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In Vitro and in Vivo Stability of Caffeic Acid Phenethyl Ester, a Bioactive Compound of Propolis

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The in vitro biochemical stability of caffeic acid phenethyl ester in rat and human plasma was investigated and compared with the stability of other caffeic acid esters (chlorogenic acid and rosmarinic acid). The incubation of the compounds in rat plasma for up to 6 h showed that caffeic acid phenethyl ester, but not the other compounds, was hydrolyzed, whereas human plasma did not affect the stability of all the assayed compounds. The products in rat plasma were caffeic acid and an unknown compound, which was identified by mass spectrometry as caffeic acid ethyl ester, produced by transesterification in the presence of ethanol used as vehicle for standard compounds. Specific inhibitors of different plasma esterases allowed the identification of a carboxylesterase as the enzyme involved in the metabolism of caffeic acid phenethyl ester. The oral administration in rats of caffeic acid phenethyl ester in the presence of both ethanol and 2-(2-ethoxyethoxy)ethanol gave rise to a dramatic increase of caffeic acid, as well as low levels of caffeic acid phenethyl ester, caffeic acid ethyl ester, and caffeic acid 2-(2-ethoxyethoxy)ethyl ester, in urine collected within 24 h after treatment. These results suggest that caffeic acid phenethyl ester is hydrolyzed also in vivo to caffeic acid as the major metabolite and that its biological activities should be more properly assayed and compared with those of caffeic acid, its bioactive hydrolysis product. Moreover, alcohols should be carefully used in vivo as solvents for caffeic acid phenethyl ester, since they can give rise to new bioactive caffeic acid esters.

KEYWORDS: Caffeic acid phenethyl ester; rat plasma; human plasma; carboxylesterase; ethyl transesterification.

INTRODUCTION

Caffeic acid phenethyl ester (**2**, **Figure 1**), identified for the first time in 1987 in propolis ("bee glue") and poplar bud exudates (*1*, *2*), is one of the most important bioactive compounds of propolis, widely investigated for its potential therapeutic properties (*3*, *4*). The compound mediates the antioxidant and anti-inflammatory properties of propolis extracts (*5*). Several investigations reported its antitumoral activities both in vitro (*6*, *7*) and in vivo (*8*), its antibiotic and antiviral properties (*9*−*11*), and its ability to inhibit acetylcholinesterase activity (*12*). Moreover, an increasing number of in vivo studies with a systemic administration of **2** in different animal models have been reported (*8*, *13*−*17*).

We recently investigated the bioavailability of **2** after oral administration in rats (*18*), observing a limited stability in plasma ascribed to the activity of plasma esterases. These enzymes can be basically classified into three families: paraoxonases (PONs), cholinesterases (ChEs), and carboxylesterases (CEs) (*19*).

PONs (EC 3.1.1.2), which are calcium-dependent serum enzymes, were so named for their ability to hydrolyze organophosphorus pesticides but are primarily involved in the metabolism of lipids and in the prevention of atherosclerotic processes (*20*). PONs are also responsible for plasma arylesterase (*21*) and lactonase (*22*) activities. ChEs are serine-hydrolases; among them, the acetylcholinesterase (AChE; EC 3.1.1.7) and the butyrylcholinesterase (BChE; EC 3.1.1.8) are the most studied. The AChE, involved in the deactivation of the neurotransmitter acetylcholine, is present also in erythrocytes, whereas the BChE (or "pseudocholinesterase") is mainly a plasma enzyme involved in the metabolism and detoxification of many pesticides and drugs, even if its function is not completely clear (*23*, *24*). CEs (EC 3.1.1.1) are broad-spectrum serine-hydrolases, active toward a wide range of ester- and amide-containing substrates (*25*). Although the highest activity occurs in the liver, they have been identified in a variety of mammalian organs and tissues, including blood. They are responsible for the hydrolysis of many endogenous compounds (e.g., short- and long-chain acyl glycerols) as well as exogenous compounds (e.g., drugs and pesticides) (*26*). In addition to these "classical" hydrolases, an esterase-like activity has been reported for serum albumin. In spite of its low enzymatic activity, the high plasma concentration of albumin makes its contribution to the overall plasma esterases not negligible (*27*).

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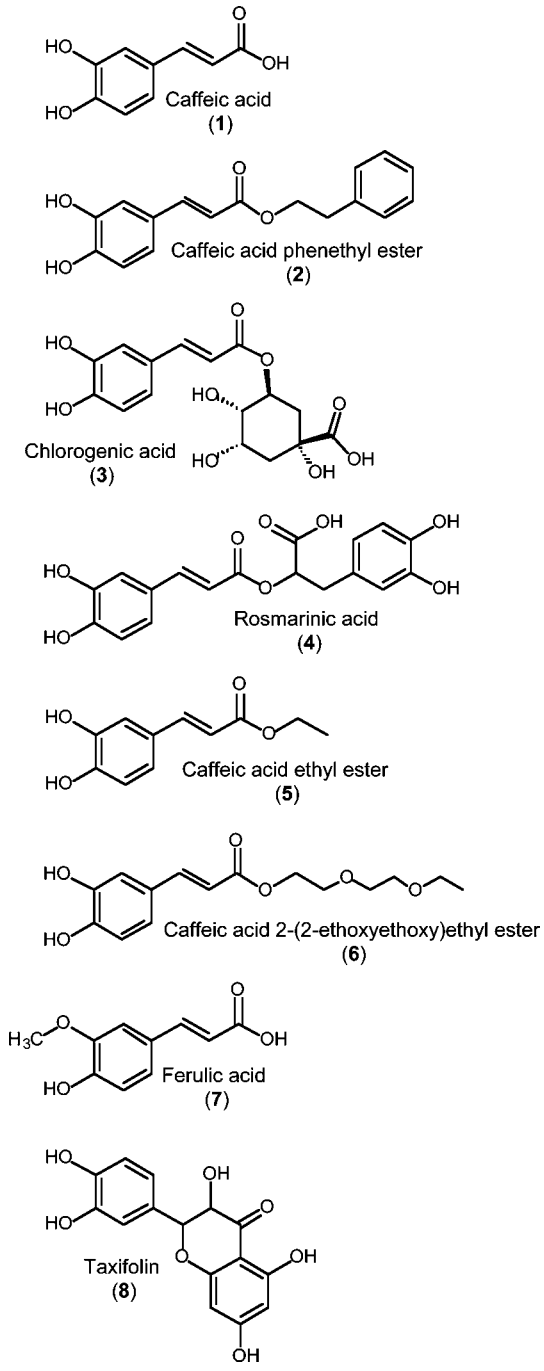

Figure 1. Chemical structures of (**1**) caffeic acid, (**2**) caffeic acid phenethyl ester, (**3**) chlorogenic acid, (**4**) rosmarinic acid, (**5**) caffeic acid ethyl ester, (**6**) caffeic acid 2-(2-ethoxyethoxy)ethyl ester, and the internal standards (**7**) ferulic acid and (**8**) taxifolin.

