LC/ES−MS Detection of Hydroxycinnamates in Human Plasma and Urine

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Hydroxycinnamates are components of many fruits and vegetables, being present in particularly high concentrations in prunes. An abundance of phenolic compounds in the diet has been associated with reduced heart disease mortality. However, little is known about the absorption and metabolism of these metabolites after normal foods are consumed. An LC−electrospray−MS method was developed to measure the concentration of caffeic acid in human plasma and urine, but it can also be applied to ferulic acid and chlorogenic acid. The limit of detection was found to be 10.0 nmol/L for caffeic acid and 12.5 nmol/L for ferulic and chlorogenic acids. The method was tested on samples of plasma and urine collected from volunteers who consumed a single dose of 100 g of prunes and increased levels were observed, demonstrating that the method is capable of detecting changes in hydroxycinnamate levels induced by dietary consumption.

Keywords: *Caffeic acid; prunes; plums; Prunus domestica; ferulic acid; chlorogenic acid; antioxidants; phenolics; absorption; metabolism*

INTRODUCTION

Hydroxycinnamic acids are among the most widely distributed phenylpropanoids in the plant kingdom, with fruits, vegetables, beverages, and grains being abundant dietary sources. From epidemiological studies, there is increasing evidence of the positive health benefits of a high dietary intake of such compounds (*1*). Their in vitro activities (in particular their antioxidant activities) have been studied intensively and have been reviewed by Rice-Evans et al.(*2*). Caffeic acid in particular has been identified as one of the active antioxidant constituents of red wine showing a dose-dependent ability to protect against oxidation of low-density lipoprotein (*3*). Further knowledge of the absorption, bioavailability, and metabolism of these compounds after normal dietary intake is essential in increasing our understanding of their mechanism of action. A recent study on the uptake of phenolic acids upon wine consumption showed significant increases in plasma concentrations of caffeic acid, although this increase was found to have no effect on ex vivo serum or LDL oxidation (*4*). LC−UV methods have been used in studying the pharmacokinetics of caffeic acid in rats (*5*) and for the analysis of these compounds in human urine (*6*). Although LC−UV is applicable to the study of these compounds at the μM/L level, such as is present in human urine, a method of greater selectivity and sensitivity is necessary to analyze for these compounds at lower levels such as might be expected in human plasma. The aim of this work was to develop and apply a sensitive method that utilizes HPLC with electrospray mass spectrometric detection to simultaneously measure caffeic, ferulic, and chlorogenic acids in human plasma and urine. This method was then tested using samples of plasma and urine collected from three volunteers who consumed a single dose of 100 g of California prunes. Prunes are characterized by high concentrations of hydroxycinnamic acids, and prune extracts have shown antioxidant activity toward isolated human LDL similar to that shown by other foods that contain phenolic phytochemicals such as grapes, wine, and chocolate (*7*).

MATERIALS AND METHODS

Hydroxycinnamic Standards, Reagents, and Prune Samples. Caffeic, ferulic, and chlorogenic (5′-caffeoylquinic) acids were obtained from Aldrich Chemical Co. (Milwaukee, WI). β-Glucoronidase (G-0251; EC 3.2.31) and sulfatase (S-9754; EC 3.1.6.1) were purchased from Sigma Chemical (St. Louis, MO). All solvents and reagents were Fisher HPLC or Optima grade; other reagents were purchases from either Fisher (Pittsburgh, PA) or Aldrich (Milwaukee, WI). Samples of pitted prunes were obtained from the California Prune Board (Pleasanton, CA).

Sample Collection. The subjects were three healthy volunteers (two females and one male) aged between 24 and 35 who followed a hydroxycinnamate-reduced diet containing no fruits, vegetables, chocolate, coffee, or tea for 2 days before the experiment. The subjects then consumed 100 g of prunes and continued the dietary restrictions for a further 4 h. By HPLC analysis the prunes were found to contain 76 ± 6 mg of hydroxycinnamates (*8*). Blood was drawn into 10-mL EDTA-containing tubes (Becton Dickinson, Franklin Lanes, NJ) before prune consumption and at 2 h after prune consumption. Plasma was prepared and 4.0-mL aliquots were mixed with 100 μL of phosphate buffered ascorbic acid (PBA 200 g/L ascorbic acid, 0.4 mol/L NaH_2PO_4, pH 3.6), flushed with nitrogen, and frozen at −70 °C. Urine samples were also collected before prune consumption and at 2 and 4 h afterward, and treated similarly to the plasma samples. The clinical protocol was approved by the Human Subjects Committee at the University of California, Davis, CA.

