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The Fate of *trans*-Caftaric Acid Administered into the Rat Stomach

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trans-Caftaric acid is the most abundant nonflavonoid phenolic compound in grapes and wines. It occurs in chicory and is one of the bioactive components of *Echinacea purpurea*. In order to fill the gap of knowledge about its bioavailability in mammals, we investigated its absorption, tissue distribution, and metabolism in rats. Assuming that the stomach is a relevant site of absorption of dietary polyphenols, a solution of *trans*-caftaric acid was maintained in the ligated stomach of anaesthetized rats for 20 min. Intact *trans*-caftaric acid was detected in rat plasma at both 10 and 20 min (293 ± 45 and 334 ± 49 ng/mL, respectively), along with its O-methylated derivative *trans*-fertaric acid, whose concentration rose over time (from 92 ± 12 to 185 ± 24 ng/mL). At 20 min, both *trans*-caftaric acid and *trans*-fertaric acid were detected in the kidney (443 ± 78 and 2506 ± 514 ng/g, respectively) but not in the liver. Only *trans*-fertaric acid was found in the urine (33.3 ± 12.8 μg/mL). In some rats, *trans*-caftaric acid was detected in the brain (180 ± 20 ng/g).

KEYWORDS: ***Trans*-caftaric acid; *Vitis vinifera*; *Cichorium intybus*; *Echinacea purpurea*; gastric absorption; peripheral tissues; metabolism; rat**

INTRODUCTION

Wine is one of the most ancient beverages, produced from grape berries by a complex technological procedure, which has undergone uninterrupted development over the ages in order to fulfill the evolving demands of consumers. In recent times, wine has been recognized as a rich source of dietary polyphenols, which are being closely scrutinized due to their pleiotropic biological effects (*1*), observed up to the level of human pathophysiology (*2*−*10*). Wine polyphenols include both flavonoid and nonflavonoid compounds. Of the latter, hydroxycinnamic acids and their tartaric esters (also called hydroxycinnamates) are the main class of phenolics in white wines and the main class of nonflavonoid phenolics in red wines (*11*). In grapes and wine, hydroxycinnamic acids occur distinctively as conjugates with tartaric acid, as first reported by Ribéreau-Gayon (*12*). The main hydroxycinnamic acids in grapes and wines are caftaric acid (caffeoyltartaric acid), *p*-coutaric acid (coumaroyltartaric acid), and fertaric acid (feruloyltartaric acid) (**Figure 1**). The presence of a double bond in the lateral side leads to the existence of two isomeric forms: cis and trans. In grape, caftaric and fertaric acids are mainly found in the trans form, while *p*-coutaric acid, although occurring predominantly as trans, also shows a not negligible fraction of its cis form. Both *trans*-caftaric acid and *trans*-fertaric acid are mainly localized in the pulp, and during the grape pressing, they are quickly released into the juice. In contrast, the trans and cis isomers of *p*-coutaric acid are less extractable since they are mostly localized in the grape skin (*13*). *trans*-Caftaric acid is the predominant hydroxycinnamic acid in both grape and wine. The free forms of these acids (caffeic, *p*-coumaric acid, and ferulic) are not present in grapes, but they can be released by cinnamoyl esterases and occur, in lower concentrations, in wine. In addition to grapes and wine, *trans*-caftaric acid is one of the major phenolics in green- and white-leaf chicory, present in both fresh and cooked leaves (*14*), and is one of the bioactive constituents of *Echinacea purpurea* (L.), one of the most commonly used medicinal plants worldwide (*15*).

There is no report about the bioavailability of *trans*-caftaric acid in the literature. However, knowledge in this field is essential to consider any possible biological activity of *trans*-caftaric acid in animal cells. The bioavailability and metabolism of structurally similar compounds such as chlorogenic acid (*trans*-5′-caffeoylquinic acid) (**Figure 1**) and neochlorogenic acid (*trans*-3′-caffeoylquinic acid) were studied in humans (*16*−*19*) and in rats (*20*, *21*). It was reported that chlorogenic acid

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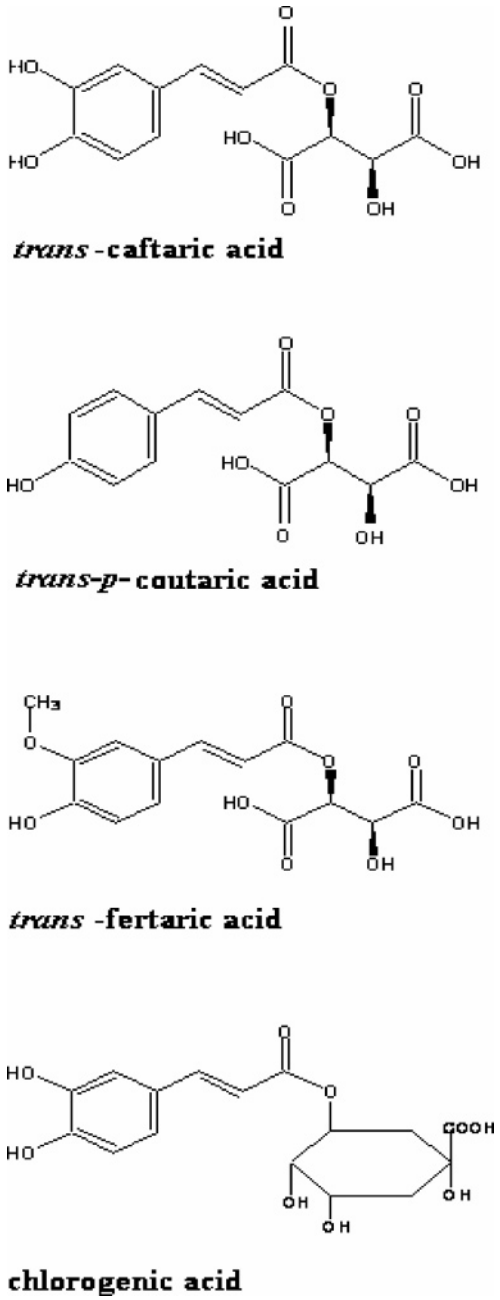