The aim of the present study was to investigate the in vitro stability of **2** in rat and human plasma, also in comparison with other bioactive esters of caffeic acid (**1**, **Figure 1**), such as chlorogenic acid (**3**, **Figure 1**) and rosmarinic acid (**4**, **Figure 1**), having as alcoholic components quinic acid and 3-(3,4-dihydroxyphenyl)lactic acid, respectively. **3** is the main phenolic compound in coffee and a common source of **1** in fruits and vegetables (*28*), while **4**, so named for the plant from which it was isolated (rosemary), was identified in various culinary and medicinal herbs (mint, salvia, melissa, etc.) (*29*). Moreover, the stability of **2** was investigated after oral administration in the rat.

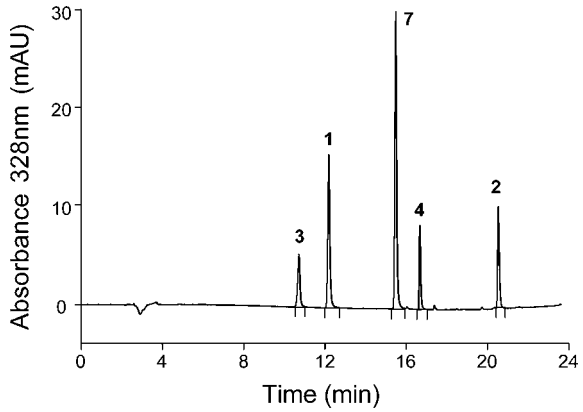

Figure 2. Representative HPLC-UV chromatogram (328 nm) of rat plasma spiked with 10 μg/mL of (**1**) caffeic acid, (**2**) caffeic acid phenethyl ester, (**3**) chlorogenic acid, (**4**) rosmarinic acid, and 25 μg/mL of (**7**) ferulic acid (internal standard).

MATERIALS AND METHODS

Chemicals and Stock Solutions. Compounds **2** and **8** were from Sigma (St. Louis, MO), whereas **3** and **4** were from Extrasynthese (Genay, France) and Fluka (Buchs, Switzerland), respectively. **1** and **7** were both obtained from Acros Organics (Geel, Belgium). The solvents acetonitrile, methanol, and ethanol, all HPLC grade, were from Carlo Erba Reagenti (Milan, Italy). Substrates and inhibitors used in the assays of esterase activity were from Sigma, except phenylacetate, NaF, and diisopropylfluorophosphate (DFP) (Fluka), paraoxon-ethyl (POX) (Riedel-de Haën, Seelze, Germany), and EDTA disodium salt (Carlo Erba Reagenti). 2-(2-Ethoxyethoxy)ethanol (Transcutol HP) was kindly provided by Gattefossé Italia (Milan, Italy). Deionized water was from Laboratori Diaco Biomedicali (Trieste, Italy). All other chemicals were from Carlo Erba Reagenti.

Standard stock solutions of **1**, **2**, **3**, **4**, **7**, and **8** were prepared in ethanol at the concentration of 1 mg/mL, unless otherwise specified. Internal standard working solutions of **7** and **8** (6.25 μg/mL and 1 μg/mL, respectively) were prepared diluting their stock solutions with methanol containing 1% formic acid. All standard solutions were stored at −20 °C.

Animals. All procedures involving animals and their care were conducted in compliance with National and International laws (Italian Legislative Decree 92/116; EEC Council Directive 86/609). Male CD-(SD) rats (Charles River Laboratories, Calco, Italy), weighting 350 ± 50 g, were housed in a temperature-controlled room (21 ± 2 °C) with 12 h of daily artificial illumination. Food (Altromin-MT) (Rieper, Vandoies, Italy) and water were given ad libitum. Rats were allowed to acclimate for at least one week before their use.

Preparation of Rat and Human Plasma. After anaesthesia of rats with pentobarbital sodium salt (Siegfried CMS, Zofingen, Switzerland) given by intraperitoneal administration (40 mg/kg in saline), blood was withdrawn by cardiac puncture and was collected in polypropylene tubes containing 10 μL of sodium heparin (Liquemin 5000 IU/mL) (Roche, Milan, Italy). Plasma samples, obtained by centrifugation at 3000*g* for 15 min at 4 °C, were pooled (n = 5), were aliquoted, and then were stored at −20 °C.

Human blood was obtained from healthy donors by arm venipuncture and was collected and prepared as described above. Plasma samples (n = 15) were first pooled and then were aliquoted and stored at −20 °C. Informed consent was obtained from all blood donors.

In Vitro Stability Assay of Caffeic Acid Phenethyl Ester, Chlorogenic Acid, and Rosmarinic Acid. Rat plasma, human plasma, and 100 mM phosphate buffer (pH 7.4) used as control were spiked with the standard compounds (**2**, **3**, or **4**) to obtain a final concentration of 50 μg/mL, were vortexed for 1 min, and were incubated in a water bath at 37 °C. Aliquots (100 μL) of each reaction mixture were withdrawn after 0, 10, 20, 30, 45, 60, 120, 180, and 360 min incubation. Samples were extracted and analyzed by high-performance liquid chromatography (HPLC)-UV. Experiments were performed in triplicate. Samples were handled in ice before incubation.

