Plasma Levels of Caffeic Acid and Antioxidant Status after Red Wine Intake

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The aim of this study was to evaluate the bioavailability of caffeic acid and the modification of plasma antioxidant status following red wine intake. Five healthy male volunteers consumed 100, 200, and 300 mL of red wine corresponding to ≈0.9, 1.8, and 2.7 mg of caffeic acid, respectively. Plasma samples collected at different times (0−300 min) were evaluated for their content of caffeic acid and their total antioxidant status. Both these parameters, i.e., plasma concentration of caffeic acid and antioxidant potential, were dose-dependent and the C_{max} was reached at about 60 min after red wine intake. The results indicate that caffeic acid is bioavailable and it may be correlated with the antioxidant potential of plasma.

Keywords: *Caffeic acid; red wine; bioavailability; plasma; total antioxidant activity; human*

INTRODUCTION

Polyphenols are widespread, in virtually all plant foods (often at high levels) and in different medicinal plants, and they include phenols, phenolic acids, flavonoids, tannins, and lignans. They are important components in the human diet, as their daily intake can reach up to 800 mg depending on the consumption of specific beverages, such as red wine and tea (*1*). Besides vitamins C, E, and A, carotenoids, some trace elements, and fiber, polyphenols may account for at least part of the health benefits associated with the consumption of fresh fruit, vegetables, red wine, and tea (*2*). In particular, flavonoids have been described to exert a large array of biological activities. The ability of flavonoids to act as antioxidants in vitro has been the subject of several studies in the past years, and important structure−activity relationships have been established (*3*). By contrast, the antioxidant activity of flavonoids in vivo in humans is less documented and far from conclusive. Indeed, the relationship between antioxidant status and intake of dietary antioxidants is complex, because it involves absorption, metabolism, distribution, and homeostatic mechanisms. These aspects have been well investigated in the case of antioxidant vitamins, some carotenoids, and the minerals selenium, copper, and zinc (*4*). On the other hand, systemic studies of the absorption, metabolism, and excretion of dietary polyphenols are limited. In recent years many efforts have been made to compensate for this lack (*5*), and presently the following facts are quite well accepted: (1) a small fraction (as by measuring the plasma level) of the ingested flavonoids are taken up in the conjugated aglycon form and, partly, in the glycoside form (*6*,*7*); (2) the major part is degraded by gut microflora to different phenolic acids, some of which maintain antioxidant activity (*8*); and (3) the absorbed flavonoids and some metabolites are regarded as possible co-antioxidants in vivo, in that they can act in synergy with endogenous reductants to preserve the body antioxidant status (*9*, *10*).

Plasma epigallocatechingallate (EGCg) has been shown to be useful as a biomarker of tea intake, and its time-course concentration was found to be in good agreement with the variation of plasma ascorbate, total glutathione, and total antioxidant status (*11*).

Extending this work, another beverage commonly consumed at meals in Mediterranean countries, i.e., red wine, was investigated. This beverage affords ethanol and polyphenols, and its moderate and regular consumption is described to be associated with lower risk for coronary heart disease (*12*, *13*, *14*). Apart from ethanol, which is known to exert potentially beneficial effects on fibrinolytic factors and HDL cholesterol (*15*), the polyphenols are involved in the cardioprotective capacity of red wine (*16*, *17*). These compounds comprise flavonoids and nonflavonoids, the latter being represented by hydroxybenzoate, hydroxycinnamate, and stilbene derivatives (i.e., resveratrol isomers) (*18*). Most of these polyphenols have free radical scavenging ability (*19*), and are able to interrupt the lipid peroxidation (*20*). Among flavonoids, anthocyanins have been detected in human urine (*21*), and (+)-catechin with its metabolites has been measured in human plasma (*22*, *23*). Concerning hydroxycinnamates, a recent paper deals with the GC−MS analysis of caffeic, protocatechuic, and 4-*O*-methylgallic acids in human plasma (*24*). In all cases the levels of the specific classes were estimated after consumption of a single dose of red wine. In this study, the levels of caffeic acid were determined in human plasma after the ingestion of different amounts of red wine. In addition, the variation of plasma total antioxidant capacity was estimated and correlated with the time course of plasma caffeic acid concentrations.