Figure 1. Structures of *trans*-caftaric acid, *trans*-*p*-coutaric acid, *trans*-fertaric acid, and chlorogenic acid.

was quickly taken up from the stomach of rats in its intact form (*22*); as it reached the intestine, it was intensively metabolized by the gut microflora (*20*). However, some structural differences between the tartaric and the quinic esters could possibly induce a different metabolic fate. Quinic acid is a monocarboxylic acid with pK_a 3.40 (*23*), while tartaric acid is a bicarboxylic acid with pK_{a1} 2.98 and pK_{a2} 4.34 (*24*). A monocarboxylic acid transporter-mediated mechanism of absorption was recently suggested as a possible explanation of the different relative gastric absorption efficiencies of chlorogenic acid and free phenolic acids in rats (*25*).

The aim of this work was to investigate if *trans*-caftaric acid is also absorbed by the stomach and, if so, to investigate its tissue distribution and metabolism. A solution of *trans*-caftaric acid was introduced for 20 min into the ligated stomach of anaesthetized rats. Samples of gastric content, blood, brain, liver, kidneys, and urine were investigated.

MATERIALS AND METHODS

Isolation of Grape Hydroxycinnamic Acids. Hydroxycinnamic acids were extracted by pressing grape berries (*Vitis vinifera*, cv. Rhine Riesling) in the presence of ascorbic acid (1 g/kg) and sodium *meta*-bisulphite (1 g/kg). The juice was treated with bentonite (1 g/L, 12 h at 4 °C) and centrifuged (4000 rpm, 15 min) in order to remove proteins. Hydroxycinnamic acids were then adsorbed by stirring for 1 h with activated carbon and diatomaceous earth (10 g/L each). The adsorbent was washed out with water, while hydroxycinnamic acids were eluted with methanol:acetic acid (99:1) and brought to dryness in a rotary evaporator. The purification of the hydroxycinnamic acids was obtained by flash chromatography on TSK gel Toyopearl HW-40S (Tosoh Corp., Tokyo, Japan). The preparative flash column (37 mm diameter, 150 mL capacity) was preconditioned with acidic methanol (0.5% acetic acid) and acidic water (0.5% acetic acid). Hydroxycinnamic acids were dissolved in acidic water (0.5% acetic acid), loaded on the column, and selectively eluted with the mixture (1:5) of methanol and acidified water (0.5% acetic acid). This fraction was transferred into diethyl ether and crystallized with *n*-hexane, leading to a purified grape hydroxycinnamic acids fraction. The recovery from grape was estimated to be 73.7, 69.2, and 34.5% for *trans*-caftaric acid, *trans*-*p*-coutaric acid, and *trans*-fertaric acid, respectively.

Isolation of *trans*-caftaric acid from the purified hydroxycinnamic acids fraction was done by preparative high-performance liquid chromatography (HPLC) with a Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV/vis detector, 8A pumps, and Class VP Software (Shimadzu Corp., Kyoto, Japan). Isolated *trans*-caftaric acid was dissolved in diethyl ether and crystallized with *n*-hexane. Pure *trans*-caftaric (purity > 99%, HPLC) was used for the in vivo trials.

Other Materials. A 2.5 mL amount of a 2.5% (w/v) solution of 2,2,2-tribromoethanol (Sigma-Aldrich, Steinheim, Germany) in ethanol:0.15 M NaCl (1:9, v/v) was used to anaesthetize rats. Heparin (Clarisco, Schwarz Pharma S.p.A., Milano, Italy) was used to prevent blood clotting. Methanol (HPLC grade) (Carlo Erba, Milano, Italy) was used for the extraction of hydroxycinnamic acids from rat tissues. Formic acid (HPLC grade) (Carlo Erba) was used for the HPLC analysis. Enzymes β-glucuronidase from bovine liver (type B-1) (EC 3.2.1.31) and sulfatase from abalone entrails (type VIII) (EC 3.1.6.1.) were from Sigma Chemical (St. Louis, MO).

Animals. Male, Wistar rats (250−270 g) were fed standard laboratory chow (Harlan Teklad 2018) and tap water ad libitum, both of which were assessed to be free of either *trans*-caftaric acid or *trans*-fertaric acid (data not shown); rats were housed in temperature-controlled rooms at 22−24 °C and 50−60% humidity, in accordance with the provisions of the European Community Council Directive 86-609. They were fasted for 24 h before the experiment and were handled with as much care as possible (*26*).