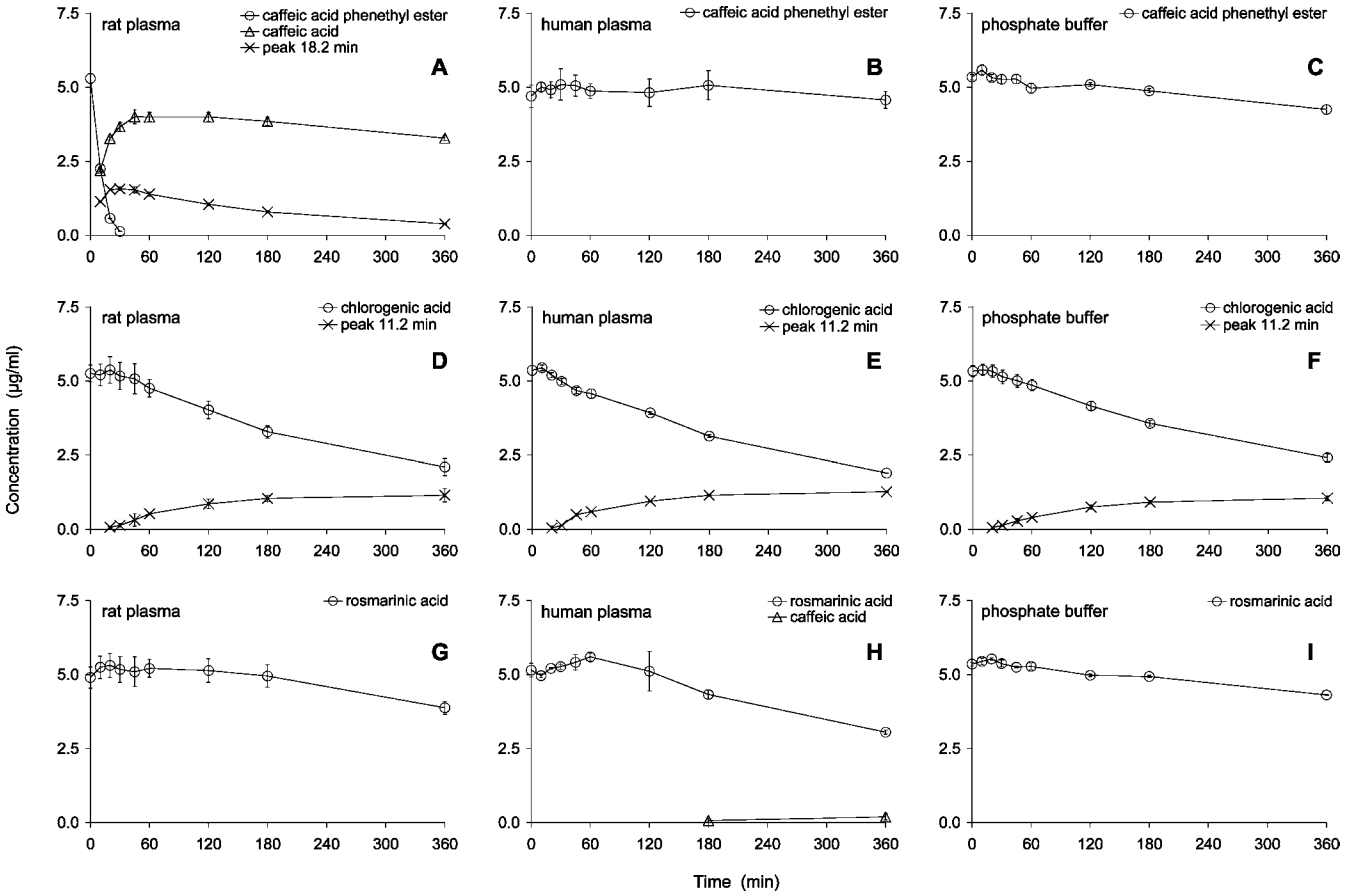

Figure 3. Time–concentration profile of (**A**–**C**) caffeic acid phenethyl ester, (**D**–**F**) chlorogenic acid, and (**G**–**I**) rosmarinic acid, incubated at 37 °C in rat plasma, human plasma, and 100 mM phosphate buffer, pH 7.4 ($n = 3$ for each experiment).

Stability of Caffeic Acid Phenethyl Ester at Different Ethanol Concentrations. A stock solution of **2** was prepared in ethanol at the concentration of 100 mg/mL. Diluted solutions in ethanol were then prepared to spike rat plasma at levels 50 μg/mL for **2** and 5, 1, 0.5, 0.1, and 0.05% (v/v) for ethanol. Aliquots (100 μL) were extracted after 0 and 30 min incubation at 37 °C and were analyzed by HPLC-UV. Experiments were performed in triplicate. Samples were handled in ice before incubation.

Preparation and HPLC-UV Analysis of Plasma Samples. Immediately after the incubations, samples were extracted in a 1.5 mL polypropylene conical tube by adding 400 μL of the internal standard (**7**) working solution to 100 μL of plasma and then by vigorous vortexing for 10 min. The protein precipitate was separated by centrifugation at 20800*g* for 5 min at 4 °C, and 250 μL of the supernatant was transferred to a new 1.5 mL polypropylene conical tube. Then, after the addition of 250 μL of water and 1 min vortexing, samples were stored at −20 °C until their analysis.

Analyses were performed using a Varian HPLC system (Palo Alto, CA) equipped with a 9010 pump and a 9050 UV/vis detector set at 328 nm. The separation was obtained with a 250 × 3.0 mm i.d., 5 μm, Luna C18(2) column provided with a 4.0 × 2.0 mm i.d., 5 μm, Luna C18(2) SecurityGuard System, both from Phenomenex (Torrance, CA). The HPLC column was thermostated at 35 °C and samples were injected using a 20 μL sample loop. Separations were carried out at the flow rate of 0.5 mL/min using a linear gradient of acetonitrile (A) in water (B), both containing 0.5% formic acid (from 10% to 30% A in 10 min, then to 100% A in 10 min with 5 min hold). A Star 4 Chromatography Workstation (Varian) was used for instrument control, data acquisition, and data handling.

To perform quantitative analyses, rat plasma, containing 0.1 mM DFP, was spiked with a standard mixture of **1**, **2**, **3**, and **4** to obtain calibration curves in the range 1−100 μg/mL. Since plasma samples were 10-fold diluted after the extraction step, the injected concentrations resulted in the range 0.1−10 μg/mL. Standard curve equations were calculated using weighted (1/*x*) linear regressions of internal ratios (analyte/internal standard peak areas) versus analyte concentrations. The linearity of the method was assessed as the range of concentrations in which the correlation coefficient (r^2) was ≥0.985 with precision and accuracy ≤15%, except for the lower limit of quantification, for which precision and accuracy ≤20% were accepted. The limit of detection, assessed as the lowest concentration for which a signal intensity at least 3 times greater than the background level can be detected, was 0.05 μg/mL for all the compounds.

Mass Spectrometry (MS) Analysis. A PE Sciex 365 triple quadrupole mass spectrometer (Toronto, Canada) was used. It was equipped with a Sciex turbo ionspray source, operated in electrospray ionization (ESI) (negative ion mode). The mass spectrometer was calibrated with polypropylene glycol (PE Sciex, Foster City, CA) with the resolution set at 0.7 ± 0.1 u (as peak width at half-height). Instrument control and data acquisition were performed using Masschrom 1.1.1 software (PE Sciex).

Mass spectrometric parameters were optimized on the standard compounds infused into the source at the flow rate of 5 μL/min by a model 11 syringe pump (Harvard Apparatus, South Natick, MA). When the standard compounds were not available, the conditions optimized for **2** were used. Both full-scan and product ion spectra were acquired using a step size of 0.1 u and a dwell time of 1 ms in the range *m*/*z* 50−300.

