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Phenolic acids from beer are absorbed and extensively metabolized in humans☆

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Abstract

In spite of the wide literature describing the biological effects of phenolic compounds, scarce data are available on their absorption from diet. In the present work, we studied the absorption in humans of phenolic acids from beer, a common beverage rich in different phenolic acids with related chemical structures. Beer was analyzed for free and total (free+bound) phenolic acids. Ferulic, caffeic and sinapic acids were present in beer mainly as bound forms, while 4-hydroxyphenylacetic acid and *p*-coumaric acid were present mainly as free forms. Vanillic acid was present equally in the free and bound forms. Plasma samples were collected before and 30 and 60 min after beer administration and analyzed for free and conjugated phenolic acid content. A significant two- to fourfold increase in plasma levels of phenolic acids was detected with peak concentrations at 30 min after beer ingestion. 4-Hydroxyphenylacetic acid was present in plasma mainly as nonconjugated forms while *p*-coumaric acid was present equally as nonconjugated and conjugated forms. Ferulic, vanillic and caffeic acids were present in plasma predominantly as conjugated forms, with a slight prevalence of sulfates with respect to glucuronates. Our results indicate that phenolic acids from beer are absorbed from the gastrointestinal tract and are present in blood after being largely metabolized to the form of glucuronide and sulfate conjugates. The extent of conjugation is related to the chemical structure of phenolic acids: the monohydroxy derivatives showing the lowest conjugation degree and the dihydroxy derivatives showing the highest one.

Keywords: Beer; Phenolic acids; Human plasma; Glucuronides; Sulfates

1. Introduction

Oxidative stress is involved in the pathology of many diseases, such as atherosclerosis, diabetes, neurodegenerative diseases, aging and cancer. Dietary antioxidants may afford protection against oxidative stress-related diseases.

Beverages account for a very high proportion of dietary antioxidant intake in the Mediterranean diet [1]. Coffee is the main contributor, followed by red wine, fruit juice, beer, tea and milk.

Among dietary antioxidants, phenolics are by far the most abundant in most of diets. Epidemiological studies have suggested associations between the consumption of phenolics-rich food and the prevention of many human diseases associated with oxidative stress [2–5]. Based on their daily intake, which greatly exceeds that of other antioxidants (vitamin E, vitamin C, β-carotene), phenolic compounds may be a major factor in assuring the antioxidant potential of the diet and may contribute in maintaining the endogenous redox balance in humans. A major class of phenolic compounds is phenolic acids, which are widely distributed in the diet, mostly in fruits, vegetables, coffee, wine, beer and olive oil [6,7]. They occur in food mainly in esterified forms with organic acids, sugars and lipids [7]. The average phenolic acid intake has been reported to be in the order of 200 mg/day within a large range, depending on nutritional habits and preferences [2,6,7]. For individuals regularly consuming wine, coffee, beer and tea, these beverages will likely be the major sources of phenolics. However, the significance of this intake for metabolic effects and antioxidant status in vivo is affected by the bioavailability of these compounds.

Recently, a large amount of research has been done about the absorption of phenolic compounds [8]. Most of the studies concerning the absorption of phenolic acids from diet

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are carried out on rats, while relatively, few papers deal with the measurements of phenolic acids and their metabolites in human urine. The measurements of human plasma levels of phenolic acids after phenolics or phenolics-rich food administration are reported by a limited number of studies, as well as the measurements of intestinal absorption. Indirect evidence of phenolics absorption through the gut barrier is the increase in plasma antioxidant capacity after the consumption of phenolics-rich foods. This has been observed for a wide array of foodstuffs such as red wine [9–11], beer [12] and coffee [13]. Indeed, the knowledge of the effective, in vivo reachable concentrations is crucial to understand the significance of phenolic acids intake on human health. Moreover, phenolic acids undergo conjugation reactions in vivo with sulfate, glucuronate, *S*-adenosylmethionine or a combination [2]. The formation of the conjugated can dramatically alter the biological properties of the circulating metabolites [14–17]. Therefore, the characterization and measure of the circulating conjugated forms of phenolics should be accomplished as well. A very limited number of human studies have been carried out in which the nature of the conjugates has been established [18–24].

Recently, a renewal interest has been focused on beer, a common beverage rich in phenolic compounds with a moderate antioxidant activity coupled with a low ethanol content. Mild to moderate alcohol consumption is associated with beneficial healthy effects on the cardiovascular system [25–29]. The majority of more recent, large, population-based studies have observed that moderate drinking in the range of one to three drinks daily is associated with a rate of coronary disease 30–40% lower compared with nondrinking. The association between alcohol consumption and cardiovascular disease is not linear but "U shaped," with higher death rates found among those who abstain as well as those who drink an excess of six drinks a day [25]. Ethanol, in addition to its direct effect on platelet aggregation, HDL metabolism and fibrinolysis (all involved in the pathogenesis of cardiovascular diseases) could play an important role in the absorption of phenolic compounds [12]. Moreover, beer consumption seems to have no effect or even an inverse effect on total homocysteine concentration [30–32].