Administration of *trans*-Caftaric Acid to Anaesthetized Rats, Blood Sampling, and Excision of Organs. The surgical procedure for administering an acidified solution of *trans*-caftaric acid into the stomach of anaesthetized rats was previously described (*27*, *28*). That procedure is compliant with both Italian (D.L.vo. 116/92) and European (n.86/609/CEE) laws. Briefly, it consisted of the dissection of the abdominal wall of the rat, the fixing of a ligature around the cardias, and the sliding of plastic tubing (connected to a syringe) into the stomach through a slit in the duodenum. After extensive rinsing, an aliquot of 1 mL of blood was sampled from the inferior cava vein by means of an insulin syringe containing 0.05 mL of heparin, in order to obtain a control blood sample. Then, the stomach was filled with 4 mL of an acidified saline solution (10 mM HCl/0.15 M NaCl, pH 3) containing 1.2 mg of *trans*-caftaric acid corresponding to approximately 4.6 mg/kg of body mass. The concentration of *trans*-caftaric in red and white grapes varies from some tens up to a few hundreds mg/kg (*13*, *29*). Also, the concentration of *trans*-caftaric acid in wines varies from a few up to some hundreds mg/L, depending strongly on the grape variety and wine-making practice (*30*, *31*). Thus, the dose administered to rats in our experiments was meaningful from a nutritional point of view. The *trans*-caftaric acid solution was introduced in the stomach by means of a syringe and gently pumped in and out for 20 min. In the meantime, 100 μL of it was sampled at time 0, 10, and 20 min for

analysis, in order to check the stability of the intragastric solution. After 10 min of administration, a second aliquot of 1 mL blood was sampled at a different site of the inferior cava vein. At the end of experiment (after 20 min), the gastric contents were withdrawn; urine was sampled from the bladder, and then, 0.1 mL (500 IU) of sodium heparin was injected into the inferior cava vein. All available blood (up to 5 mL) was drained. Then, a phosphate-buffered (pH 7.4) saline solution (about 20 mL) was injected into the cava vein. This step, lasting less than 1 min, resulted in the washing out of the blood from the right kidney, most of the liver, and the brain. The rat was euthanized by decapitation. Immediately thereafter, the whole brain, the right kidney, and one lobe of the liver were quickly excised and weighed before being homogenized.

Preparation of Tissue Extracts. *Gastric Contents.* Samples (100 μL) of the *trans*-caftaric acid solution administered intragastrically were added with 9 volumes of methanol and centrifuged (at 14000*g* for 1 min). The supernatants were diluted and acidified with formic acid so to obtain the initial HPLC gradient conditions and directly analyzed by HPLC.

Plasma. This was obtained by centrifugation (at 14000*g* for 1 min) of blood samples. *trans*-Caftaric acid was extracted by adding plasma to 9 volumes of ice-cold methanol saturated with nitrogen. Screw-capped tubes were centrifuged at 3640*g* for 10 min at 4 °C. Supernatants were decanted into glass tubes under a stream of nitrogen. Extracts were stored at −20 °C until HPLC analysis within a few days.

Liver, Kidney, and Brain. Samples of those organs were homogenized in 5 volumes of ice-cold phosphate-buffered (pH 7.4) saline solution. Aliquots (1 mL) were extracted in 9 volumes of ice-cold methanol saturated with nitrogen. Extracts were centrifuged at 3640*g* for 10 min at 4 °C. Supernatants were decanted into glass tubes under a stream of nitrogen. Extracts were stored at −20 °C until analysis.

Urine. Urine was transferred into 1.5 mL polypropylene tubes, and the volume was estimated gravimetrically; 4 volumes of methanol was added, and the tubes were centrifuged at 14000*g* for 1 min. The supernatants were stored at −20 °C until analysis.

Enzymatic Hydrolysis of Tissue Extracts. Methanolic extracts of plasma, brain, liver, and kidney were evaporated. Residues (about 1 mL, pH 7.4) were incubated in nitrogen-flushed tubes at 37 °C for 2.5 h either without (as the control) or with β-glucuronidase (500 U) or sulfatase (25 U). Longer incubations (up to 24 h) were found not to change the results. After incubation, residues were diluted in 5 mL of 5 mM H_2SO_4, cleaned up, and analyzed as described below.

Cleanup of Extracts. *Selection of Solid Phase Adsorbent.* The solid phase adsorption capacity of three different matrix type columns was tested by evaluating the recovery of 3.7 mg/L *trans*-caftaric acid. The recoveries were (i) 90.1 ± 2.9% ($N = 6$) by using StrataX 33 μm Polymeric Sorbent cartridge (60 mg/3 mL) (Phenomenex, Torrance); (ii) 84.2 ± 3.9% ($N = 6$) by using styrene divinyl benzene Bakerbond cartridge (100 mg/3 mL) (J.T. Baker, Deventer, Holland); and (iii) 82.1 ± 11.6% ($N = 9$) by using C18 Sep-Pak cartridge (0.35 g) (Waters, Milford).