Esterase Activity Assays. The activity of rat plasma esterases was measured at 37 °C using a Biomate 5 spectrophotometer (Thermo Spectronic, Cambridge, U.K.). Reactions were monitored continuously for 2 min after a 10 s delay and were performed in parallel without plasma to correct for the nonenzymatic hydrolysis of the substrates. All the assays were performed in triplicate.

Basal PONs activity was assayed as previously described by Eckerson et al. (*30*) using 1 mM POX as substrate in a final volume of 800 μL of 50 mM Tris buffer (pH 8.0) containing 1 mM $CaCl_2$. The hydrolysis of POX, started by the addition of 50 μL of rat plasma, was

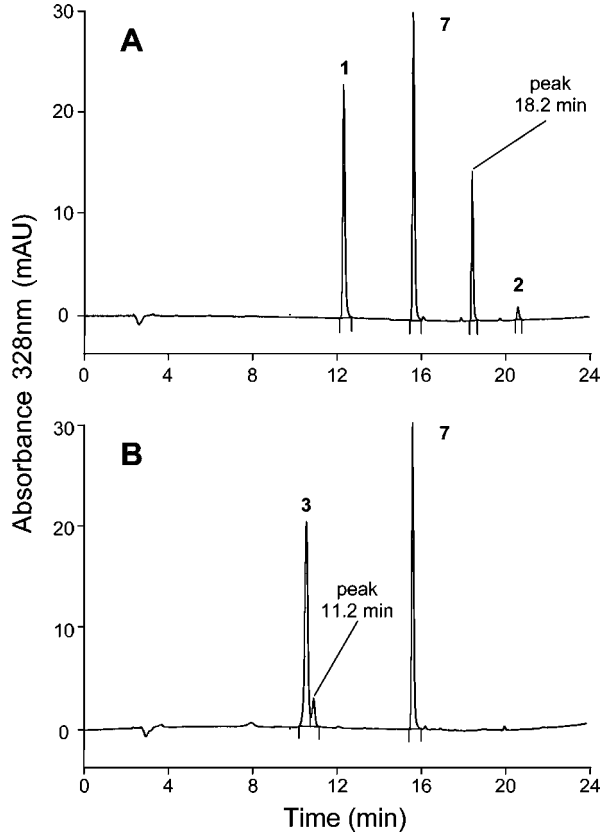

Figure 4. Representative HPLC-UV chromatograms (328 nm) obtained after incubation in rat plasma at 37 °C of (**A**) caffeic acid phenethyl ester for 30 min and (**B**) chlorogenic acid for 1 h.

monitored at 412 nm, and the activity was calculated using the molar extinction coefficient of the product (4-nitrophenol) at pH 8.0 (16900 M^{-1} cm^{-1}). Similarly, arylesterase activity was measured (*30*) using 1 mM phenylacetate as substrate and 5 μL of rat plasma, monitoring the absorbance of phenol at 270 nm. The activity was calculated using the difference between the molar extinction coefficients of phenol and phenylacetate (1310 M^{-1}·cm^{-1} at pH 8.0).

AChE and BChE activities were measured by the method described by Ellman et al. (*31*) with minor modifications. The reaction was started by adding 125 μL of the substrate (acetylthiocholine or butyrylthiocholine, both 4 mM) to 775 μL of 100 mM phosphate buffer (pH 8.0) containing 0.4 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 100 μL of rat plasma. The activity was monitored at 412 nm and was calculated using the molar extinction coefficient of 5-thio-2-nitrobenzoic acid at pH 8.0 (13600 M^{-1} cm^{-1}).

CEs activity was assayed according to a previously described method (*32*). Briefly, 10 μL of plasma was added to 970 μL of 100 mM phosphate buffer (pH 7.4), and reactions were started by the addition of 20 μL of the substrate (50 mM 1-naphthylacetate), following the formation of 1-naphtol at 322 nm (extinction coefficient 2200 M^{-1} cm^{-1} at pH 7.4).

Plasma esterases activity was also assayed in the presence of esterase inhibitors in the reaction mixture, at the following final concentrations: 0.1 mM DFP, 0.25% (w/v) NaF, 1 mM bis(*p*-nitrophenyl)-phosphate (BNPP), 10 μM eserine, 5 mM EDTA, and 0.1 mM POX.

These esterase inhibitors were used at the same concentrations to assay the hydrolysis of **2**. Rat plasma samples were preincubated with inhibitors for 10 min at room temperature before the addition of **2** at the final concentration of 50 μg/mL with 5% (v/v) ethanol. Samples (100 μL) were extracted, after 0 and 30 min incubation at 37 °C, and were analyzed by HPLC-UV.

In Vivo Study. The stability of **2** in rats was assayed in vivo as previously reported (*18*). Briefly, rats ($n = 4$) were housed in metabolic cages and urine was collected in glass tubes containing 20 μL 25% sodium azide and 500 μL 2 M acetate buffer pH 5.0, within 24 h before

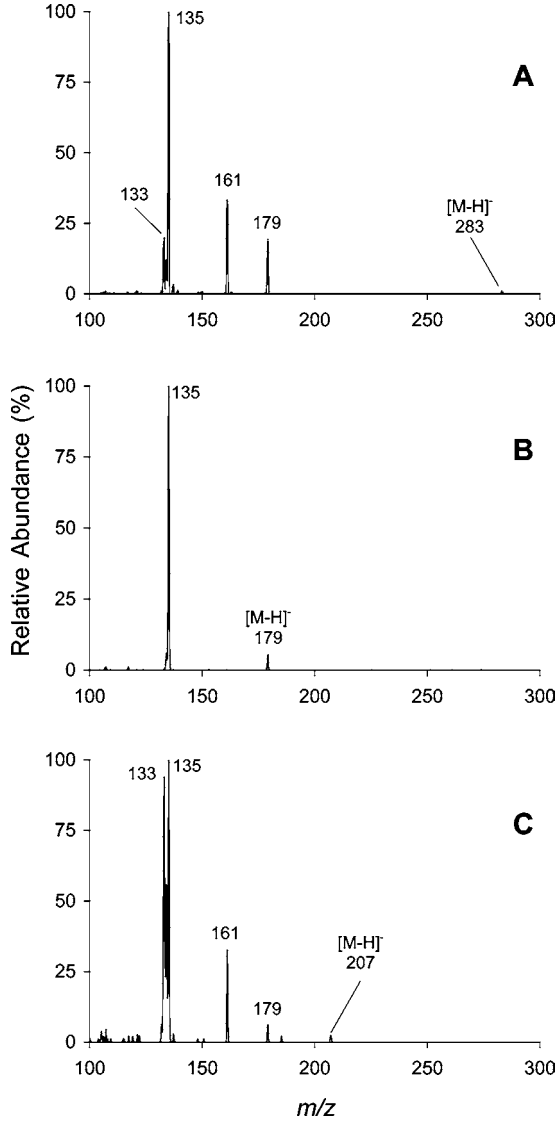

Figure 5. Product ion spectra of (**A**) caffeic acid phenethyl ester (*m*/*z* 283), (**B**) caffeic acid (*m*/*z* 179), and (**C**) the compound eluting at 18.2 min (*m*/*z* 207).

treatment and for 2 days at 24 h intervals after treatment. Urine samples were centrifuged at 3000*g* for 15 min at 4 °C to remove particulate material and were stored at −20 °C until analysis.

