidase). The solution was acidified with HCl 37% to pH 1 and extracted twice with 100 mL of ethyl acetate. The ethyl acetate phases were combined, dehydrated with anhydrous sodium sulfate, and filtered through a Gelman No. 1 filter. The filtrate was evaporated to dryness at 50 °C under vacuum. The residue was dissolved with 1 mL of methanol and filtered on a 0.2 μm filter for HPLC analysis.

HPLC Analysis. *Identification of cis and trans Isomers of FA.* *cis*- and *trans*-ferulic acids were followed using an HPLC 600 E Waters system and an ESA Coulochem II coulometric detector equipped with a guard cell model 5021 and an analytical cell model 5010 with two working electrodes. The electrochemical settings were as follows: guard channel potential, −250 mV; channel 1 (working electrode 1) potential, 0 mV, and sensitivity, 1 μA; channel 2 (working electrode 2) potential, 450 mV, and sensitivity, from 1 to 20 μA. Aliquots of 20 μL were injected along a C_{18} Interchrom column (250 × 4.6 mm i.d.; 10 μm particle size) (Interchim). The mobile phase was acetonitrile/methanol/sodium acetate buffer 50 mM, pH 4.8 (10/10/80, v/v/v) at a flow rate of 0.8 mL/min.

trans-Ferulic acid has been identified by comparing its retention time and its electrochemical behavior with those of a commercial standard. The retention time of this compound was 17 min with a maximal electrochemical response at 450 mV. *cis*-Ferulic acid was identified by comparison with the *cis* isomer prepared by photoisomerization according to the procedure of Maillard and Berset (*14*): its retention time is 9 min, its UV spectrum shows a maximum at 268 nm and a shoulder at 306 nm, its electrochemical behavior is identical to that of *trans*-ferulic acid,and its mass spectrum shows a molecular peak $(M - H)^-$ at *m*/*z* 193. This last result was obtained by an HPLC system (Perkin-Elmer 200LC) coupled to an Applied Biosystems API 100 mass detector by a turbospray interface; the separation of *cis*- and *trans*-ferulic acids was realized on a C18 symmetry column (100 × 2.1 mm i.d.; 3.5 μm particle size) (Waters) with a mixture of methanol/water 40/60 (v/v) at a flow rate of 0.2 mL/min.

Quantification of FA in Plasma. Ferulic acid solutions were prepared at different concentrations in acetone. Two hundred and fifty microliters of each solution was added to 100 μL of blank plasma. The calibration curve was linear between 0 and 2×10^{-4} mol/L ($R^2 = 0.995$).

Quantification of FA in Urine. A linear calibration curve was established from standard solutions of *trans*-ferulic acid in methanol, in the range of $0-2.5 \times 10^{-4}$ mol/L ($R^2 = 0.997$).

The extraction yield of FA in bladder urine was determined on a blank urine containing *trans*-ferulic acid at a final concentration of 1×10^{-4} mol/L (three replicates) and submitted to the different extraction procedures. Yields were, respectively, 99.4 ± 0.3% in the experiment without enzyme at pH 6.8 and 94.3 ± 4.2% at pH 4.9, 90.9 ± 6.3% in the experiment with β-glucuronidase, and 94.5 ± 0.7% in the experiment with sulfatase.

The recovery of FA from the paper sheets was determined as follows: 5 mL of *trans*-ferulic acid, 1.5×10^{-3} mol/L in water, was poured on two sheets of absorbent paper, dried for 4 h at ambient temperature, and frozen at −30 °C. Ferulic acid was then extracted as previously described. The yield was 75.7 ± 2.5% (average of three assays).

Data Evaluation and Statistics. The concentrations of the different metabolites of FA in plasma and urine were calculated by comparing the enzymatically hydrolyzed samples to the nonhydrolyzed samples as described below:

$$\text{glucuronidated FA} = \text{FA after } \beta\text{-glucuronidase treatment} - \text{free FA} \quad (1)$$

$$(\text{sulfated} + \text{sulfoglucuronidated})\ \text{FA} = \text{FA after sulfatase}/\beta\text{-glucuronidase treatment} - \text{glucuronidated FA} \quad (2)$$

$$\text{sulfated FA} = \text{FA after sulfatase}/\beta\text{-glucuronidase treatment in the presence of saccharic acid 1,4-lactone} - \text{free FA} \quad (3)$$

$$\text{sulfoglucuronidated FA} = (\text{sulfated} + \text{sulfoglucuronidated})\ \text{FA} - \text{sulfated FA} \quad (4)$$