Cleanup of Methanolic Extracts of Tissues. Methanol was evaporated to about the initial volume of the sample (1/10); the residue was diluted in 5 mL of 5 mM H_2SO_4 and loaded onto a StrataX cartridge, previously conditioned with 3 mL of methanol followed by 5 mL of 5 mM H_2SO_4. The cartridge was washed with 5 mL of 5 mM H_2SO_4 and dried with nitrogen before elution with 4 mL of methanol. The eluate was evaporated to dryness under reduced pressure at 38 °C and dissolved in 0.5 mL of aqueous methanol, identical to the initial HPLC gradient. The sample was filtered through a 0.22 μm PVDF filter (Millipore, Bedford) into a HPLC vial and analyzed by HPLC.

HPLC Analysis. An Agilent 1100 HPLC with DAD connected to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto) was used for hydroxycinnamic acids detection and quantification. The method was developed for monitoring grape hydroxycinnamic acids, both free and conjugated with tartaric acid. Separation was performed using a 250 mm × 2.1 mm i.d., 5 μm ODS Hypersil C18 column with a 20 mm × 2.1 mm i.d., 5 μm ODS Hypersil guard column (Agilent Technologies). The mobile phase consisted of A, 0.5% formic acid in water, and B, 2% formic acid in methanol. Separation was carried out at 40 °C for 33 min. The gradient conditions were linear starting at 16% B, to 25% B in 15 min, to 43% B in 13 min, to 100% B in 0.1 min, 100% B for 4.9 min, and back to 16% B in 0.1 min. The column was equilibrated for 10 min prior to each analysis. The flow rate was 0.4 mL/min, and the injection volume was 70 μL. The UV/vis spectra were recorded from 220 to 700 nm, with detection at 320 nm.

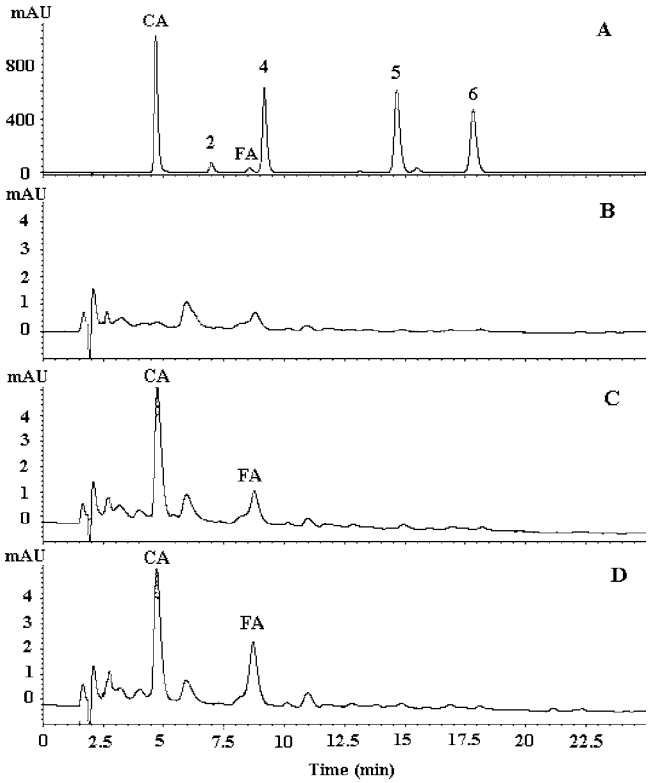

Figure 2. (**A**) HPLC chromatogram at 320 nm of standard hydroxycinnamate tartaric esters isolated from (grape) *V. vinifera* grape (cv. Rhine Riesling), *trans*-caftaric (CA), *p*-coutaric (**2**), and *trans*-fertaric acid (FA), and their corresponding free forms, caffeic (**4**), *p*-coumaric (**5**), and ferulic acids (**6**). HPLC chromatograms at 320 nm of plasma extracts obtained from the same rat. (**B**) Before, (**C**) 10 min after, and (**D**) 20 min after the intragastric administration of a *trans*-caftaric acid solution. *trans*-Caftaric acid (CA); *trans*-fertaric acid (FA).

RESULTS AND DISCUSSION

Detection and Identification of Grape Hydroxycinnamic Acids. The HPLC method enabled us to separate *trans*-caftaric acid from its O-methylated derivative, fertaric acid, and both of them from their respective products of hydrolysis, i.e., caffeic acid and ferulic acid (**Figure 2A**). The identity of each compound was based on their UV/vis spectra and retention times, which were compared to the respective standards. Quantification of compounds was based on peak areas at λ 320 nm, and the respective concentrations in samples were expressed as *trans*-caftaric acid equivalents. Calibration curves were prepared by injecting aliquots of standard *trans*-caftaric acid solution in the ranges 0.05−5 and 5−50 mg/L. The calibration curves were linear over the studied ranges with correlation coefficients 0.99938 and 0.99946, respectively. The limit of *trans*-caftaric acid detection (LOD), corresponding to a signal-to-noise ratio (S/N) of 3, was 0.015 mg/L, whereas the limit of quantification (LOQ, S/N = 10) was 0.048 mg/L. To assess the repeatability properties of the method, 1.81 mg/L of *trans*-caftaric acid and 1.18 mg/L of fertaric acid were sequentially analyzed by HPLC ($N = 10$). The coefficients of variation (CV) of their concentrations were 1.75% for *trans*-caftaric acid and

0.15% for *trans*-fertaric acid. The CV of the retention time was 0.29% for *trans*-caftaric acid and 0.19% for *trans*-fertaric acid.