Previous study from our group indicated that beer is able to improve human plasma antioxidant capacity, likely through its phenolic component [12]. In the present work, we studied the absorption in humans of several phenolic acids, with related chemical structure, from beer, focusing on the measurements of plasma levels of free and conjugated forms of phenolic acids.

2. Materials and methods

2.1. Materials

β-Glucuronidase (EC 3.2.1.31, type IX A from *E. coli*), sulfatase (type H-1 from helix pomatia, containing β-glucuronidase), D-saccharic acid 1,4-lactone monohydrate, chlorogenic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were from Sigma (St. Louis, MO, USA). *o*-Coumaric acid and 4-hydroxyphenylacetic acid were from Extrasynthese (Genay Cedex, France). Supelclean LC-SAX SPE cartridges (1-ml tubes) were from Supelco (Bellefonte, PA, USA). All organic solvents were obtained from Carlo Erba (Milan, Italy). For HPLC analysis, ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) was used.

Stock solutions of standard phenolic acids were prepared in methanol (1 mg/ml), stored at −80 °C and used within 1 week. Working standard solutions were prepared daily by dilution in sample buffer (1.25% glacial acetic acid, 7% methanol in water).

2.2. HPLC instrumentation

Phenolic acids in food and human plasma extracts are routinely detected in our laboratory by HPLC-ECD [33,34]. The HPLC consists of a Perkin-Elmer Series 4 Liquid Chromatograph (Perkin-Elmer, Norwalk, CT, USA) with gradient pump, column thermoregulator and autosampling injector (Gilson, Beltline, Middleton, WI, USA) equipped with electrochemical coulometric detector (Coulochem II, ESA, Bedford, MA, USA). A Turbochrom chromatography work station software was used for data processing. Operating conditions were as follows: column temperature, 30 °C; flow rate, 1 ml/min; injection volume, 50 μl; electrochemical detection at +600 mV; sensitivity range, 100 nA; filter, 2 s.

Chromatographic separations were performed on a Supelcosil LC-18 C_{18} column (5.0-μm particle size, 250×4.6 mm ID) including a guard column (C_{18}, 5.0-μm particle size, 20×4.0 mm ID; both Supelco). For gradient elution, mobile phase A and B were employed. Solution A contained 1.25% glacial acetic acid in water; solution B was absolute methanol. The following gradient was used: 0–25 min, from 93% A, 7% B to 76% A, 24% B, linear gradient; 26–45 min, 76% A, 24% B; 46–53 min, from 76% A, 24% B to 55% A, 45% B, linear gradient; 54–55 min, 55% A, 45% B; 56–86 min, 93% A, 7% B. Prior to HPLC analysis, all samples were filtered using Millex-HV filters (Millipore) with 0.45-μm pore size.

2.3. Study design and samples collection

The study was approved by the Ethical committee of National Institute for Food and Nutrition Research. Ten healthy subjects (six males, four females), aged between 25 and 45 years, with a body mass index between 19 and 29 kg/m^2, were recruited. Subjects were nonsmokers, either nondrinkers or social drinkers (<28 and <14 g ethanol per day, for males and females, respectively) and they were not taking dietary antioxidant supplements. They were asked to avoid coffee, wine, beer, tea and fruit juices the day preceding the experiment. Fasting subjects received in the morning 500 ml of beer in combination with 27 g of

Table 1
Phenolic acids content (mg/L) of beer before and after hydrolysis[a]

	Free phenolic acids	Total phenolic acids	Increase[b] (fold)
Protocathecuic acid	tr[c]	tr[c]	–
4-OH-phenylacetic acid	0.64±0.06	0.84±0.10[d]	1.3
Chlorogenic acid	tr[c]	–	–
Vanillic acid	0.63±0.03	1.2±0.22[d]	1.9
Caffeic acid	0.21±0.03	1.13±0.13[d]	5.4
Syringic acid	tr[c]	tr[c]	–
p-Coumaric acid	0.74±0.05	1.06±0.17[d]	1.4
Ferulic acid	2.34±0.06	13.50±0.75[d]	5.8
Sinapic acid	0.22±0.03	2.81±0.06[d]	12.8

[a] Phenolic acids content was measured in nonhydrolyzed samples and in samples submitted to alkaline hydrolysis as reported in Materials and methods. Values are means±S.D. ($n=3$).

[b] The increase in phenolic acids content measured after hydrolysis is expressed as fold increase in respect to the value measured in non-hydrolyzed samples.