Since **2** is almost insoluble in water and high ethanol concentrations should not be used for in vivo experiments, **2** was dissolved in water/2-(2-ethoxyethoxy)ethanol/ethanol (50:40:10 v/v/v) at the concentration of 30 mg/mL and was administered by gavage at the dose of 100 mg/kg.

Preparation and LC-ESI-MS/MS Analysis of Urine Samples. Samples were prepared as previously described (*18*). Two hundred microliters of urine was added with 50 μL internal standard (**8**) working solution and 500 μL 10 mM HCl and then was extracted by adding 3 mL ethyl acetate. After 15 min vortexing followed by 15 min centrifugation at 3000*g*, the organic phase was evaporated to dryness under nitrogen flow and was stored at −20 °C. Samples were reconstituted just before analysis with 200 μL water/methanol/formic acid (49.5:49.5:1 v/v/v).

Analyses were performed using a Perkin-Elmer 200 micro HPLC pump system (Norwalk, CT) directly connected to the mass spectrometer through a turbo ionspray source. Samples were injected using a Perkin-Elmer 200 autosampler (thermostated at 4 °C) equipped with a 20 μL sample loop, and separations were achieved using the same column and chromatographic conditions described for the HPLC-UV method. Analyses were performed in negative ion mode by selected

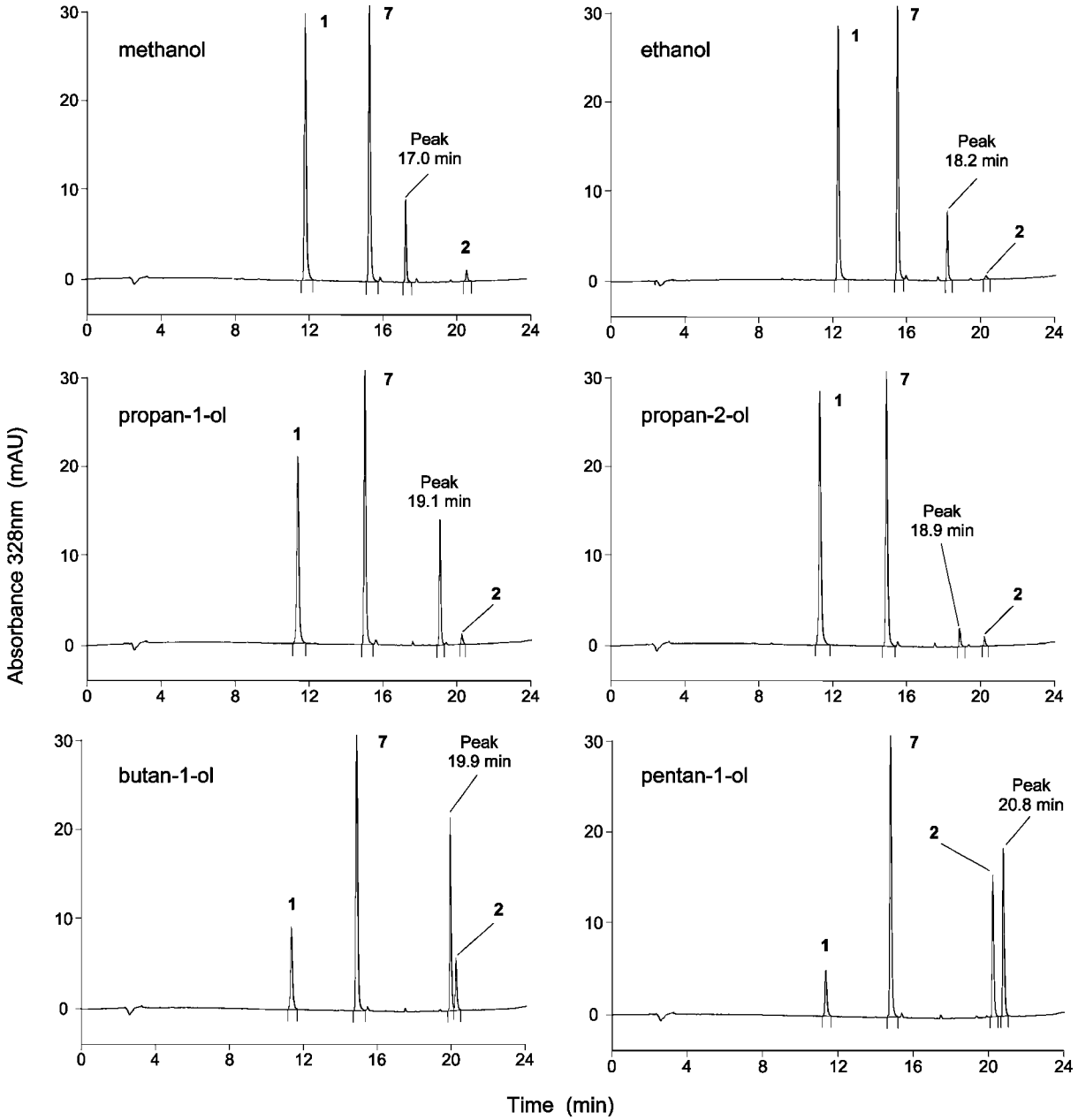

Figure 6. Representative HPLC-UV chromatograms (328 nm) obtained after incubation, in rat plasma at 37 °C for 30 min, of caffeic acid phenethyl ester vehicled with different alcohols (1% v/v, as final concentration).