Enzymatic Hydrolysis, Sample Preparation, and Extraction. To prevent oxidation of the analytes, all solvents

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and reagents were deoxygenated by purging with nitrogen and kept on ice. The 4.0-mL aliquots of plasma were thawed and an additional 400 μL of PBA was added. The plasma was divided into two 2-mL samples for duplication, and 1000 μL of a 0.6 M solution of $CaCl_2$ solution was added to each sample. The plasma was incubated at 37 °C in a shaking water bath for 45 min in nitrogen-flushed tubes containing 400 U of sulfatase and 10 000 U of β-glucoronidase dissolved in 480 μL of water. For estimation of free metabolites, a set of samples was also incubated without enzymes. After incubation, the plasma was acidified with 40 μL of orthophosphoric acid, extracted with 4 mL of methylene chloride, vortexed for 1 min, and centrifuged at 4500*g* for 10 min at 4 °C. The aqueous supernatant was removed and the remaining portion was extracted a second time with 2 mL of H_2O. The aqueous extracts were combined and extracted twice with 3.0 mL of ethyl acetate. The combined ethyl acetate extracts were evaporated under nitrogen and then redissolved in 500 μL of 50% aq. methanol for analysis.

The urine samples were thawed and divided into two 2.0-mL aliquots for duplication. These were then mixed with 450 μL of 50 mM sodium acetate buffer (pH 5.5) and 1000 μL of 0.6 mol/L $CaCl_2$ solution. These aliquots were incubated in a manner similar to that of the plasma samples, followed by acidification with 20 μL of 6N HCl. The urine was then extracted with 4 mL of ethyl acetate, vortexed, and centrifuged at 4500*g* for 10 min at 4 °C. The supernatant was removed and the remaining portion was extracted a second time with 3.0 mL of ethyl acetate. The combined ethyl acetate extracts were evaporated under nitrogen and then redissolved in 500 μL of 50% aq. methanol for analysis.

LC−MS Analysis. Analyses were performed on a Hewlett-Packard HPLC system equipped with DAD and MS detection (HP-1100 LC−MS). The HPLC column was a Hamilton PRP-1. The solvents were A, water with 1% acetic acid, and B, acetonitrile. The solvent gradient began with 4 min at 20% B, a linear ramp to 50% B at 6 min, than a linear ramp to 100% B at 15 min which continued to the end of the run, 20 min total. The flow rate was 0.5 mL/min. The retention times were 6.1 min for chlorogenic acid,, 10.1 min for caffeic acid, and 12.9 min for ferulic acid. Ionization was conducted at 350 °C with a nebulizer pressure of 60 psi, a drying gas flow of 12.0 L/min, a fragmentor voltage of 95 V, and a capillary voltage of 3500 V. The MS data were collected in SIM mode, monitoring ions at *m*/*z* 179 and 135 for caffeic acid, *m*/*z* 193 and 149 for ferulic acid, and *m*/*z* 353 and 191 for chlorogenic acid using a dwell time of 439 ms.

Calibration Curves, Limit of Detection, and Analyte Recoveries. Calibration curves were prepared by injecting aliquots of standard solutions in the ranges of 25−250 nmol/L and 1.0−10.0 μmol/L and then plotting the peak areas of selected ions monitored against concentration. The calibration curves were linear over the range studied and had mean r^2 values of ≥ 0.997. The limit of detection (signal-to-noise = 3) was 10.0 nmol/L for caffeic acid and 12.5 nmol/L for ferulic and chlorogenic acids. Recoveries were determined by spiking samples at four concentrations between 25 and 250 nmol/L in blank plasma and six concentrations between 0.9 and 10.0 μmol/L in blank urine and subjecting them to the described extraction procedure. These recovery experiments were repeated six times and gave values for caffeic acid of 98.0 ± 6.1% in plasma and 99.1 ± 4.3% in urine. Recoveries for ferulic were 47.5 ± 3.9% in plasma and 49.8 ± 2.9% in urine by this method. Recovery for chlorogenic acid varied with the level of concentration. At low levels (plasma, 25.0−50.0 nmol/L; urine, 0.9−4.5 μmol/L) it was found to be 22.8 ± 8.8% in plasma and 27.4 ± 1.8% in urine. At higher concentrations (plasma, 100−250 nmol/L; urine, 4.5−10 μmol/mL), recoveries were found to be 67.5 ± 13.4% and 47.0 ± 1.8% in plasma and urine, respectively.