[c] tr, traces (<0.1 mg/L).

[d] Values are significantly different from those measured in non-hydrolyzed samples ($P<.05$ by ANOVA).

crackers. Crackers were given to avoid undesirable effects of ethanol in fasting conditions. The beer used in this study was an Italian brand containing 4.5% ethanol. Crackers were from an Italian brand (wheat flour, brewer's yeast, salt, sodium bicarbonate) and did not contain added fat. The content of phenolic acids of crackers was not relevant with respect to the content of phenolic acids of beer, being in the range 0.9–6.8% of the different ingested phenolic acids. In another set of experiments on a subsample of three subjects, 500 ml of beer, without crackers, or 27 g of crackers without beer were administered in fasting conditions, as control. Blood was collected into EDTA-containing vacutainers (1 mg/ml) just before ($t=0$) and 30 and 60 min after ingestion of beer, crackers or beer plus crackers. Plasma was immediately prepared by centrifugation at 1000×*g* for 20 min at 4 °C. Plasma aliquots (0.5 ml) were acidified at pH 3.0 with 18 μl of 4 N HCl and stored at −80 °C.

2.4. Beer and crackers analyses

Beer samples were degassed by sonication and treated for free and total (free + bound) phenolic acid determination as already reported [33]. The total antioxidant activity of beer was measured as previously described [34]. The antioxidant activity of beer is expressed (as conventional for food) as Trolox equivalents (TE), defined as the antioxidant capacity of 1.0 mM Trolox, a well-known antioxidant.

Crackers were smashed and powdered in a Waring Blendor. Aliquots of 250 mg of powdered crackers were suspended in 1 ml of distilled water and treated for free and total (free + bound) phenolic acid content [33].

2.5. Treatment of plasma samples

Plasma samples were treated for phenolic acid extraction essentially as already described [35], with minor modifications concerning the enzymes used to hydrolyze conjugated phenolic acids. Plasma samples (1-ml aliquots) from each subject were thawed and treated according to one of the four following procedures: no hydrolytic treatment, to detect free, nonconjugated phenolic acids; β-glucuronidase treatment, to hydrolyze glucuronide conjugates; sulfatase treatment, to hydrolyze sulfate conjugates; β-glucuronidase plus sulfatase treatment, to hydrolyze glucuronide conjugates, sulfate conjugates and sulfoglucuronide conjugates.

o-Coumaric acid was selected as an internal standard due to the absence of detectable amounts of this compound in human plasma samples before and after beer administration, with or without enzymatic hydrolysis treatments.

2.5.1. Nonconjugated phenolic acids

Plasma sample was added with *o*-coumaric acid (200 ng) as an internal standard and treated as already described for free phenolic acid detection [35]. After deproteinization and extraction, the residue was dissolved in 0.5 ml of water, vortexed for 5 min, then processed for solid-phase extraction (SPE) as described in the following discussions. This procedure allows the detection of free, nonconjugated phenolic acids in plasma sample.

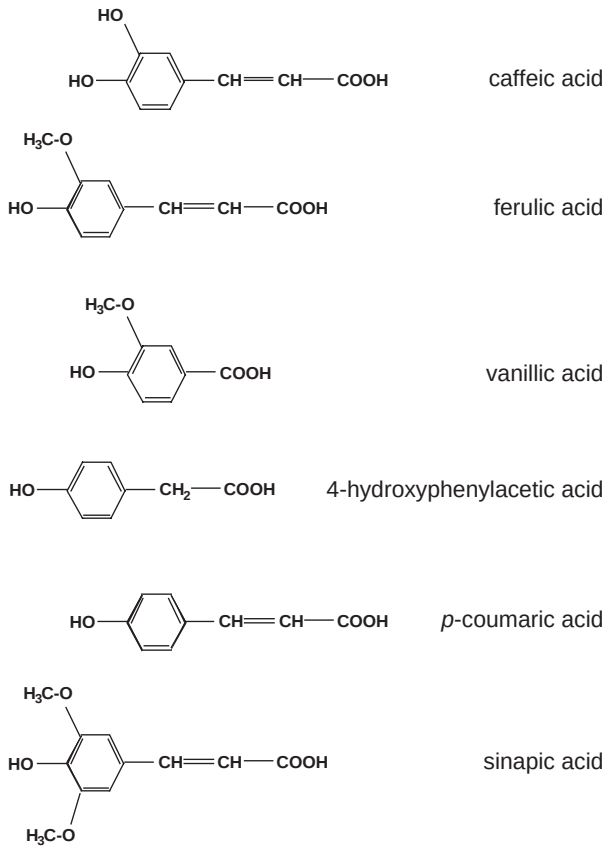


Fig. 1. Chemical structures of the main phenolic acids present in beer. Caffeic acid: 3,4-dihydroxycinnamic acid; ferulic acid: 4-hydroxy-3-methoxycinnamic acid; vanillic acid: 4-hydroxy-3-methoxybenzoic acid; *p*-coumaric acid: 4-hydroxycinnamic acid; sinapic acid: 3,5-dimethoxy-4-hydroxycinnamic acid.