MATERIALS AND METHODS

Subjects and Diet. Five healthy subjects (males), mean age 35 ± 6 years, BMI 24.1 ± 1.4 kg/m^2, received 100, 200,

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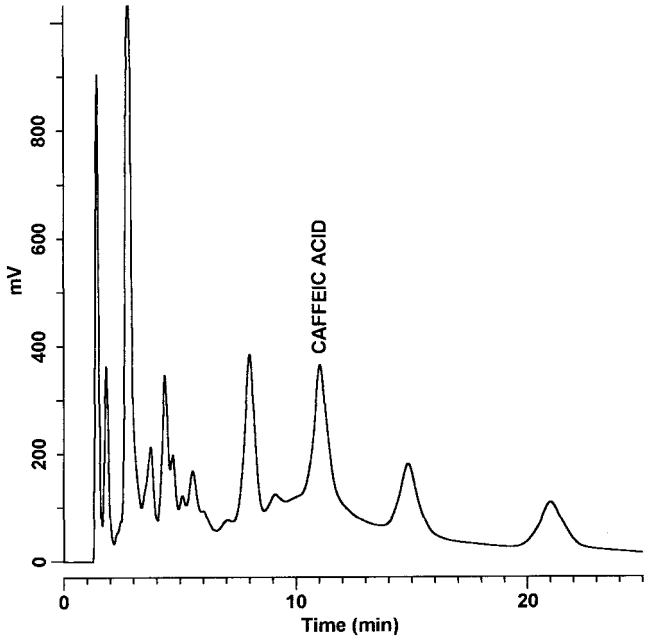

Figure 1. HPLC chromatogram of red wine. Column: 5 μm Symmetry C_{18} (250 × 4.6 mm, i.d.). Eluents: 30 mmol/L NaH_2PO_4, pH 3/acetonitrile (88:12, v/v). Flow rate: 1.5 mL/min. Electrochemical parameters: GC, −350 mV; Cell 1, −50 mV; analytical cell, +350 mV.

and 300 mL of red wine (RW). Volunteers participated on three occasions one week apart, consuming one of each beverage each time. Volunteers refrained from consuming specific phenolic-rich foods and beverages for 3 days prior to the study, including bran cereals; vegetables such as onions, spinach, tomatoes, and lettuce; fruits such as apples, apricots, blackberries, cranberries, grapes, grapefruit, and strawberries; and beverages such as beer, coffee, fruit juices, tea, and wine.

Chemicals. The HPLC-grade methanol and acetonitrile were obtained from BDH Laboratory Supplies (Poole, England). 2-2′-diazobis-(2-amidinopropane)-dihydrochloride (ABAP) was from Wako Chemicals (Richmond, VA). R-phycoeritrin (R-PE) was from Molecular Probes (Eugene, OR). Caffeic acid and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were from Aldrich Chemical (Milwaukee, WI). All other chemicals were from Merck (Darmstdadt, Germany). The red wine (Cabernet, 1998, Friuli, Italy) was obtained from a local commercial winery.

Standards. A stock solution of standard caffeic acid was prepared by dissolving 20 mg of sample in 200 mL of methanol. This solution was stored at 0−4 °C and used within 4 weeks after preparation. Working solutions were prepared by diluting the stock solution with the mobile phase (1−100 ng/mL).

Wine Sample Preparation. A 1-ml sample of red wine was diluted to 3 mL with water, and the resulting solution was applied to a previously activated (washed with 3 mL of methanol and 6 mL of water) Sep-Pak C_{18} cartridge (Waters, Milford, MA). After washing with 6 mL of 10 mM HCl and 3 mL of 10 mM HCl/methanol (85:15, v/v), the caffeic acid containing fraction was eluted with 3 mL of 10 mM HCl/methanol (50:50, v/v). The eluate was evaporated to dryness under nitrogen, and the residue was dissolved in 1 mL of 30 mM NaH_2PO_4 (pH 3)/acetonitrile (88:12, v/v).

Plasma Sample Preparation. Fasting venous blood samples were taken in vacutainer tubes containing sodium heparin before RW intake and at 20, 40, 60, 120, 180, 240, and 300 min after RW intake. Plasma was separated by centrifugation at 10,000*g* for 1 min.

An aliquot of plasma (250 μL) was added to 2 mL of 10 mM HCl; and the resulting solution was applied to a previously activated (3 mL of methanol followed by 6 mL of water) Sep-Pak C_{18} cartridge.