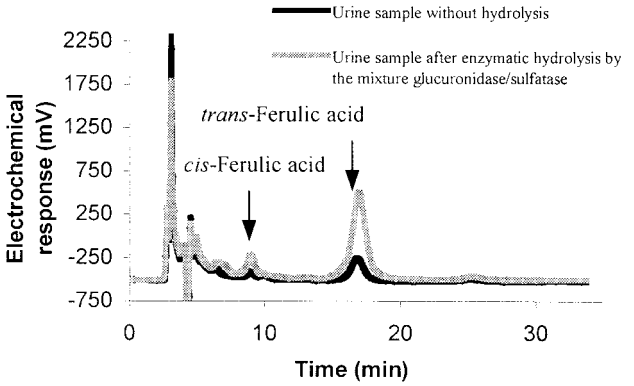

Figure 1. HPLC chromatograms of bladder urine samples treated or not by the mixture β-glucuronidase/sulfatase, obtained by HPLC coupled to an electrochemical detector. Chromatographic conditions: C_{18} Interchrom column (250 × 4.6 mm i.d.; 10 μm particle size); mobile phase, acetonitrile/methanol/sodium acetate buffer, 50 mM, pH 4.8 (10/10/80, v/v/v); flow rate, 0.8 mL/min; Electrochemical settings: guard channel, −250 mV; channel 1, 0 mV, 1 μA (sensitivity); channel 2, 450 mV, 20 μA.

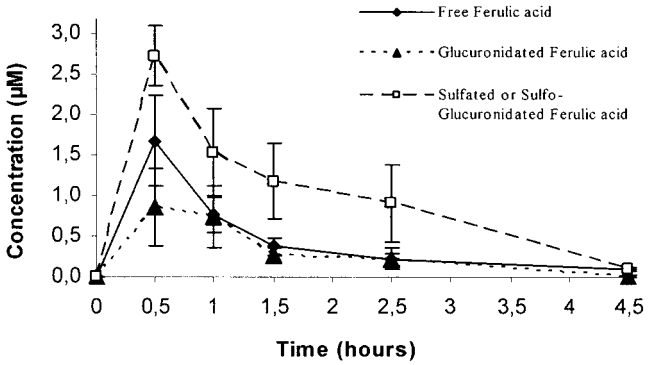

Figure 2. Concentration of free ferulic acid and metabolites in rat plasma after an oral short-term ingestion of FA at a concentration of 5.15 mg/kg of body weight. (Values are mean ± SEM, $n = 6$.)

Results are expressed as mean ± SEM. Kinetic studies involved six rats per time. Data were analyzed for the statistical significance using the mathematical derivate of the Student *t* test or the Kruskal−Wallis test (xlstat 4.4, shareware version).

The extraction of free FA in plasma and urine was performed under two conditions: pH 6.8 (optimal pH for β-glucuronidase) and pH 4.9 (optimal pH for the mixture of β-glucuronidase/sulfatase). No significant difference ($p < 0.001$) was observed between these two pH conditions in the plasma or urine assay.

The purification procedure of urine, according to classical procedures used for the analysis of polyphenolic metabolites (*12*), conducted to a high recovery (>90%) of FA with a good repeatability (variation coefficient < 7%) and the electrochemical method allowed the detection of traces (3×10^{-7} mol/L) of FA and metabolites. Nevertheless, some isomerization of *trans*-ferulic acid in *cis*-ferulic acid may occur along the procedure as shown in **Figure 1**.

RESULTS

Plasma Level of FA and Metabolites. No FA or conjugated forms were detected in plasma of the control group. In the supplemented one, the plasmatic concentration of FA reached a maximum 30 min after ingestion and decreased quickly between 0.5 and 1.5 h and then more slowly until 4.5 h (**Figure 2**). At this time, the concentration was lower than 0.2 μM, a value corresponding to the detection limit of the method. The different metabolic forms followed similar kinetics. The maximum concentrations, obtained 30 min after ingestion of the diet supplemented in FA, were 1.68 ± 0.55 μM for free FA, 0.87 ± 0.48 μM for glucuronidated FA, and 2.73 ± 0.37 μM for sulfated plus sulfoglucuronidated FA. At 0.5 h, the difference between free FA and glucuronidated FA was not significant (Kruskal−Wallis test, $p < 0.05$), and the ratio of the different chemical forms remained constant between 0.5 and 2.5 h: sulfated metabolites were the main forms (58% of the total FA forms), followed by free FA (24%) and glucuronidated metabolites (18%) (**Table 2**).