2.5.2. Glucuronides

Plasma sample was added with *o*-coumaric acid (200 ng) as an internal standard and deproteinized with ethanol [35]. The dried residue obtained after deproteinization was dissolved in 0.5 ml of 0.2 M K-phosphate buffer, pH 6.8, and vortexed for 5 min. The sample was added with 50 μl of β-glucuronidase (776 U) dissolved in the same buffer and incubated in a water bath at 37 °C for 2 h. At the end of incubation, the pH was brought to 3.0 with 52 μl of 1 N HCl. After addition of 300 mg NaCl, sample was extracted three times with ethyl acetate as already reported [35]. The final residue was dissolved in 0.5 ml of water, vortexed for 5 min, then processed for SPE as described below. This procedure allows the measure of free plus glucuronide conjugates of phenolic acids. The amount of glucuronides in plasma samples was calculated by subtracting the value of free, nonconjugated phenolic acid content obtained with the above reported procedure.

2.5.3. Sulfates

To hydrolyze sulfate conjugates, sulfatase type H-1 from Sigma, which also contains β-glucuronidase activity, was preferred to a pure sulfatase, due to its high cost and large number of experiments. Moreover, according to Hackett and Griffiths [36], the activity of β-glucuronidase can be totally inhibited by performing the enzymatic reaction in the presence of D-saccharic acid 1,4-lactone. Plasma sample was added with *o*-coumaric acid (200 ng) as an internal standard and deproteinized with ethanol [35]. The dried residue obtained after deproteinization was dissolved in 0.5 ml of 0.1 M Na-acetate buffer, pH 5.0, and vortexed for 5 min. The sample was added with 50 μl of D-saccharic acid 1,4-lactone monohydrate (26 mM final concentration) and 50 μl of sulfatase (containing 70 U sulfatase and 2325 U β-glucuronidase), dissolved in the same buffer, and incubated in a water bath at 37 °C for 2 h. At the end of incubation, the pH was brought to 3.0 with 28 μl of 1 N HCl. After addition of 300 mg NaCl, sample was extracted three times with ethyl acetate as already reported [35]. The final residue was dissolved in 0.5 ml of water and vortexed for 5 min then processed for SPE as reported below. This procedure allows the measure of free plus sulfate conjugates of phenolic acids. The amount of sulfates in plasma samples was calculated by subtracting the value of free, nonconjugated phenolic acid content obtained with the above reported procedure.

2.5.4. Total phenolic acids

Plasma samples were submitted to sequential enzymatic hydrolysis with β-glucuronidase, followed by sulfatase treatment. Plasma sample was added with *o*-coumaric acid (200 ng) as an internal standard and deproteinized with ethanol as previously described [35]. The dried residue obtained after deproteinization was dissolved in 0.5 ml of 0.2 M K-phosphate buffer, pH 6.8, and vortexed for 5 min. The sample was added with 50 μl of β-glucuronidase dissolved in the same buffer (corresponding to 776 U) and incubated in a water bath at 37 °C for 2 h. At the end of incubation, the pH was brought to 5.0 with 30 μl of 1 N HCl. After addition of 50 μl of sulfatase (containing 70 U sulfatase and 2325 U β-glucuronidase), sample was incubated in a water bath at 37 °C for 2 h. At the end of incubation, the pH was brought to 3.0 with 28 μl of 1 N HCl. After addition of 300 mg NaCl, sample was extracted three times with ethyl acetate as already reported [35]. The final residue was dissolved in 0.5 ml of water, vortexed for 5 min, then processed for SPE as reported below. This procedure allows the detection of total phenolic acids (nonconjugated, glucuronides, sulfates and possible mixed sulfate/glucuronide conjugates). For each single phenolic acid, the amount obtained with this incubation, subtracted the amount of the respective nonconjugated, glucuronides and sulfates obtained with the above reported procedures, should represent the amount of mixed sulfate/glucuronide conjugates in the sample.

2.5.5. Solid-phase extraction

Solid-phase extraction of all treated plasma samples was carried out as follows. The residue dissolved in 0.5 ml of water obtained as described in the previous discussions was brought to pH 7–8 with 2 μl of 1 N NaOH and passed through the LC-SAX tube preconditioned with 1 ml of absolute methanol and 2 ml of water. The tube was then washed with 1 ml of water. Phenolic acid elution was obtained with 1 ml of buffer containing 1 N acetic acid/MeOH (90:10). The eluant was immediately brought to pH 3 with 6 μl of 4 N NaOH, filtered and aliquot (50 μl) injected into the LC system.