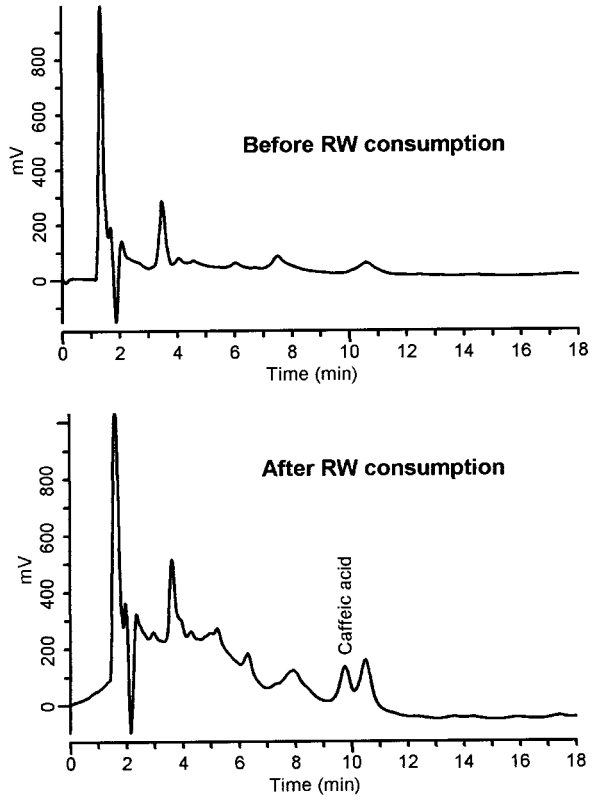

Figure 2. HPLC analysis of the human plasma before and after red wine consumption. See Figure 1 for analytical conditions.

After washing the cartridge with 6 mL of 10 mM HCl and 3 mL of 10 mM HCl/methanol (85:15, v/v), the caffeic acid fraction was eluted with 3 mL of 10 mM HCl/methanol (50:50, v/v), dried under nitrogen, and dissolved in 120 μL of 30 mM NaH_2PO_4 (pH 3)/acetonitrile (88:12, v/v).

HPLC Analysis of Caffeic Acid. The HPLC system consisted of a model 510 pump (Waters), connected with a Coulochem II detector (ESA, Chelmsford, MA), and a Millenium work station (Waters).

The injection was by means of a model 7125 Rheodyne injector (Waters), and the volume injected was 50 μL. A Symmetry C_{18} column (220 × 4.6 mm) with a 5-μm particle size (Waters) was used. Elution (1.3 mL/min) was performed with a mobile phase of 30 mM NaH_2PO_4 (pH 3)/acetonitrile (88:12, v/v).

Chromatograms were obtained with detection under the following conditions: guard cell, −250 mV; control cell, −50 mV; analytical cell, +350 mV.

All injections were performed in duplicate. Quantification was carried out using a caffeic acid calibration plot obtained by calibration runs with caffeic acid (range 1−20 ng/mL).

Percent Recovery of Caffeic Acid. Blank plasma samples (1 mL) were added with increasing amounts of caffeic acid standard (1−10 ng). An aliquot (250 μL) was treated and analyzed by HPLC, as described above. The recovery of caffeic acid was 96 ± 2%.

Determination of Plasma Total Radical-Trapping Antioxidant Parameter (TRAP). Plasma total radical-trapping antioxidant parameter (TRAP) was determined by the method of Ghiselli et al. (*25*).

Plasma peroxidation induced by ABAP was monitored by the loss of fluorescence of the protein R-phycoeritrin. The lag phase of the plasma was compared to that of a known concentration of the internal standard Trolox, and then quantitatively related to the antioxidant capacity of the plasma.

Plasma (100 μL) was diluted 10 times with a phosphate buffer (75 mM at pH 7), and 80 μL of this solution was added

Table 1. Plasma Caffeic Acid (ng/mL) Before and After Drinking 100, 200, and 300 mL of Red Wine (values are means ± SE, $n = 5$)

amount of RW	time after consumption of red wine (min)							
	0	20	40	60	120	180	240	300
100 mL	0.00 ± 0.00	0.49 ± 0.10	0.79 ± 0.30	1.19 ± 0.38	0.46 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
200 mL	0.00 ± 0.00	0.75 ± 0.08	1.97 ± 0.14[a]	3.23 ± 0.11[a]	1.02 ± 0.14	0.11 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
300 mL	0.00 ± 0.00	2.48 ± 0.67[a,b]	2.96 ± 0.33[a,b]	4.90 ± 0.33[a,b]	2.66 ± 0.90[a,c]	0.70 ± 0.10[a,b]	0.00 ± 0.00	0.00 ± 0.00

[a] $p < 0.01$ vs 100 mL RW intake at different times. [b] $p < 0.01$ vs 200 mL RW intake at different times. [c] $p < 0.05$ vs 200 mL RW intake at different times.