RESULTS AND DISCUSSION

Analysis Method. Although caffeic acid showed excellent recovery by this method, the recovery of ferulic and chlorogenic acids was less satisfactory. The difference in polarity of chlorogenic acid due to the attached sugar moiety accounts for its lower and more variable extraction into organic solvents as compared with caffeic acid. This difficulty may be overcome by developing a separate extraction route utilizing solid-phase extraction cartridges (*9*). Even though the recovery of ferulic acid is quite reproducible by this method, it is significantly lower than that of caffeic acid, suggesting that polarity differences between these metabolites can greatly affect extraction efficiency.

ESI−MS Behavior of Hydroxycinnamates. Prior to the LC−MS experiments, the optimization of ionization mode and associated parameters, namely drying gas flow and temperature, nebulizer pressure, capillary voltage, and fragmentor voltage, were investigated. This was achieved by flow injection analysis of concentrations varying from 10^{-3} to 10^{-6} M of caffeic, ferulic, and chlorogenic acids. The optimal parameters found to give maximum sensitivity for the characterization of the phenolic compounds studied in this work by MS with negative mode electrospray ionization were mentioned above. These conditions were optimized to produce a relatively soft ionization with the major peak for each compound being the $[M-H]^-$ molecular ion and a charcteristic loss of CO_2 to give ions at *m*/*z* 135 and 149 for caffeic acid and ferulic acid, respectively. Chlorogenic acid produced a fragment ion at *m*/*z* 191 corresponding to the cleavage of the quinic ester moiety. Of the MS parameters, fragmentor voltage was found to have the strongest effect on this ionization with values greater than 120 V leading to spectra dominated by fragment ions and spectra taken with fragmentor values of less than 75 V dominated by the molecular ion only. This variable was optimized to 95 V to allow detection of both molecular ion and characteristic fragment ions. Negative ion mode electrospray was found more informative than positive mode for these metabolites as has been similarly reported for other classes of phenolic metabolites (*10*). Both positive and negative mode APCI were also studied for the analysis of hydroxycinnamates. Although APCI negative mode provides comparable results to electrospray negative mode at concentrations of 10^{-3}−10^{-5} M, the molecular ions of these hydroxycinnamates proved difficult to detect working at lower concentrations by this method as has been previously reported (*11*). Because the sensitivity of the electrospray mode is affected by the ability of the mobile phase to support preformed ion in solution, both methanol and acetonitrile containing varying amounts of 1−5% formic or acetic acid were investigated as mobile phase. Addition of 1% acetic acid to the aqueous phase led to a better sensitivity than addition of formic acid, with an increase in the amount of acetic acid beyond 1% giving no further increase in sensitivity. The addition of ionization agents such as 10 or 5 mM sodium acetate, or postcolumn addition of 1.5 M ammonium hydroxide, led to no appreciable increase in intensity of the monitored ions. Maximum sensitivity was thus achieved with a mobile phase of acetonitrile/(1% acetic acid in water). Quantitation of hydroxy cinnamates was based on SIM monitoring in this mode observing ions at *m*/*z* 179 and 135 for caffeic acid, *m*/*z* 149 and 193 for ferulic acid, and *m*/*z* 353 and 191 for chlorogenic acid.

Levels of Hydroxycinnamates in Human Plasma. To determine whether this method was capable of detecting hydroxycinnamate levels resulting from nor-

Table 1. Levels of Free and Total Caffeic Acid Detected in Plasma Samples

		nmol/L			
		time = 0 hours		time = 2 hours	
subject	sample	free	total	free	total
1	A	nd[a]	52	nd	42
	B	nd	44	nd	42
2	A	nd	nd	nd	42
	B	nd	nd	nd	46
3	A	25	55	32	86
	B	25	45	33	96

[a] nd, not detected.