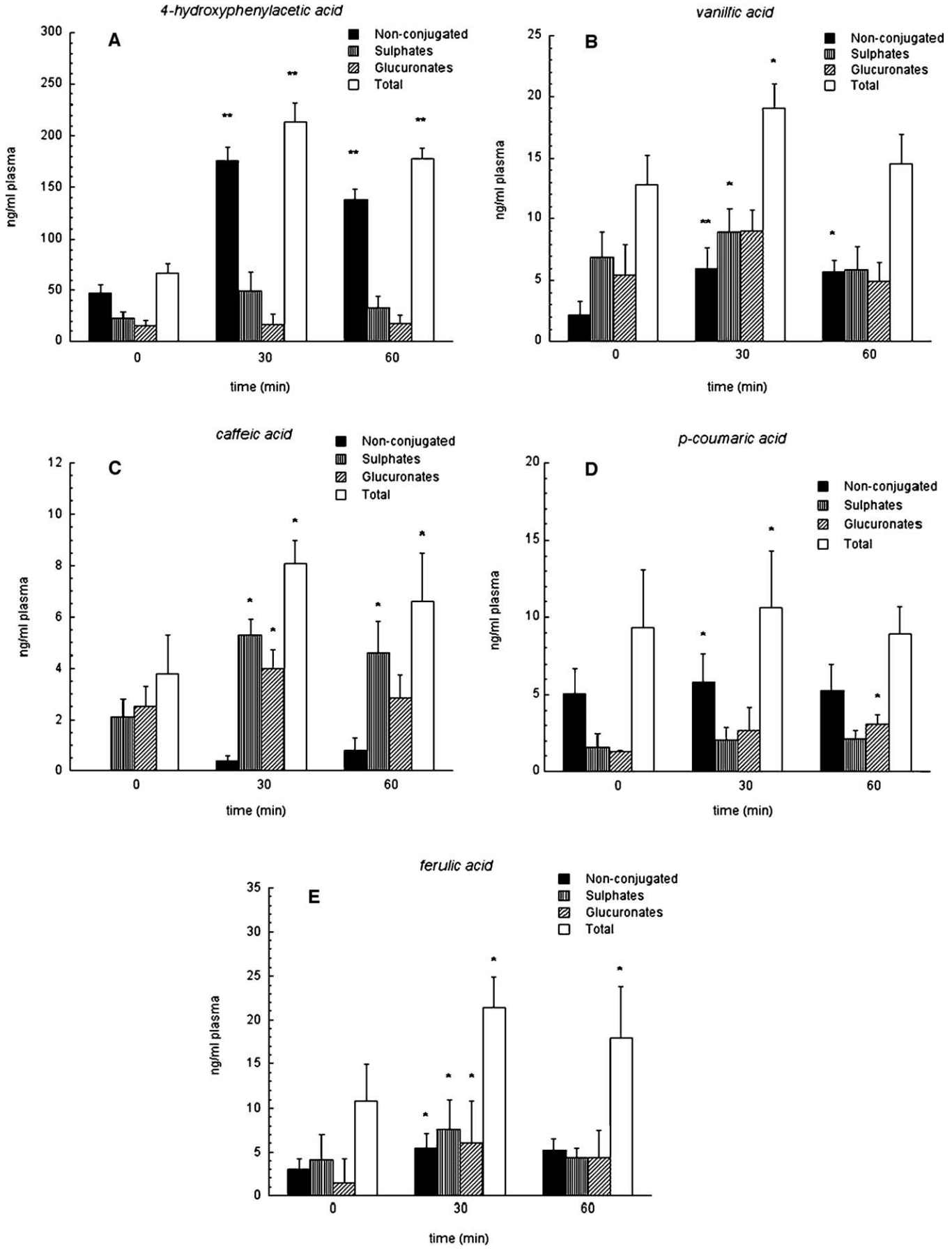
2.5.6. Recovery

The overall procedures allow an almost complete recovery of all phenolic acids under study, as found by recovery experiments performed adding known amounts of pure compounds to plasma samples. In particular, recovery was in the range 86.2±8.6% to 102.1±5.4% for all phenolic acids tested (4-hydroxyphenylacetic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, isoferulic acid and *o*-coumaric acid) ($n=4$).

2.6. Data evaluation, quantitation and statistical analysis

For calibration curve, appropriate volumes of the stock standard solutions were diluted with sample buffer. Three replicates of standards at four concentration levels (20, 100, 200 and 500 ng/ml) were analyzed. Calibration curve was determined on each day of analysis. For quantitative determination, peak areas in the sample chromatograms were correlated with the concentrations according to the calibration curve.