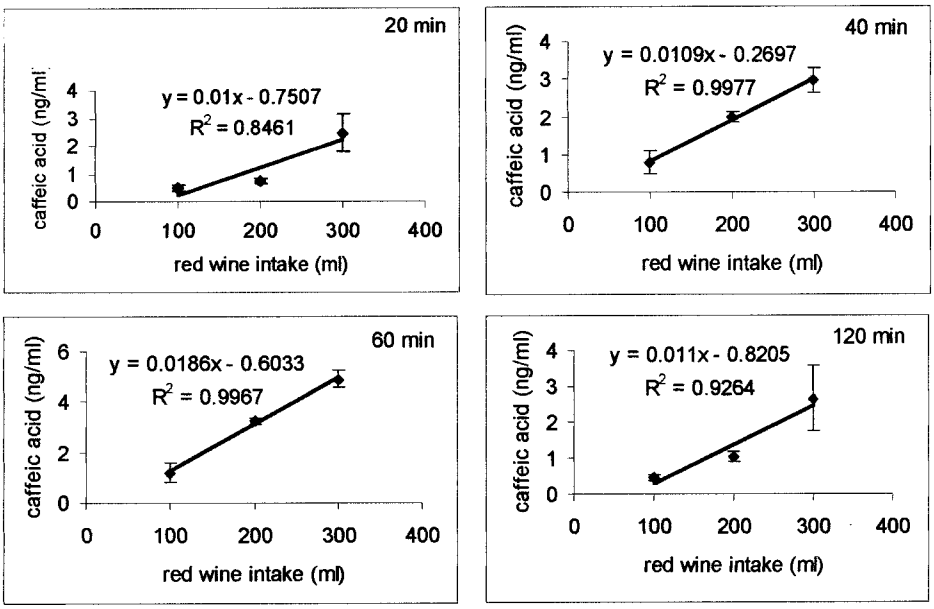

Figure 3. Correlation between plasma level of caffeic acid and red wine intake.

with 250 μL of distilled water, 1.55 mL of phosphate buffer (75 mM at pH 7), and 20 μL of 1.5×10^{-8} M R-PE in the same phosphate buffer. The resulting mixture was maintained at 37 °C for 5 min in a quartz fluorometer cell. After being stabilized, the oxidation reaction was started by adding 100 μL of ABAP (with a final concentration in the fluorescence spectrometer of 4.0 mM that corresponds to 538 mg in 10 mL of phosphate buffer). After 25 min, 60 μL of 120 μM Trolox (7.51 mg in 250 mL water) was added. The decay of R-PE fluorescence was monitored by a model LS50B fluorescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, England). The detector was set at an excitation wavelength of 495 nm and an emission wavelength of 575 nm.

The TRAP value was calculated according to the following formula:

$$\text{TRAP } (\mu\text{M Trolox}) = \frac{\text{plasma lag phase} \times 250 \times 120 \times 2}{\text{Trolox lag phase} \times 34.333}$$

where 2 = Trolox factor, 250 = dilution in cuvette, 120 = Trolox concentration, and 34.333 = Trolox dilution in cuvette.

Statistical Analysis. Data are reported as mean ± SE. Pearson's univariate correlation and analysis of variance (RM-ANOVA) were performed using a statistical package running on a PC (Statistical Statsoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

A typical HPLC trace of red wine is shown in Figure 1. Caffeic acid eluted at around 11 min, and its identity was established by spiking the sample with a standard. Quantitation of caffeic acid in red wine was obtained from the following calibration curve:

$$y = 13.656x - 2.7119; \quad r^2 = 0.9998$$

The limit of detection was 0.3 ng/mL. Caffeic acid in the selected wine was 9.01 ± 0.29 mg/L. Thus, ingestion of 100, 200, and 300 mL of red wine provided about 0.90, 1.80, and 2.70 mg of caffeic acid, respectively.

Figure 2 shows the HPLC analysis of the human plasma before and after RW consumption. Caffeic acid was not detected in the plasma of volunteers before consumption of RW, and this is due to the low polyphenols content of their diets during the 3 days prior to the study. The highest plasma level of caffeic acid (C_{max}) was reached 60 min after ingestion. These levels decreased to basal values within 180 min in the case of 100 and 200 mL of RW intake, and 240 min after ingestion of 300 mL of RW (Table 1). In addition, the increase in plasma concentration of caffeic acid was significant ($p < 0.01$) after the ingestion of 300 mL of RW as compared with the increase achieved with 100 and 200 mL.

The plasma levels of caffeic acid were linear with the dose of ingested red wine with highest correlation coefficients at 40 and 60 min after consumption (Figure 3). Both the absence of caffeic acid in plasma before the trial and the significant increment of this acid after RW ingestion suggest that it may be a possible marker of consumption of beverages containing this acid.