Table 1. Activity of Rat Plasma Esterases as Affected by a Selection of Inhibitors[a]

	% activity[b]					
	DFP	NaF	BNPP	eserine	EDTA	POX
caffeic acid phenethyl ester hydrolase	nd[c]	24.3 ± 2.9	1.1 ± 0.2	76.2 ± 1.5	86.0 ± 3.5	nd[c]
caffeic acid phenethyl ester transesterase	nd[c]	36.0 ± 4.5	7.0 ± 0.6	90.8 ± 3.6	81.0 ± 7.4	nd[c]
paraoxonase	50.0 ± 2.2	109.6 ± 5.8	47.4 ± 1.7	99.7 ± 6.3	1.6 ± 1.2	45.8 ± 7.5
arylesterase	69.4 ± 3.1	3.2 ± 0.1	[d]	97.0 ± 6.6	20.5 ± 1.0	91.3 ± 13.5
acetylcholinesterase	0.5 ± 0.4	13.7 ± 0.5	97.0 ± 0.1	15.6 ± 0.7	92.5 ± 1.0	13.7 ± 0.3
butyrylcholinesterase	1.9 ± 0.8	15.5 ± 2.3	99.0 ± 7.7	27.1 ± 3.1	85.0 ± 9.2	32.5 ± 0.8
carboxylesterase	1.0 ± 0.4	24.7 ± 1.1	[d]	97.3 ± 3.8	100.4 ± 6.6	1.1 ± 0.5

[a] Results are expressed as mean values ± SD of three independent assays. [b] Enzymatic activity in the absence of inhibitors was taken as 100%. [c] nd, product not detected. [d] Not determined, because of the background interference caused by the high absorbance of BNPP at the selected wavelength.

reaction monitoring (SRM) using precursor to product ion transitions *m/z* 179 → 135 for **1**, 283 → 135 for **2**, 207 → 135 for **5**, 295 → 161 for **6**, and *m/z* 303 → 285 for the internal standard (**8**). Dwell time was set at 300 ms for each compound. All the other mass spectrometric parameters are reported elsewhere (*18*).

Peak integration of extracted ion chromatograms and all calculations of concentrations and regression parameters were performed using PE Sciex TurboQuan 1.0 software. For quantitative analyses, calibration curves in the range 0.1−10 μg/mL for **1** and 0.01−1 μg/mL for **2** were prepared in water/methanol (50:50 v/v) and were extracted as described

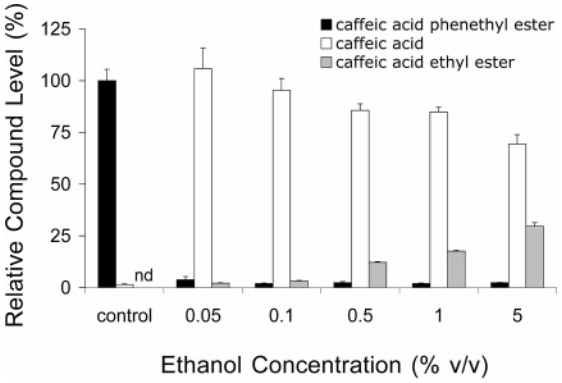

Figure 7. Effect of ethanol concentration on the hydrolysis and transesterification of caffeic acid phenethyl ester in rat plasma after 30 min incubation at 37 °C. Results, reported as mean ± SD ($n = 3$), are expressed as % of control (initial concentration of caffeic acid phenethyl ester). nd: not detected.

above. Standard curve equations were calculated using weighted ($1/y$) linear regressions of internal ratios (analyte/internal standard peak areas) versus analyte concentrations. The acceptance criteria were the same as reported for the HPLC-UV method. The limits of detection were 0.01 μg/mL for **1** and 0.002 μg/mL for **2**. Urine levels of **5** and **6** were estimated using the calibration curve of **2**.

RESULTS AND DISCUSSION

In our previous study (*18*), the bioavailability of **2** in the rat was investigated and its rapid degradation in plasma was observed and attributed to plasma esterases. The aim of this study was to validate this hypothesis and to verify if other caffeic acid esters (**3** and **4**) are rapidly hydrolyzed in both rat and human plasma. For this purpose, an HPLC-UV method was developed that allowed a good separation of the three caffeic acid derivatives and **1**, their expected hydrolysis product (**Figure 2**). **Figure 3** shows a 6 h time course analysis of **2**, **3**, and **4** levels upon incubation at 37 °C in rat plasma, human plasma, and phosphate buffer. Among the assayed caffeic acid esters, **2** was the only compound hydrolyzed under our experimental conditions and only in rat plasma. Moreover, **Figure 3** shows that **2** is enzymatically hydrolyzed, since it is stable in phosphate buffer (**Figure 3C**) and that the hydrolysis is due to an enzyme which is present in rat but not in human plasma (**Figure 3A** and **3B**). The stability of **3** and **4** even in rat plasma (**Figure 3D** and **3G**) could be attributed to a higher affinity of the hydrolytic enzyme toward the phenethyl moiety of **2** rather than **1** or the alcoholic components of **3** and **4** (quinic acid and 3-(3,4-hydroxyphenyl)lactic acid, respectively). As also shown in **Figure 4A**, in parallel with the disappearance of **2**, the formation of **1** was observed in rat plasma. In addition, an unidentified chromatographic peak, related to the degradation of **2**, appeared at a retention time of 18.2 min. To identify this compound, samples of the reaction mixture after 30 min incubation of **2** were separated by HPLC and the fractions eluting at 18.2 min were collected and characterized by mass spectrometry. The full-scan spectrum obtained by infusion of the unidentified compound showed *m*/*z* 207 as base peak. When product ion scan of *m*/*z* 207 was acquired, the mass spectrum (**Figure 5C**) showed a fragmentation pattern that was very similar to that of **2** (product ion scan of *m*/*z* 283) (**Figure 5A**). The product ion spectrum of **2** is characterized by the caffeate anion (*m*/*z* 179) and, as for **1** (product ion scan of *m*/*z* 179) (**Figure 5B**), by its decarboxylated product (*m*/*z* 135) and by ions at *m*/*z* 161 and 133, with the last two not resulting from the fragmentation pattern of **1**. These two ions can be supposed as products formed after cleavage of the ester bond with a rearrangement of the caffeoyl residue (*m*/*z* 161) and a subsequent loss of carbon monoxide (*m*/*z* 133). Product ion scan of *m*/*z* 179 and 161, after in-source collision-induced dissociation of **2**, showed *m*/*z* 135 and 133, respectively, as the only products obtained by their fragmentation (data not shown). These mass spectrometric data let us hypothesize that the unidentified compound was caffeic acid ethyl ester (**5**, **Figure 1**), formed by transesterification of **2** with ethanol, that was used as vehicle for the standard compound (5% v/v, as final concentration in the reaction mixture). This hypothesis was further confirmed when acetonitrile and dimethyl sulfoxide (1% v/v, as final concentration) were used as vehicles for **2**, and **1** was the only resulting product of the incubation in rat plasma (data not shown). It was also observed that incubation of **1** in rat plasma, under the same assay conditions used for **2**, did not produce any product. Furthermore, when in preliminary experiments **2** was incubated in rat plasma using aliphatic alcohols other than ethanol as vehicles (1% v/v, as final concentration), HPLC-UV analysis of each reaction mixture showed the formation of an unidentified compound eluting after **1** at a retention time which increased with longer chain alcohols (**Figure 6**). When mass spectrometric analyses were performed, the full-scan spectrum of each unidentified compound showed the base peak corresponding to the deprotonated molecule of the expected caffeic acid ester, and product ion spectra of all base peaks resulted in the same fragmentation pattern of **5** (data not shown).