Table 2. Ratio of Ferulic Acid Metabolites in Plasma, Urine from Bladder, and Excreted Urine

metabolite	plasma (0.5 h after meal, %)	urine from bladder (2.5 h after meal, %)	excreted urine (4.5 h after meal, %)
glucuronidated FA	18	3	2
free FA	24	11	14
sulfated or sulfo-glucuronidated FA	58	86	84

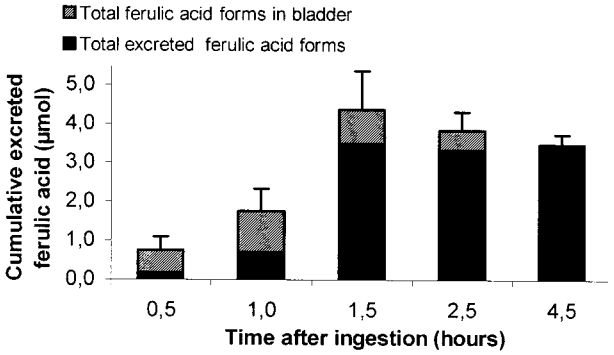

Figure 3. Cumulative excretion of free ferulic acid and metabolites after an oral short-term ingestion of FA at a concentration of 5.15 mg/kg of body weight. (Values are mean ± SEM, $n = 6$.)

Urinary Excretion of FA and Metabolites. Hydrolysis by β-glucuronidase and sulfatase was conducted on excreted and bladder urine in order to follow the excretion kinetics of FA in rat. A plateau was reached 1.5 h after ingestion (**Figure 3**). The level of total FA forms was logically more important in excreted urine than in bladder urine for higher times (≥1.5 h). Thus, in total urine, 43.4 ± 4.6% of the ingested dose of FA was recovered. The analysis of the different forms showed that sulfates and sulfoglucuronidates were the main forms in both bladder and excreted urine followed by free FA and glucuronidates (**Table 2**). However, if the ratio of free FA was constant ($p < 0.001$) whatever the time after meal, the ratio of glucuronidates decreased with time to profit by sulfates and sulfoglucuronidates (**Figure 4**). Moreover, only a slight part (~8.5%) of the sulfated forms was also glucuronidated, considering that the inhibition of β-glucuronidase by saccharic acid 1,4-lactone was total.

It must be noted that an isomerization of *trans*-FA to *cis*-FA occurred in urine samples, during the extraction procedure, whereas this isomerization was negligible in plasma samples: the isomerization rates were 17 ± 1% for the bladder urine samples and 44 ± 5% for the extracted urine samples. The high level of isomerization for the excreted urine is probably due to the supplementary evaporation step of ethyl acetate under vacuum at 50 °C. Therefore, all of the concentrations of FA in urine and plasma were expressed as the sum of *cis*- and *trans*-ferulic acid concentrations, these two isomers showing the same behavior in the coulometric detection method.

The recovery of FA was thus >90%, which is higher than previous results shown in the literature (*8*).

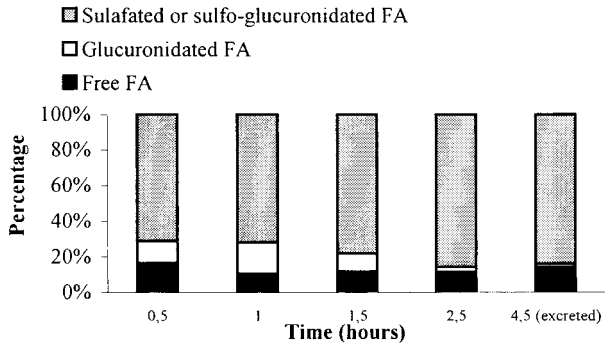

Figure 4. Ratio of free ferulic acid and its metabolites recovered in bladder urine between 0.5 and 2.5 h and in excreted urine 4.5 h after the meal ($n = 6$).