Recovery of *trans*-Caftaric and *trans*-Fertaric Acid from Relevant Tissues of Control Rats. Tissues from fasted control rats were used to assess both the occurrence of either *trans*-caftaric acid or *trans*-fertaric acid in control animals and their recoveries from spiked samples. Neither *trans*-caftaric nor *trans*-fertaric acid were detected in plasma, brain, liver, kidney, and urine of control samples.

To assess recoveries of both *trans*-caftaric acid and *trans*-fertaric acid from samples of whole blood, plasma, brain, liver, and kidney homogenates of control, rats were spiked with the pure compounds at similar concentrations to those detected in rat tissues. Spiked blood was centrifuged in order to separate plasma from the particulate fraction. *trans*-Caftaric acid was analyzed in extracts of both the plasma and the cell pellet. The recoveries were 49.4 ± 1.2 and 1.4 ± 0.2% ($N = 10$), respectively. The recovery from spiked plasma was 57.1 ± 1.9% ($N = 7$), thus similar to the recovery from whole blood. The recovery of *trans*-fertaric acid from rat blood was 78.2 ± 1.9% ($N = 6$). The different recoveries of either *trans*-caftaric acid or *trans*-fertaric acid might possibly be related not only to their different polarity but also to different binding ratios to albumin, as noted in studies of caffeic acid and ferulic acid binding to albumin (*32*), which is the main protein involved in the plasmatic transport of phenolics.

The recoveries of *trans*-caftaric acid from brain, liver, and kidney homogenates were 84.2 ± 1.9 ($N = 4$), 52.1 ± 2.4 ($N = 6$), and 56.2 ± 3.5% ($N = 4$), respectively. The recoveries of *trans*-fertaric acid from brain, liver, and kidney homogenates were 93.2 ± 3.1 ($N = 4$), 64.9 ± 5.9 ($N = 5$), and 62.7 ± 2.4% ($N = 4$), respectively.

Absorption of *trans*-Caftaric Acid from the Stomach into the Blood. The absorption of *trans*-caftaric acid from the stomach lumen was assessed in anaesthetized rats, using a minimally invasive surgical procedure used in previous works (*27*, *28*). Samples of blood were taken from the inferior cava vein at 0, 10, and 20 min. Plasma extracts were analyzed for their *trans*-caftaric acid content. The chromatograms of all three plasma samples obtained from the same rat are shown in **Figure 2**. At time zero, plasma was free of *trans*-caftaric acid (**Figure 2B**); however, the latter appeared at 10 min (**Figure 2C**) and was maintained to a similar extent at 20 min (**Figure 2D**). In addition, a peak with retention time corresponding to *trans*-fertaric acid rose with time. Its UV/vis absorption spectrum was identical to that of pure *trans*-fertaric acid. Similar chromatograms were obtained with all other 14 examined rats. The pooled data of *trans*-caftaric and *trans*-fertaric acid concentration in plasma after both 10 and 20 min of *trans*-caftaric administration are presented in **Table 1**.

Origin of *trans*-Fertaric Acid. *trans*-Fertaric acid can be regarded as the specific product of the reaction catalyzed by the enzyme catechol-O-methyl transferase (EC 2.1.1.6) (*33*). This enzyme, ubiquitously expressed in mammalian tissues, is most abundant in the liver, the kidney, and the gastrointestinal tract (*33*). Thus, *trans*-caftaric acid could be O-methylated already in the gastric lumen. In order to check this possibility, samples of the gastric content were withdrawn from the stomach at times 0, 10, and 20 min. It was found that *trans*-caftaric acid was stable with respect to both its concentration and its composition. In particular, neither *trans*-fertaric acid nor caffeic acid appeared, thus ruling out both methyl transferase and esterase activities at the gastric level.

Table 1. Occurrence of *trans*-Caftaric Acid and *trans*-Fertaric Acid in Some Rat Tissues Following Intragastric Administration of *trans*-Caftaric Acid

	means ± SEM[a] (min–max)	
tissue	*trans*-caftaric acid	*trans*-fertaric acid
	plasma ($N = 15$)[b] (ng/mL)	
0 min	ND[c]	ND
10 min	293.0 ± 45.0 (78.9–590.1)	92.0 ± 11.6 (34.4–182.8)
20 min	334.0 ± 48.8 (74.7–585.0)	185.1 ± 24.0 (47.9–362.6)
	brain ($N = 5$)[d] (ng/g)	
20 min	179.6 ± 20.2 (103.9–221.5)	ND
	liver ($N = 12$) (ng/g)	
20 min	ND	ND
	kidney ($N = 3$) (ng/g)	
20 min	442.5 ± 78.4 (361.2–599.2)	2505.5 ± 514.0 (1810.4–3508.9)
	urine ($N = 3$) (ng/mL)	
20 min	ND	33286 ± 12809 (9334–53133)

[a] Standard error of means. [b] Number of samples analyzed. [c] Not detected. [d] Data refer to five positive observations out of 15 trials (no *trans*-caftaric acid was detected in 10 rats).