Data presented are mean±S.D. or standard error as specified. Statistical analysis was performed by a one-factor analysis of variance (Scheffe's method) for multiple comparison or paired *t* test as specified, using a statistical


4-hydroxyphenylacetic acid
A
Non-conjugated
Sulphates
Glucuronates
Total
ng/ml plasma
time (min)
vanillic acid
B
caffeic acid
C
p-coumaric acid
D
ferulic acid
E

package running on a PC (Stat View 4.01, Abacus Concepts, Berkeley, CA, USA). Probability value of .05 was considered statistically significant.

3. Results

Beer contains an appreciable amount of phenolic compounds that contribute to the overall antioxidant activity of the product. The beer used in this study has an antioxidant activity of 0.93±0.07 TE, of the same order reported in the literature [12]. Table 1 shows the content of free (nonhydrolyzed beer) and total (free+bound, hydrolyzed beer) phenolic acids and their derivatives in beer. 4-Hydroxyphenylacetic acid and *p*-coumaric acid were present mainly as free forms (76.2% and 69.8% of total, respectively). On the contrary, caffeic, ferulic and sinapic acids were present mainly as conjugated forms, the free, nonconjugated forms amounting to 18.6%, 17.3% and 7.8% of total, respectively. Vanillic acid was present in beer equally as bound and free forms, the latter amounting to 52.5% of total. Ferulic acid was by far the most abundant phenolic acid in beer, both as free and bound forms. Our results are in agreement with the data from the literature [37,38]. Fig. 1 shows the chemical structures of phenolic acids under study.

In order to study the absorption of phenolic acids from beer, plasma samples were collected before and after beer plus cracker administration and analyzed for content of both free (nonconjugated) and conjugated phenolic acids. Four different procedures were used in order to detect (a) nonconjugated phenolic acids, (b) glucuronide conjugates, (c) sulfate conjugates and (d) total phenolic acids (nonconjugated+glucuronide conjugates+sulfate conjugates+sulfoglucuronide conjugates).

Beer plus cracker administration resulted in a fast increase of phenolic acid concentrations in plasma (Figs. 2A–E). Fig. 2A shows the levels of 4-hydroxyphenylacetic acid before and 30 and 60 min after administration. The concentration of total 4-hydroxyphenylacetic acid increased three- to fourfold at both 30 and 60 min with respect to time 0. Most of the 4-hydroxyphenylacetic acid was present as nonconjugated form (70.8%, 82.2%, 77.4% of total at time 0, 30 and 60 min, respectively). A slight, although not significant, increase in the concentration of sulfate conjugates of 4-hydroxyphenylacetic acid was also measured at 30 and 60 min, with respect to time 0, while the small amount of glucuronate conjugates measured at time 0 remained unchanged during the time.

Fig. 2B shows the plasma levels of vanillic acid as measured before and 30 and 60 min after beer plus cracker consumption. A significant increase in nonconjugated form, sulfate conjugates and total vanillic acid was observed at 30 min after beer plus cracker ingestion with respect to time 0. A slight, although not significant, increase in the concentration of glucuronate conjugates of vanillic acid was also observed at 30 and 60 min with respect to time 0. Unlike 4-hydroxyphenylacetic acid, vanillic acid was present in plasma mainly as conjugated forms, the nonconjugated forms amounting to 17.2%, 31.6% and 39.3% of total at time 0, 30 and 60 min, respectively.

As shown in Fig. 2C, the bulk of caffeic acid was present in plasma as sulfate and glucuronate conjugates, the nonconjugated forms amounting to 0%, 4.4% and 12.7% of total at time 0, 30 and 60 min, respectively. At 30 min after beer plus cracker administration, a significant increase in sulfate conjugates, glucuronate conjugates and total caffeic acid was observed with respect to time 0, while at 60 min after ingestion, a significant increase in sulfate conjugates and total caffeic acid was measured with respect to time 0.

A significant increase in nonconjugated and total *p*-coumaric acid was observed 30 min after beer plus cracker administration with respect to time 0, the nonconjugated forms amounting to 54.4%, 54.7% and 59.5% of total at time 0, 30 and 60 min, respectively (Fig. 2D). Among conjugated derivatives, a significant increase in glucuronate derivatives was observed at 60 min after ingestion with respect to time 0, while the increase of sulfate conjugates measured at 30 and 60 min after ingestion did not reach statistical significance.

Finally, Fig. 2E shows the plasma levels of ferulic acid as measured before and 30 and 60 min after beer plus cracker consumption. A significant increase in nonconjugated form, sulfate conjugates, glucuronate conjugates and total ferulic acid was observed at 30 min after beer plus cracker administration with respect to time 0, the nonconjugated forms amounting to 27.8%, 25.2% and 29.0% of total at time 0, 30 and 60 min, respectively. Further, total ferulic acid was significantly higher at 60 min after ingestion with respect to time 0.