Contrary to **2**, compounds **3** and **4** were almost stable in rat plasma, human plasma, and phosphate buffer (**Figure 3D**−**I**). Also for **3** (**Figure 3D**−**F**), incubations gave rise to an unidentified compound, eluting at 11.2 min close to the peak of **3** (11−14% of the initial concentration of **3** after 60 min) (**Figure 4B**). Since this peak was obtained with the same area ratio when **3** was incubated in rat and human plasma as well as in phosphate buffer, and since it showed the same base peak of **3** with a very similar fragmentation pattern when analyzed by mass spectrometry (data not shown), it cannot be attributed to an enzymatic reaction but it is probably due to an isomerization of **3**. Concerning **4**, a very low amount of **1** was produced in human plasma beginning at 180 min incubation (<4% of the initial concentration of **4** at 360 min) (**Figure 3H**), whereas **1** was not produced when **4** was incubated in rat plasma as well as in phosphate buffer (**Figure 3G** and **3I**).

Since 5% (v/v) ethanol is not a physiological concentration in plasma, ethyl transesterification was assayed by incubating **2** in rat plasma with decreasing ethanol concentrations (down to 0.05% v/v). **Figure 7** shows that **2** was almost completely hydrolyzed after 30 min incubation, independent of ethanol concentration, and that levels of **5** increased dose dependently with increasing ethanol concentrations, showing a contemporary decrease of **1**. Compound **5** was also obtained (2.2% of the initial amount of **2**) at the lowest tested ethanol concentration (0.05% v/v).

To identify the esterase involved, hydrolase/transesterase activities on **2** were assayed in the presence of a selection of specific esterase inhibitors and were compared with those of "classical" plasma esterases (PONs/arylesterases, ChEs, and CEs) (**Table 1**). Hydrolase/transesterase activities on **2** were only slightly affected by the presence of a PONs inhibitor such as EDTA (86% and 81% of residual activity, respectively). Moreover, although NaF slightly enhanced PONs activity (110%), probably because of a salt-stimulation effect (*33*), it decreased hydrolase/transesterase activities on **2** to 24% and

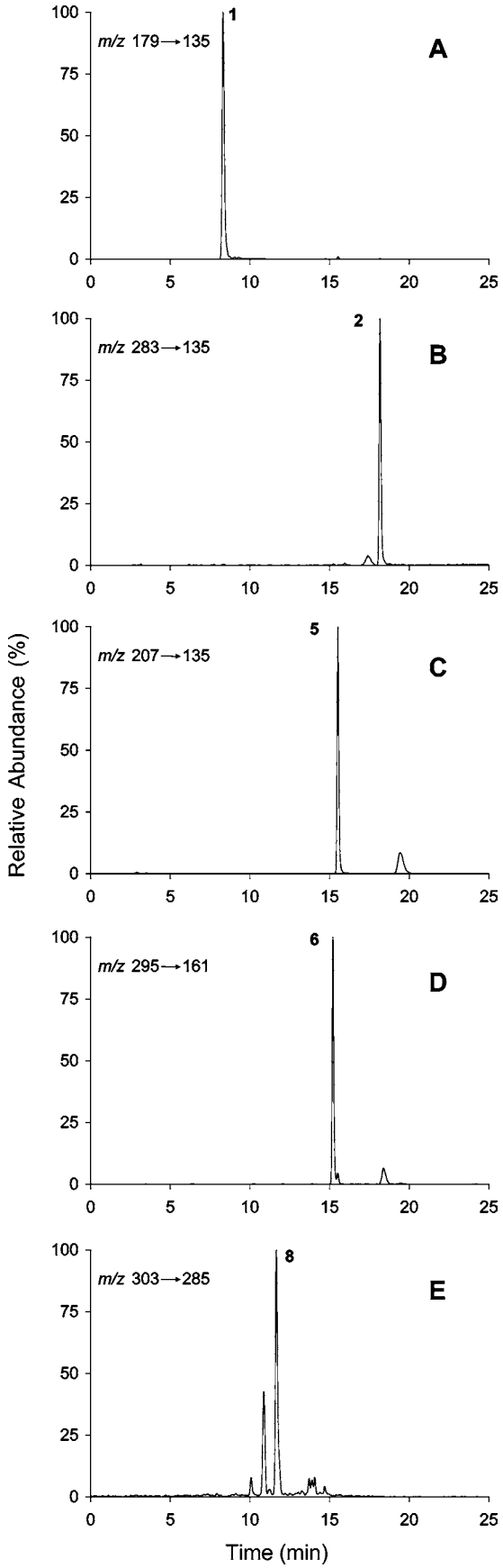

Figure 8. Representative LC-MS/MS (SRM) chromatograms of rat urine samples collected within 24 h after treatment with caffeic acid phenethyl ester at the dose 100 mg/kg: (**A**) caffeic acid, (**B**) caffeic acid phenethyl ester, (**C**) caffeic acid ethyl ester, (**D**) caffeic acid 2-(2-ethoxyethoxy)ethyl ester, and (**E**) taxifolin (internal standard).

Table 2. Levels of Caffeic Acid Phenethyl Ester, Caffeic Acid, Caffeic Acid Ethyl Ester, and Caffeic Acid 2-(2-Ethoxyethoxy)Ethyl Ester in Urine of Rats Treated with Caffeic Acid Phenethyl Ester at the Dose 100 mg/kg[a]

	control[b] (μg)	0–24 h (μg)	24–48 h (μg)
caffeic acid (**1**)	67.0 ± 21.9	3673 ± 1094	346.3 ± 39.0
caffeic acid phenethyl ester (**2**)	nd[c]	5.0 ± 2.3	0.4 ± 0.2
caffeic acid ethyl ester (**5**)[d]	nd[c]	18.5 ± 8.3	0.4 ± 0.2
caffeic acid 2-(2-ethoxyethoxy)-ethyl ester (**6**)[d]	nd[c]	8.2 ± 4.5	0.03[e]

[a] Results are expressed as mean values ± SD of four rats. [b] Urine samples collected within 24 h before treatment. [c] nd, not detected. [d] Estimated with the calibration curve of caffeic acid phenethyl ester. [e] Detected and estimated only in one sample.