Relatively high amounts of catechol-*O*-methyl transferase also occur in rat erythrocytes (*33*). To check if O-methylation of *trans*-caftaric acid occurred in the blood, aliquots (1 mL) of rat blood were incubated in vitro at 37 °C up to 60 min in the presence of 9.2 μg of *trans*-caftaric acid in duplicate. Blood samples were withdrawn at time intervals (0, 10, 30, 45, and 60 min), and plasma was analyzed. The *trans*-caftaric acid concentration was found to be stable, i.e., 9.08 ± 0.7 μg/mL plasma (pooled data, $N = 10$; value not corrected by recovery factor), and no other possible metabolite appeared to some significant extent. These data indicate that the compound is both chemically stable in blood and unable to be adsorbed onto and/or to penetrate into blood cells. It can be concluded therefore that *trans*-fertaric acid observed in the plasma of rats receiving *trans*-caftaric acid intragastrically should have originated from some other peripheral tissue.

Tissue Distribution of *trans*-Caftaric Acid. *Brain.* In five out of 15 treated rats, *trans*-caftaric acid was detected (**Figure 3A** and **Table 1**). In no case were any of its possible derivatives detected, such as *trans*-fertaric acid or caffeic acid. Such results demonstrate that the blood−brain barrier was selectively permeable only to *trans*-caftaric acid and that no appreciable metabolism thereof occurred in the brain during the time frame of the experiment. Although *trans*-caftaric acid might have been lost in 10 out of 15 brains, we thought that this interpretation might be misleading, overlooking the possibility that these findings could rather reflect a specific interindividual genetic variability, so that *trans*-caftaric acid could quickly pass through the blood−brain barrier in only some of the investigated rats. Because this possibility is biologically plausible, the positive data obtained in five subjects were averaged and the mean is presented in **Table 1**.

Liver. Neither *trans*-caftaric nor any of its possible derivatives mentioned above were detected in the livers of 12 rats after 20 min of intragastric administration of *trans*-caftaric acid (**Figure 3B** and **Table 1**). Because the bile compartment is intrinsically connected to the hepatic cell compartment and therefore the liver homogenate comprises both cells and bile, our results show not only that *trans*-caftaric acid is not taken up by the liver but also that it is neither metabolized nor excreted into the bile. In particular, in the case of bile excretion of either *trans*-caftaric acid or its derivatives, at least minimum quantities thereof should

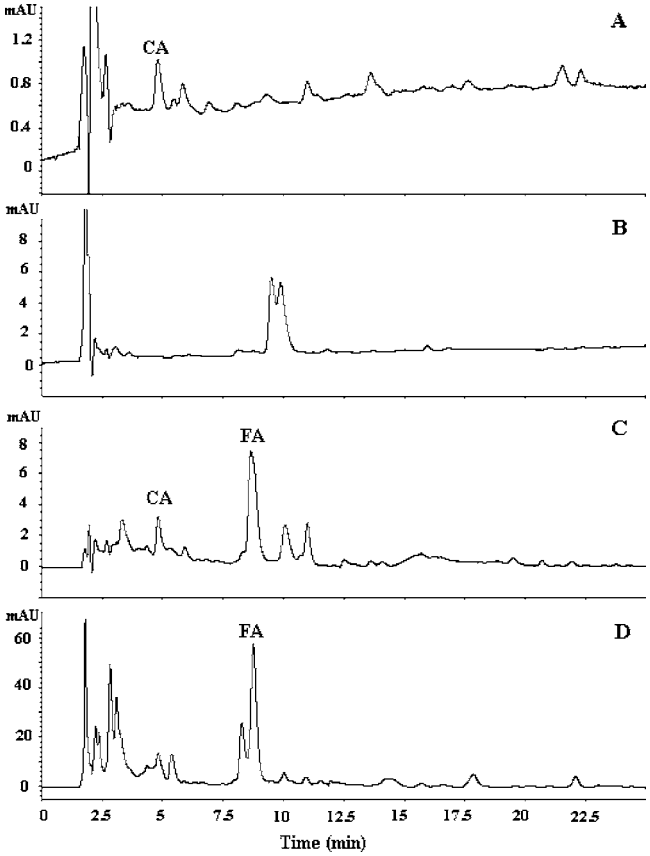

Figure 3. HPLC chromatograms at 320 nm of extracts from (**A**) brain, (**B**) liver, (**C**) kidney, and (**D**) urine obtained from rats that received an intragastric solution of *trans*-caftaric acid for 20 min. *trans*-Caftaric acid (CA); *trans*-fertaric acid (FA).

have been detected in liver homogenates. However, this does not exclude that, under different experimental conditions (e.g., longer time of exposure to a higher amount of *trans*-caftaric acid), the liver might be found to be a site of uptake, metabolism, and excretion of *trans*-caftaric acid.

Kidney and Urine. In a further experiment, the kidneys and urine of three treated rats were analyzed. In the kidneys, *trans*-caftaric acid was found at comparable amounts to those detected in plasma, whereas *trans*-fertaric acid was found at concentrations 13-fold higher in the kidneys than in plasma (**Figure 3C** and **Table 1**). Both compounds were characterized not only by their UV/vis spectra but also by control analyses done by LC/ESI-MS (data not shown). The free acids, which are the hydrolytic products of *trans*-caftaric acid or *trans*-fertaric acid, were below the detection limit.