Table 2. Levels of Free and Total Ferulic Acid Detected in Plasma Samples

		nmol/L			
		time = 0 hours		time = 2 hours	
subject	sample	free	total	free	total
1	A	77	118	39	78
	B	57	77	34	50
2	A	36	31	30	36
	B	35	nd[a]	26	30
3	A	34	nd	20	nd
	B	nd	nd	28	nd

[a] nd, not detected.

mal dietary consumption, it was applied to the analysis of samples from three human subjects who consumed a single dose of 100 g of prunes, containing 76 ± 6 mg of hydroxycinnamates ($n = 3$). The levels of these metabolites detected in duplicate plasma samples (A and B) of the three volunteers is shown in Tables 1 and 2, where total metabolite refers to those samples subjected to glucuronidase and sulfatase hydrolysis and free metabolite refers to samples which were not hydrolyzed. The level of free caffeic acid present before prune consumption and 2 h afterward is below the limit of detection in two of the three subjects. Caffeic acid is, however, clearly visible in all samples taken at 2 h and subjected to enzymatic hydrolysis. This indicates that caffeic acid is present mostly in the conjugated form in plasma. In two of the subjects there is a clear increase in the amount of total caffeic present at 2 h relative to that before prune consumption, but the other subject shows a slight decrease. The amount of total caffeic acid present in duplicate samples from subject 3 undergoes an almost 2-fold increase upon prune consumption. The maximum level detected in duplicates of any one sample was 86 and 96 nmol/L found in the sample taken at 2 h from subject 3 after enzymatic hydrolysis. These values are in a range similar to the maximum value of 84 nmol/L which was recently reported in a study on the absorption of caffeic acid upon red wine consumption (*3*). The levels of free and total ferulic acid detected are shown in Table 1. The increase in metabolite concentration upon hydrolysis is clearly evident in only one of the subjects studied in this case. No increase in the amount of ferulic acid upon consumption of prunes is evident for these plasma samples. However, the low levels of ferulic acid in prunes, the low recovery of this metabolite by this method, and the difficulty of establishing a dietary regime devoid of ferulic acid (which is one of the universal components of plant cell walls) restrict any possible conclusions on the bioavailability of this metabolite in plasma upon prune consumption. Chlorogenic acid was below the limit of detection in the plasma samples studied which may also be due to its lower recovery at these concentrations. The presence of hydroxycinnamates at time zero in some cases demonstrates the difficulty in eliminating these substances from the diet, as they are found in all plant foods. A diet that totally eliminates plant foods was not attempted here, as it is too aberrant compared to a normal diet.

Levels of Hydroxycinnamates in Human Urine. The chromatogram of an extract of a urine sample taken 2 h after prune consumption with SIM traces for caffeic, chlorogenic, and ferulic acid is shown in Figure 1. The urinary concentrations of caffeic, ferulic, and chlorogenic acids are shown in Table 3. The cumulative excretion of caffeic acid is illustrated graphically in the case of one subject in Figure 2. The ingestion of prunes leads to an increase in the amount of free caffeic acid excreted in all samples. This increase becomes more evident upon enzyme hydrolysis. For example, the level of caffeic acid detected in a sample collected from subject 1 at time zero was 0.143 μmol upon hydrolysis, whereas at 2 h this value increases to 0.448 μmol. This 1.5−2- or 3-fold increase in the amount of caffeic acid detected upon hydrolysis is evident in the urine samples collected at

Table 3. Levels of Free and Total Hydroxycinnamates Detected in Urine at Times 0, 2, and 4 Hours

			time = 0 hours		time = 2 hours		time = 4 hours	
metabolite	subject	sample	free μmol	total μmol	free μmol	total μmol	free μmol	total μmol
caffeic acid	1	A	0.154	0.123	0.181	0.496	0.148	0.267
		B	0.137	0.143	0.147	0.448	0.098	0.286
	2	A	0.064	0.092	0.070	0.175	0.111	0.215
		B	0.057	0.099	0.059	0.146	0.085	0.316
	3	A	0.209	0.188	0.299	0.345	0.412	0.397
		B	0.189	0.124	0.282	0.334	0.386	0.392
ferulic acid	1	A	nd[a]	nd	0.001	0.031	0.003	0.011
		B	nd	nd	0.003	0.032	0.002	0.013
	2	A	nd	nd	nd	0.008	0.005	0.016
		B	nd	nd	nd	0.009	0.005	0.018
	3	A	nd	nd	0.001	0.009	0.002	0.008
		B	nd	nd	0.006	0.009	nd	0.007
chlorogenic acid	1	A	0.008	nd	0.019	0.014	0.004	0.009
		B	0.006	nd	0.019	0.016	0.008	0.004
	2	A	0.006	nd	0.019	0.016	0.008	0.004
		B	0.018	nd	0.023	0.005	0.006	0.006
	3	A	0.003	nd	0.045	0.006	0.039	0.016
		B	0.001	nd	0.025	0.004	0.026	0.016