36%, respectively. As for EDTA, hydrolase and transesterase activities on **2** were only slightly inhibited by eserine (76% and 91%, respectively), a typical ChEs inhibitor. These results suggest that hydrolysis and transesterification of **2** are not due to enzymes belonging to PONs and ChEs families. On the contrary, hydrolase/transesterase activities on **2** were completely inhibited by DFP and POX, two serine-hydrolase inhibitors, and by BNPP, a specific CEs inhibitor, indicating that both hydrolysis and ethyl transesterification activities are due to a rat plasma enzyme belonging to the CE family. This was further proved by the lack of hydrolysis products of **2** in human plasma, which normally contains PONs/arylesterases and ChEs but does not contain CEs (*27*).

To assess if **2** is hydrolyzed and transesterified also in vivo, rats were treated by gavage with **2** in the presence of 10% ethanol and 40% 2-(2-ethoxyethoxy)ethanol, and urine was collected up to 48 h and was analyzed by LC-MS/MS. A preliminary in vitro assay was performed using 2-(2-ethoxy-ethoxy)ethanol as solvent for **2** (1% v/v, as final concentration in rat plasma) to verify if the compound is transesterified also in the presence of this solvent. As a result, the HPLC-UV chromatogram showed the formation of **1** and a peak at 17.8 min, subsequently identified by mass spectrometry as caffeic acid 2-(2-ethoxyethoxy)ethyl ester (**6**, **Figure 1**). Therefore, in addition to compounds **1, 2**, and **5**, also **6** was determined in rat urine by LC-MS/MS (**Figure 8**). As shown in **Table 2**, compound **1** was found in pretreatment samples (urine of rats fed the standard diet), and its excretion dramatically increased in samples collected within 24 h after treatment and decreased thereafter. **2**, **5**, and **6**, not detected in the pretreatment samples, showed a peak level within 24 h after treatment, even if at a negligible ratio compared to **1**, and their levels decreased in the 24−48 h interval.

The enzymatic hydrolysis of esters, taken as drugs or as toxic environmental compounds, is a widely described reaction (*25*−*27*). In the present study, the enzymatic inhibition assay (**Table 1**) clearly showed that hydrolase/transesterase activities are both due to a rat plasma CE. The action of CEs, as well as of other esterases, is based on a two-step mechanism (*25*, *26*). In the first step, the carbonyl carbon of the substrate is attacked by the serine of the enzyme active site, forming a covalent acyl-enzyme intermediate with a consequential alcohol release. In the second step, the hydrolysis of the intermediate leads to the carboxylic acid product, returning the enzyme active site in its original state. During the hydrolysis, the presence of alcohols, which could potentially compete with water for the acyl-enzyme intermediate, may determine the formation of a new ester compound. The active site of CEs is placed inside a hydrophobic catalytic gorge (*25*). This may explain the formation of **5** also

in the presence of ethanol at low concentrations (0.05% v/v), since alcohols are generally preferred to water in hydrophobic interactions.

Transesterification reactions by CEs of both endogenous and exogenous substrates have also been described. Although it is a discussed hypothesis (*34*), it has been proposed that CEs are involved in intracellular cholesterol homeostasis by an acyl-CoA:cholesterol acyltransferase-like activity (*35*). Beyond this possible physiological role, CEs have shown to contribute to the formation of fatty acid ethyl esters during acute and chronic alcohol abuse (*36*). Fatty acid ethyl esters are the products of nonoxidative ethanol metabolism and have shown to be toxic and to cause tissue damage (*37*). With regard to exogenous compounds, hydrolysis with contemporary transesterification has been described only for few drugs. Several studies reported the formation of cocaethylene (benzoylecgonine ethyl ester), in both rats and humans, when alcohol is co-abused with cocaine (benzoylecgonine methyl ester), with a consequent increased toxicity and lethality (*38*). In humans, the transesterification of cocaine occurs mainly in the liver, by the action of specific CEs (*39*, *40*), as well as in other tissues (lung, kidney, and heart), but not in brain and serum (*41*). In addition to CEs, BChEs can be involved in cocaine detoxification determining the specific hydrolysis of the benzoyl ester bond (instead of the methyl ester bond hydrolyzed by CEs) and leading to the formation of ecgonine methyl ester (*42*), a pharmacologically inactive metabolite. However, transesterification reactions due to BChEs have not been reported.

The ethyl transesterification by CEs of two psychoactive drugs (meperidine and methylphenidate), potentially co-abused with alcoholic beverages, has been described to occur in rat liver preparations when deuterated ethanol (about 0.3% v/v) is used (*43*). To our knowledge, an ethyl transesterification reaction by plasma CEs in the presence of deuterated ethanol (from 0.05 to 2.5% v/v) has been reported to occur in the rat only for a potent and short-acting growth hormone secretagogue (*44*).

In our study, the hydrolysis of **2** in rat plasma leads to the formation of **1**, which has been shown to have important biological activities (*16*, *45*, *46*). The increased amount of **1** excreted in rat urine after administration of **2**, and the low amount of **2** in its unmodified form, showed that the absorbed amount of **2** is essentially hydrolyzed, even if it is not possible to discriminate the contribution of the different CEs (plasma, liver, intestinal, or of other tissues). The presence of **5** and **6** in rat urine showed that transesterification occurs also in vivo when alcohols are coadministered with **2**. Compound **5**, as well as other caffeic acid esters, has shown to possess antioxidant, anti-inflammatory, and antitumoral properties (*47*−*50*). This clearly represents a further complication in the evaluation of the in vivo activity of **2**, since these effects may be potentially due to different compounds.

In conclusion, our study demonstrates that **2** is hydrolyzed to **1** in rats, both in vitro (plasma) and in vivo. Thus, it could be difficult to define the real contribution of **2** and **1** to the observed biological activities, when **2** is used in vivo, since the two compounds may be similarly active (*16*). Our results suggest that in vivo biological activities of **2** should be more properly assayed and compared with those of **1** and that, in these studies, alcohols should be carefully used as solvents, since their use can give rise to new caffeic acid esters, also having bioactive properties.

ABBREVIATIONS USED

AChE, acetylcholinesterase; BNPP, bis(*p*-nitrophenyl)phosphate; BChE, butyrylcholinesterase; CE, carboxylesterase; ChE, cholinesterase; DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetic acid; PON, paraoxonase; POX, paraoxon-ethyl.

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Supporting Information Available: Precision, accuracy and linearity data of HPLC-UV and LC-MS/MS methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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