In the urine, *trans*-caftaric acid was not detected, although some unidentified compound was eluted with a similar retention time. Conversely, a large peak of *trans*-fertaric acid appeared (**Figure 3D**), enabling us to calculate that the *trans*-fertaric acid concentration in urine was as much as 180-fold higher than in plasma (**Table 1**).

Enzyme Treatment of Tissue Extracts. Extracts of plasma, brain, liver, and kidney but not of urine (due to paucity of available samples), collected from rats at the end of the experiment, were treated with β-glucuronidase and sulfatase in order to induce the release of either *trans*-caftaric acid or *trans*-fertaric acid from the corresponding glucurono- and sulfoconjugates, putatively formed in vivo. However, such treatments had no effect on the peak areas of either *trans*-caftaric acid or *trans*-fertaric acid in the tissue extracts.

The absorption of dietary polyphenols from the gastric tract has been directly documented in anaesthetized rats receiving an intragastric solution of structurally diverse test compounds, such as flavonols (*34*), anthocyanins (*27*, *35*), hydroxycinnamates (*25*, *36*), and their esters (*22*). Data obtained in this study with *trans*-caftaric acid add a further piece of evidence in support of the absorbing properties of the stomach. Although passive trans-epithelial diffusion, driven by protonation of polar molecules, is a recognized mechanism of gastric absorption (*37*), a carrier-mediated type of trans-epithelial diffusion should be taken into account in the case of a structurally complex polar molecule such as *trans*-caftaric acid. In general terms, the gastric epithelium poorly expresses membrane carriers (*38*). Notwithstanding, the documented expression of some of them, for instance, the monocarboxylic acid transporter (*39*), the fatty acid transporter (*40*), the glutamate transporter (*41*), the iodide transporter (*42*), or the bilirubin transporter (*43*), suggests that some selected ingredients of the diet can be absorbed from the gastric lumen by means of specific transporters.

The hypothesis of a carrier-mediated mechanism that favors *trans*-caftaric acid bioavailability is further supported by data presented in this work. Indeed, neither *trans*-caftaric acid nor any possible metabolite thereof could be recovered from the liver, ruling out the option of passive diffusion of *trans*-caftaric acid through cell membranes. Similarly, no significant amounts of *trans*-caftaric acid could be taken up by the particulate fraction of the blood, even after a long incubation time. Likewise, it can be speculated that the occurrence of *trans*-caftaric acid in the brain, although documented in only one-third of the tested animals, reflects the activity of specific mechanism(s) of transport across the blood−brain barrier. In this special case, support to this assumption is offered by the observation that no *trans*-fertaric acid could be recovered from the brain, in spite of its occurrence at a plasmatic concentration, less than 2-fold lower than that of *trans*-caftaric acid; its lower polarity than *trans*-caftaric acid, thus allowing a higher specific permeability through cell membranes; and its optimal recovery in methanolic extracts of spiked brain homogenate.

The absence of *trans*-fertaric acid in the brain might suggest that brain catechol-*O*-methyl transferase isoforms have insignificant affinity for *trans*-caftaric acid and/or that the latter is not available in the brain regions where catechol-*O*-methyl transferase is expressed. The fact that *trans*-caftaric acid was detected in one-third of the rat brains examined might reflect the combination of low blood−brain barrier permeability with a high interindividual variability, as also reported in some studies about the occurrence of anthocyanins in rat brains (*28*, *44*).

The plasma concentration of *trans*-caftaric acid was about 300 ng/mL once it reached the steady-state level at 10 min, corresponding to ca. 1 μM; this value was quite similar to that observed in rats that received 35 μmol of chlorogenic acid (1.6 μM) (*22*), although in our experiments rats received only 3.8 μmol of *trans*-caftaric acid.

In our experiments, no hydrolysis of *trans*-caftaric acid to caffeic acid took place in the stomach nor anywhere else in the organism, since caffeic acid could be detected neither in the stomach contents nor in the plasma. This is in line with what has been observed for chlorogenic acid, which can be split only at a later stage by the colonic microflora (*45*), thus reducing its bioavailability in comparison to that of free caffeic acid (*20*).

Unlike the chlorogenic acid infusion into the stomach, which produced no other metabolite in the plasma (*22*), the administration of *trans*-caftaric acid yielded *trans*-fertaric acid in the plasma, to such an extent that the plasma steady-state concentra-

tion of *trans*-caftaric acid might be seen as being eventually determined by the rate of its conversion to *trans*-fertaric acid.