[a] nd, not detected.

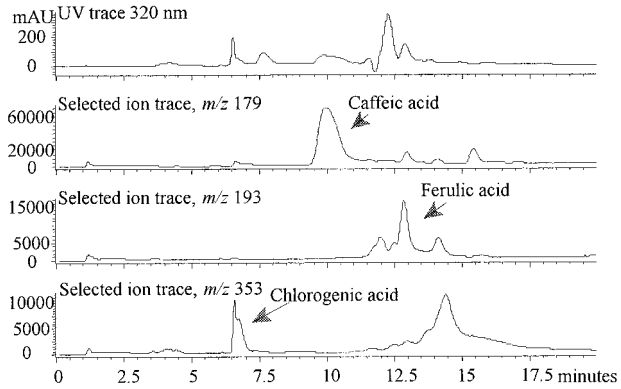

Figure 1. LC−MS chromatogram of hydrolyzed urine sample taken at 2 h.

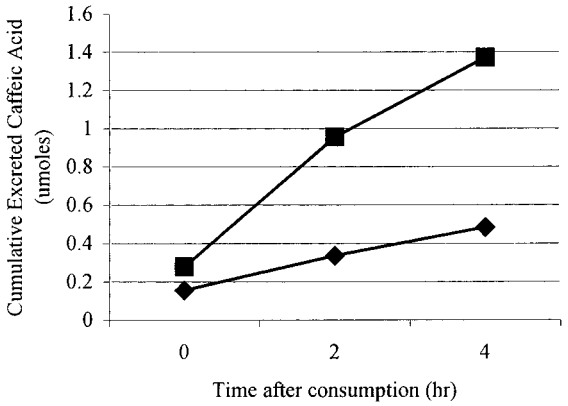

Figure 2. Average cumulative urinary excretion of caffeic acid by one subject after consumption of 100 g of prunes. Forms are free (-◆-, caffeic acid detected without hydrolysis) and total (-■-, caffeic acid detected after glucoronide and sulfatase hydrolysis).

2 and 4 h. These results show that prune consumption appears to lead to a large increase in the amount of caffeic acid present and that most of the caffeic acid is excreted in conjugated form. Ferulic acid is detected only in urine samples taken at 2 and 4 h and is much lower in concentration than caffeic acid in these samples. Recent work on the urinary excretion of this metabolite after a high dietary intake of tomato has demonstrated a low level of recovery for this metabolite (*6*). In all samples, hydrolysis led to a greater than 2-fold increase in the level of ferulic acid detected. Chlorogenic acid is also detected at a low level relative to the amount of caffeic acid present. Previous studies on the urinary recovery of chlorogenic acid (*9*) in human and rat subjects had not detected this metabolite and suggested that it may be absorbed poorly or undergo a more complete metabolism than the unesterified hydroxycinnamates. These results, however, show free chlorogenic acid already present at time zero. A 2−3-fold increase in free metabolite detected 2 h after prune consumption is evident is samples taken from two of the subjects, but the increase in free chlorogenic acid is lower in the other subject. Enzymatic hydrolysis actually appears to lead to a decrease in chlorogenic acid concentration in all samples. This suggests that some enzyme-catalyzed hydrolysis of the quinic ester is occurring in this step.

This method is useful for evaluating the dietary intake of hydroxycinnamates at typical consumption levels and also for pharmacokinetic studies. To our knowledge, this is the first report of an increase in the excretion of chlorogenic acid after normal dietary intake of a hydroxycinnamate-rich food. These results add to the increasing evidence of the bioavailability of hydroxycinnamate antioxidants in humans and again suggest that the metabolic conjugates are most important with regard to phenolics present in biological fluids.

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