Plasma antioxidant capacity (TRAP) was measured before and after the ingestion of 100, 200, and 300 mL of RW. Accordingly to previous reports (*26*, *27*), RW produced an increase in plasma antioxidant activity, and the results expressed as percent variation of TRAP are given in Table 2. The highest variation was obtained with 300 mL of RW, reaching a peak in the interval of 60−120 min. A remarkable variation was achieved also

Table 2. TRAP Levels (% variation ± SE) Before and After Drinking 100, 200, and 300 mL of Red Wine (values are means ± SE, $n = 5$)

amount of RW	time after consumption of red wine (min)							
	0	20	40	60	120	180	240	300
100 mL	0.0 ± 0.0	0.7 ± 0.5	2.9 ± 0.8	6.0 ± 1.3	6.2 ± 1.2	0.6 ± 0.4	1.8 ± 2.0	−0.6 ± 1.5
200 mL	0.0 ± 0.0	7.3 ± 1.7[a]	8.2 ± 2.2[b]	19.5 ± 2.6[a]	6.8 ± 1.9	7.5 ± 1.7[b]	4.9 ± 1.8	5.1 ± 3.3
300 mL	0.0 ± 0.0	5.9 ± 1.9[b]	8.7 ± 1.2[b]	25.4 ± 4.2[a]	28.9 ± 4.7[a,c]	7.5 ± 2.1[b]	4.8 ± 1.9	2.0 ± 4.2

[a] $p < 0.01$ vs 100 mL RW intake at different times. [b] $p < 0.05$ vs 100 mL RW intake at different times. [c] $p < 0.01$ vs 200 mL RW intake at different times.

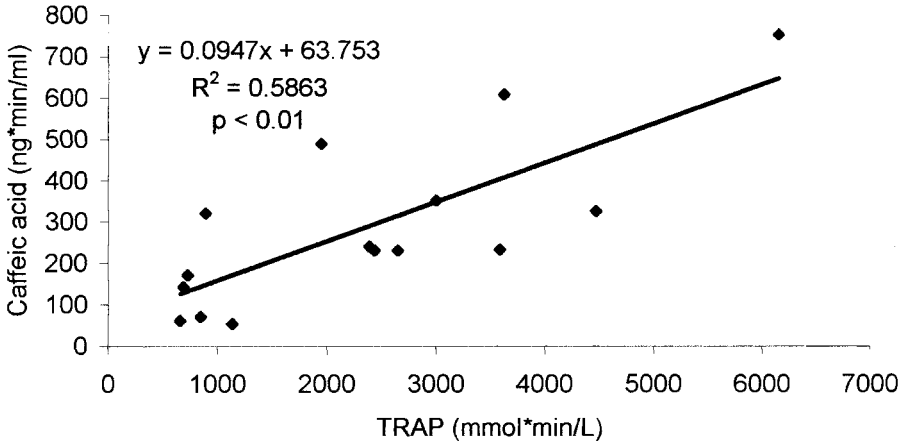

Figure 4. Correlation between areas under the curve (AUC) of TRAP and caffeic acid.

after consumption of 200 mL of RW, with a maximum at 60 min. Both these variations were statistically significant ($p < 0.01$) as compared with the increase achieved with consumption of 100 mL of RW. Conversely, the effect of 100 mL of RW on TRAP was modest and not significant. These results may suggest that a generous glass of RW (about 300 mL) seems a reasonable amount to increase the plasma antioxidant capacity.

The TRAP values were in good accordance with the time-course of plasma caffeic acid concentration. The correlations between these two variables were the following:

$$y = 0.090x + 0.167; \quad r^2 = 0.307; \quad p < 0.001 \text{ for 100 mL of RW,}$$

$$y = 0.114x + 0.039; \quad r^2 = 0.438; \quad p < 0.001 \text{ for 200 mL of RW,}$$

$$y = 0.116x + 0.509; \quad r^2 = 0.484; \quad p < 0.001 \text{ for 300 mL of RW;}$$

where y and x represent the plasma caffeic acid (ng/mL) and the percent variation of TRAP, respectively. In addition, a significant correlation between the areas under the curve (AUC) for TRAP and caffeic acid was established, as shown in Figure 4.

CONCLUSIONS

After a single ingestion of moderate amounts of red wine, a beverage commonly consumed at meals in Mediterranean countries, caffeic acid was detected in plasma. The concentrations of this hydroxycinnamic derivative were dose-dependent, and were well correlated with the variation of plasma total antioxidant capacity. These results may be helpful in explaining partly the health benefits of moderate red wine consumption.

ABBREVIATIONS USED

ABAP, 2-2′-diazobis-(2-amidinopropane)-dihydrochloride; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; TRAP, plasma total radical-trapping antioxidant parameter.

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