We failed to quantitatively measure sinapic acid in plasma samples due to either the low amount present in the samples or the interference from an unknown component in the chromatograms.

Administration of crackers alone did not result in any significant increase of plasma phenolic acid levels at both 30 and 60 min with respect to time 0. Moreover, plasma levels of phenolic acids after beer plus cracker consumption were found similar to those measured after administration of beer alone in three different subjects (data not shown).

Fig. 2. Plasma phenolic acids levels in 10 different subjects before and after beer plus cracker consumption. Plasma samples separated from blood collected just before (time 0) or after (30 min and 60 min) beer plus cracker administration were analyzed for nonconjugated and conjugated phenolic acids as reported in Materials and methods. (A) 4-Hydroxyphenylacetic acid; (B) vanillic acid; (C) caffeic acid; (D) *p*-coumaric acid; (E) ferulic acid. Values are means±S.E. * $P \leq .05$ with respect to time 0 by paired *t* test. ** $P \leq .01$ in respect to time 0 by paired *t* test.

4. Discussion

In the present study, we were able to demonstrate a significant rise in both free and conjugated (glucuronates and sulfates) phenolic acid levels in human plasma after beer administration, with maximum absorption peak at 30 min. The rapid absorption of phenolic acids indicates that it probably takes place in the proximal part of the intestine. Due to the presence of both free and bound forms of phenolic acids in beer, plasma phenolic acids may be derived from the direct absorption of the free forms present in beer, by in vivo hydrolysis of the bound forms present in beer as well, or both. On this regard, phenolic acids have been recently described to be absorbed from their bound forms present in the human diet [35,39–41]. Both glucuronidation and sulfatation occurred at various extents during absorption of phenolic acids from beer. Glucuronidation and sulfatation are two well-known ways of detoxification, leading to increased solubility of compounds, which promote their excretion in urine and bile. The linkage of glucuronic acid with phenolic acids might involve either the carboxyl group in the side chain (ester linkage) or the hydroxyl group on the aromatic ring (ether linkage), while sulfotransferases catalyze the sulfate conjugation of phenolic hydroxyl group. From our data, most of the 4-hydroxyphenylacetic acid (>70% of the total) was present in blood as nonconjugated form, and this is in agreement with the hydrophilic feature of this compound, due to the presence of a polar acetyl group and one hydroxyl group on the aromatic ring. *p*-Coumaric acid, with a propenoic side chain and a hydroxyl group on the aromatic ring, was present as free, nonconjugated form at levels lower than that observed for 4-hydroxyphenylacetic acid (54–59% of the total), but considerably higher than those observed for ferulic, vanillic and caffeic acids. These three phenolic acids were present in blood mainly as conjugated forms, with a slight prevalence of sulfates with respect to glucuronate forms. The presence of a methoxyl group in addition to the hydroxyl group on the aromatic ring of both vanillic acid and ferulic acid decreases their hydrophilicity, with respect to both 4-hydroxyphenylacetic acid and *p*-coumaric acid, and may account for the extensive conjugation observed (61–83% of the total, for vanillic acid; 71–75% of the total, for ferulic acid). Similar results have been reported after ingestion of ferulic acids in rats, with plasma conjugated ferulic acid amounting to 76% of the total ferulic acid [42]. Remarkably, caffeic acid, in spite of the presence of two hydroxyl groups in the ortho position on the aromatic ring (3,4-dihydroxyl moiety), was present in plasma mainly as conjugated forms (87–100% of the total), with a prevalence of sulfates, with respect to glucuronate, after beer ingestion. We already observed a similar behavior for plasma caffeic acid after coffee drinking in human [35].