DISCUSSION

The present study shows that a part of ferulic acid is recovered in blood circulation after a short-term ingestion and mainly circulates as metabolized forms. Sulfated and glucuronidated derivatives represent 76% of total FA, whereas the native form achieves a maximum of 24%. Such metabolic pathways have already been found as well for caffeic acid (*9*, *10*). Glucuronidation and sulfatation are well-known ways of detoxification, which increases the polarity of compounds (*15*). However, our results are quantitatively quite different from those of Azuma et al. (*10*) on caffeic acid. These authors find a maximum concentration of caffeic acid and metabolites in plasma 2 h after administration, and the native form of caffeic acid represents only 2%. Such differences could be explained as well by the structure of caffeic acid, which differs from FA by the presence of a phenolic group in the 3-position instead of a methyl group, and by the higher quantities of compound ingested by rats (25 times higher than the dose of FA used in our experiment). In particular, the higher hydrophilic property of caffeic acid could delay its crossing through the intestinal cells. Studies on prune hydroxycinnamic acids (corresponding to 76 ± 6 mg of mainly caffeic, ferulic, and chlorogenic acids), realized on three human volunteers, have also demonstrated that the conjugated forms were the most important with regard to phenolics present in biological fluids (*8*). Therefore, it seems that similar pathways are involved in humans and rats. Indeed, the quantity of phenolic acids ingested here (~1 mg/kg of body weight) is close to quantities usually ingested by consumers. These results on hydroxycinnamic acids can be compared to bioavailability studies on flavonoids, which report that flavan-3-ols from tea and quercetin are also largely glucuronidated and sulfated in blood (*16*, *17*).

In the excreted urine, 4.5 h after the intake of FA, the proportion of glucuronidated and sulfated conjugates is higher than in blood: 84 versus 76% in plasma. This significant difference ($p < 0.001$) could come from a possible conjugation in kidney. Indeed, as shown in the bladder urine, the decrease of the ratio of glucuronidated conjugates with time is correlated to the increase of sulfoglucuronidated or sulfated forms (**Figure 4**), whereas such a phenomenon is not observed in plasma (data not shown). This result could be explained by the action of phase II enzymes such as sulfatase or β-glucuronidase, which have already been detected in kidney, but only a few studies have considered this renal pathway (*15*).

Comparison of the bladder urine and the excreted urine was impossible at 4.5 h because of the too low concentration of FA and metabolites in the bladder urine. Moreover, the ratios at 2.5 h in bladder and at 4.5 h in excreted urine are similar (**Table 2**). This results confirm the low degradation of metabolites on the paper sheet, which could occur after fecal contamination.

The urinary excretion of FA and metabolites occurs quickly, with a plateau reached only 1.5 h after ingestion. The excretion is slower in humans, with a plateau of excretion between 7 and 9 h after consumption (*9*). The free FA recovered in urine samples represents $4.9 \pm 1.5\%$ of the native FA consumed by rats. This result is very similar to the value obtained with human subjects (4−5% of free FA) over 24 h after consumption of fresh tomatoes (*9*). However, the part of glucuronidated FA is smaller: 0.5% for our study versus 11−25% for the human study. Choudhury et al. (*18*) report an excretion of free FA of $5.4 \pm 4.1\%$ of the ingested dose close to our results ($4.9 \pm 1.5\%$) and $5.1 \pm 3.6\%$ of glucuronidated FA after administration to rats by force-feeding of 50 mg/kg of FA. Thus, if the excretion of free FA in urine seems to be independent from the ingested dose (for doses varying from 0.5 to 50 mg/kg), the variability is more remarkable for the conjugated forms.

Considering that less than half of the ingested dose of FA is recovered into urine, several hypotheses can be examined. First of all, even if glucuronidation and sulfatation are the most important metabolic pathways, the presence of other metabolic forms should be considered. For example, a small quantity of FA has been detected after oral administration of caffeic acid to rats (*10*), demonstrating the existence of methylation reactions (*19*). Then, FA could be eliminated by other excretion ways or could remain unabsorbed. Crespy et al. (*11*) have shown that quercetin was glucuronidated in enterocytes and then largely released in the lumen of the small intestine. Forty-two percent of ^{14}C was recovered in the biliary duct after a single injection of [U-^{14}C]catechin in the tail vein (*20*). Chlorogenic acid was only slightly absorbed by the intestine of ileostomic humans, and the main part was excreted in the feces (*21*). At least, an accumulation of FA or metabolites could occur in many tissues (lung, heart, liver, genital organs. etc.) as described for 3-palmitoyl-(+)-[^{14}C]-catechin (a synthetic ester of catechin), [^{3}H]-(−)-epigallocatechin gallate, and resveratrol. This case would be favorable for the promotion of a preventive antioxidant action in cells (*22*−*24*). Such antioxidant effects have already been reported in low-density lipoprotein oxidation systems for both FA and glucuronidated FA at a concentration of 20 μM (*25*).

Further studies should now be performed to complete the excretion pathways of ferulic acid and to determine the antioxidant activity of its metabolites in biological systems.

ABBREVIATIONS USED

FA, ferulic acid; HPLC-ECD, high-performance liquid chromatography coupled with electrochemical detection; SEM, standard error of the mean.

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