Our data show that the kidney is the site of election for uptake, metabolism, and excretion of *trans*-caftaric acid. Metabolism consists in its conversion to *trans*-fertaric acid, likely catalyzed by the II phase enzyme catechol *O*-methyl transferase. Access of *trans*-caftaric acid into the kidney tubular cell, which is one of the best examples of a polarized cell, could occur either from the luminal (tubular) side, following glomerular filtration, and/or from the basolateral (vascular) side. Considering that *trans*-caftaric acid might be bound to plasma proteins, in analogy with other hydroxycinnamic acids (*32*, *46*), and therefore might undergo poor glomerular filtration, it is possible to think that *trans*-caftaric acid is taken up at the basolateral plasma membranes of kidney cells, allowing *trans*-caftaric acid to become a substrate of catechol *O*-methyl transferase and be efficiently converted to *trans*-fertaric acid. *trans*-Caftaric acid might be transported by the organic anion transporter 1 (OAT1), a carrier expressed in the kidneys and, to a lower extent, in the brain, but not in liver (*47*), which might explain the absence of *trans*-caftaric acid in liver homogenates. In this connection, it is important to mention that the dietary polyphenol, ellagic acid, is transported by OAT1 and not by OAT2, OAT3, and oatp1, which are organic anion carriers expressed both in the liver and in the kidneys (*48*).

Apparently, O-methylation to chlorogenic acid was precluded (*22*), because of the probable absence of specific mechanism(s) of transport into the kidney tubular cells rather than due to its inability to be a substrate of catechol *O*-methyl transferase (*49*). Thus, it could be speculated that the occurrence of chlorogenic acid in the urine (*22*) might follow from glomerular filtration rather than from uptake into kidney tubular cells from peritubular capillaries.

Some of the above-mentioned carriers might also be involved in the transport of *trans*-fertaric acid from the kidney tubular cells back into the blood. This might happen because the intracellular concentration of *trans*-fertaric acid progressively increased, due to the efficiency of the methylating reaction, while excretion lagged behind. Thus, *trans*-fertaric might have been transported from the kidney cells back into the plasma. If so, it should be concluded that the excretion of *trans*-fertaric acid into the urine was rate-limiting.

In spite of a limited availability of the urine sample from the 20 min experiment, we considered it very important to analyze the small amount obtained, since it is known that the urinary excretion of ferulic acid (*17*, *50*) can be relatively high. On the contrary, a low urinary excretion of *trans*-fertaric acid would imply either strong excretion in the bile or extensive catabolism. The finding that *trans*-fertaric acid is concentrated in the urine indicates that an active mechanism of apical renal transport is involved, such as perhaps the multidrug resistance protein 2 (MRP2) (*47*). In this regard, it has been reported that naturally occurring plant polyphenols available in our normal diet can interact with and be transported by MRP2 (*51*). The tartaric moiety of *trans*-caftaric acid seems to act as a specific tag for OAT1 and/or for another so far unidentified specific transporter, diverting the compound from the liver and addressing it to the kidney instead. Thus, *trans*-caftaric acid seems to follow entirely the metabolic fate of tartaric acid, which is indeed exclusively excreted in the urine as such (*52*).

The fact that neither glucurono- nor sulfoconjugates of either *trans*-caftaric acid or *trans*-fertaric acid were found indicates that none of such transformations occurred to a detectable extent in the tissues that *trans*-caftaric acid did reach; perhaps more exactly, this piece of evidence confirms that *trans*-caftaric acid had no access to the sites where the enzymes glucuronoyl transferases and sulfotransferases are most active (*53*), i.e., the liver, which is one of the peculiar findings of this work, and the gut, which was meant by the experimental design.

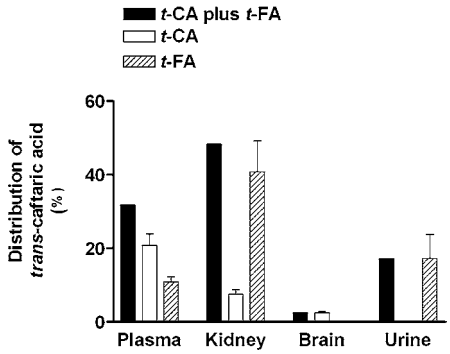

Figure 4. Distribution of *trans*-caftaric acid (CA) and *trans*-fertaric acid (FA) in plasma, kidneys, brain, and urine of rats that received an intragastric solution of *trans*-caftaric acid for 20 min. Values are expressed as percentage of the recovered dose in the investigated tissues and fluids, assuming that rat plasma is 8.8 mL (http://www.ratbehavior.org/Stats.htm) and considering that the experimental value of urine volume was 0.077 mL and the brain and kidney masses were 2.0 and 2.4 g, respectively.

The detailed description of the mechanisms of membrane transport and intracellular metabolism of both *trans*-caftaric and *trans*-fertaric acid in the kidney demands further investigation. Calculating the distribution of both *trans*-caftaric acid and its metabolite in the investigated tissues and fluids, it is possible to observe that the major percentage is present in kidneys (48.6%, 7.3% as *trans*-caftaric acid and 41.3% as *trans*-fertaric acid), followed by plasma (31.4%, 20.2% as *trans*-caftaric acid and 11.2% as *trans*-fertaric acid), urine (17.6% of *trans*-fertaric acid), and brain (2.5% of *trans*-caftaric acid) (**Figure 4**).

In conclusion, the main results of the present work are that *trans*-caftaric acid is rapidly available through the stomach, bypasses the liver, can reach the brain, and is taken up, metabolized, and excreted as *trans*-fertaric acid by the kidneys. The fate of *trans*-caftaric acid absorbed from the rat stomach provides a new example of how strongly the molecular structure affects the plasmatic levels, the tissue bioavailability, and the metabolism of phenolic acids present in the human diet.

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