Phenolic acids such as vanillic and caffeic acids have been reported to inhibit human sulfotransferases activity in in vitro experiments [43,44]. Our results clearly indicated that inhibition of sulfotransferases activity by phenolic acids did not occur in vivo, at least in the range of concentration of this study, and demonstrated that once absorbed, dietary phenolic acids are extensively conjugated to sulfate and glucuronate. Differences in the degree of conjugation are likely related to the different chemical features of the phenolic acids under study and may reflect differences in the activity and specificity of both glucuronosyltransferases and sulfotransferases family toward different phenolic acids. In particular, in our study, the monohydroxy derivatives showed the lowest degree of conjugation and the dihydroxy derivative showed the highest one, while the 3-methoxy-4-hydroxy-derivatives (vanillic and ferulic acids) fell in the middle. On this regard, the presence of caffeic acid in human plasma almost exclusively as conjugated forms, in particular as sulfates, may be explained by the fact that a sulfotransferase form present in the human intestine, SULT1A3, displays a high activity on catechol group (3,4-dihydroxy moiety) [2]. Further, human intestine glucuronosyltransferases appeared to be especially effective in conjugating 3,4-dihydroxy structure (catechol unit) [45]. Taking into account that the antioxidant action as well as the prooxidant toxicity of caffeic acid is especially related to its 3,4-dihydroxy structure [46,47], it is of interest to note that the conjugating enzymes in human intestine appear to be especially effective in conjugating this structure. This would imply that upon glucuronidation and sulfation along the transport across the intestinal border, the catechol-type phenolic compounds loose a significant part of their biological activity. The effect of the carbon side chain on conjugation might also be considered. From our data, *p*-coumaric acid, with a propenoic side chain, was conjugated to a higher degree with respect to 4-hydroxyphenylacetic acid, which possesses an acetyl group instead of the propenoic group. However, only minor differences in the extent of conjugation was observed between ferulic acid, with a propenoic side chain, and vanillic acid, with a carboxyl group instead of a propenoic side chain.

From our data, for each single compound, the amount of total phenolic acid measured was not significantly different from the theoretical amount calculated by addition of nonconjugated, glucuronide and sulfate conjugates, thus, suggesting that mixed sulfate/glucuronide conjugates are present, if any, at very low level.

Perhaps, the most relevant conclusion of our study is that the plasma concentration of conjugated forms of vanillic, ferulic and caffeic acids largely exceeds that of nonconjugated forms. Thus, for these compounds, phenolic metabolites will likely be the molecules responsible for any biological activity in vivo.

The antioxidant activity and biological effects of caffeic, ferulic, vanillic and *p*-coumaric acids have been widely studied and described in the literature in the last decade. Recently, 4-hydroxyphenylacetic acid has been described to scavenge reactive oxygen and nitrogen species both in vitro and in vivo [48–50]. However, scarce or no information is

available concerning the potential activity of the metabolites of phenolics. In fact, glucuronidation and sulfatation may modify the biological activity of the parent, nonconjugated molecules. As above mentioned, the conjugation of the catechol unit might result in a partial lost of biological activity for those phenolics bearing a 3,4-dihydroxy unit. For quercetin, one of the most abundant flavonoids in the human diet, it has been described that the conjugated forms, quercetin glucuronides and quercetin 3-*O*-sulfate, still retain antioxidant activity, although to a less extent with respect to quercetin [14]. The antioxidant activity of ferulic acid glucuronide, which has not only the hydrophobic ferulic acid moiety but also a hydrophilic sugar moiety, is stronger than that exhibited by ferulic acid [16].

Even if glucuronidation and sulfatation are the most important pathways, the presence of other metabolic forms cannot be completely ruled out. However, in a previous study on the absorption of phenolic acids from coffee in humans, we failed to demonstrate the presence of conjugated forms of caffeic acid different from glucuronate/sulfate conjugates in plasma [35].

In the present study, plasma phenolic acids reached 0.11 μM concentrations for vanillic acid and ferulic acid at 30 min after ingestion and values in the range of 0.05–0.07 μM for caffeic acid and *p*-coumaric acid at 30 min after ingestion. 4-Hydroxyphenylacetic acid reached relatively high concentration in plasma, amounting to 1.40 and 1.17 μM at 30 and 60 min, respectively. 4-Hydroxyphenylacetic acid is a metabolite of tyrosine and various phenolic compounds [48]. On this regard, beer has been reported to contain discrete amount of tyrosine [51]. Therefore, 4-hydroxyphenylacetic acid recovered in plasma at 30 and 60 min might derive not only from the direct absorption of 4-hydroxyphenylacetic acid present in beer, but also from the postabsorption metabolism of phenolic acids and tyrosine present in beer as well. Noteworthy, no appreciable increase in plasma level of 4-hydroxyphenylacetic acid was observed at 30 and 60 min after ingestion of crackers alone with respect to time 0, as well as for all the other phenolic acids under study.

Although the concentrations of phenolic acids measured in plasma seems to be quite low, it must be considered that they were obtained after a single dose of 500 ml of beverage, containing about 10 mg in total of the phenolic acids under study. This value is quite far from the average total daily intake of phenolic acids, which has been calculated to be in the order of 200 mg/day [2,7]. Synergistic effects might also occur in vivo. Moreover, an accumulation of phenolic acids and their metabolites might occur in many tissues (lung, hearth, liver) as described for 3-palmitoyl-catechin, epigallocatechin gallate and resveratrol [52–54].

The antioxidant and biological activity of the different metabolites of phenolic acids with respect to the parent compounds and possible synergistic effects in vivo will be focused in further studies.

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