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Structural Characterization of the Metabolites of Hydroxytyrosol, the Principal Phenolic Component in Olive Oil, in Rats

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Hydroxytyrosol is quantitatively and qualitatively the principal phenolic antioxidant in olive oil. Recently it was shown that hydroxytyrosol and five metabolites were excreted in urine when hydroxytyrosol was dosed intravenously or orally in an olive oil solution to rats. The conclusive identification of three metabolites of hydroxytyrosol by MS/MS as a monosulfate conjugate, a 3-*O*-glucuronide conjugate, and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) has been established in this investigation. The structural configurations of the glucuronide conjugate and 4-hydroxy-3-methoxyphenylacetic acid were confirmed by ^{1}H NMR. The radical scavenging potencies of homovanillic acid, homovanillic alcohol, hydroxytyrosol, and the metabolites were examined with the radical 2,2-diphenyl-1-picrylhydrazyl. These studies showed them to be potent antioxidants with SC_{50} values of 14.8 and 11.4 μM for homovanillic acid and homovanillic alcohol, respectively. The 3-*O*-glucuronide conjugate was more potent than hydroxytyrosol, with an SC_{50} of 2.3 in comparison to 11.0 μM, and the monosulfate conjugate was almost devoid of radical scavenging activity.

KEYWORDS: Hydroxytyrosol; olive oil; metabolites; antioxidant activity; Mediterranean diet

INTRODUCTION

Epidemiological studies have shown that a lower incidence of coronary heart disease (*1*) and prostate and colon cancers (*2*) has been attributed to the Mediterranean diet, which is largely vegetarian in nature and includes the consumption of large quantities of olive oil (*1*). Olive oil is primarily composed of triglycerols and ~0.5−1.0% of nonglyceridic constituents, most of which are phenolic compounds (*3*). Phenolic compounds are potent in vitro inhibitors of low-density lipoprotein (LDL) oxidation and are capable of breaking peroxidative chain reactions (*4*). The in vivo oxidation of LDL is strongly linked to the formation of atherosclerotic plaques, which in turn contribute to the development of coronary heart disease. Peroxidative chain reactions have been linked to the pathogenesis of coronary heart disease and cancer (*4*). Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol, **Figure 1**] is the principal phenolic compound found in olive oil and is known to possess strong antioxidant scavenging abilities (*6*). Hydroxytyrosol has also been suggested to contribute to the prevention of cardiovascular disease. It has been recently found that hydroxytyrosol decreases the amount of isoprostane excreted in urine (*7*) and decreases the oxidative stress in rats exposed to passive smoking (*8*).

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However, despite the wide body of evidence linking the in vitro properties of hydroxytyrosol with positive health outcomes, there are limited data on its metabolism in the body. Recently, two studies have investigated the fate of hydroxytyrosol after the oral consumption of olive oil by humans (*9*, *10*). It was initially postulated (*9*) that hydroxytyrosol was eliminated in urine unmetabolized and as a glucuronide conjugate. Two more metabolites, homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) and homovanillic alcohol, were subsequently identified upon re-examination of the urine samples obtained from the earlier cited human study (*10*). The formation of homovanillic alcohol has been reported previously in Caco-2 cells (*11*).

Recently, we reported that hydroxytyrosol was extensively absorbed by rats when dosed as an olive oil solution and that it and five metabolites were excreted in urine (*12*). We did not attempt to identify the metabolites in that study. In this study we describe the identification of three metabolites of hydroxytyrosol by mass spectrometry as a monosulfate conjugate, a monoglucuronide conjugate, and 3-hydroxy-4-methoxyphenylacetic acid (homovanillic acid). The position of the substituent on the aromatic ring of the monoglucuronide conjugate and 3-hydroxy-4-methoxyphenylacetic acid was confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The radical scavaging ability of each of these metabolites and also authentic homovanillic acid and homovanillic alcohol was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test.

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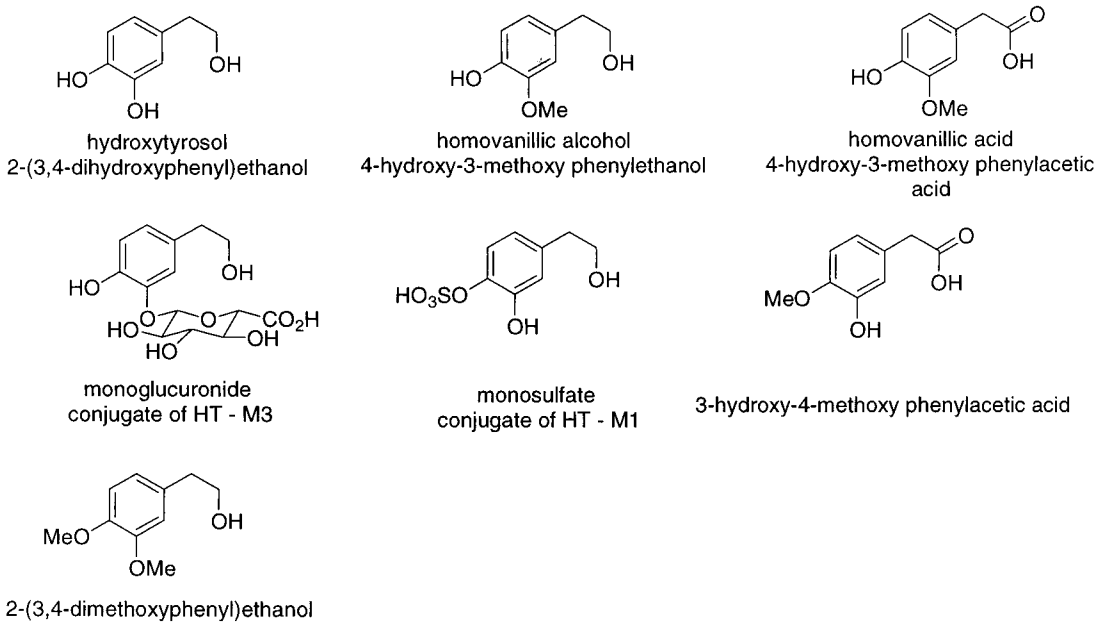

Figure 1. Structures of hydroxytyrosol and metabolites.

MATERIALS AND METHODS

Reagents. β-Glucuronidase type VII-A (bacterial from *Escherichia coli*, 1000 units per vial), sulfatase type VI (from *Aerobacter aerogenes*, 50 units/3.5 mL), homovanillic acid, homovanillic alcohol, 2-(3,4-dimethoxyphenyl)ethanol, and DPPH radical were purchased from Sigma-Aldrich Chemical Co. (Sydney, Australia). Hydroxytyrosol-[*ring*-2,5,6-^{3}H] was synthesized and purified according to previously published procedures (specific activity of hydroxytyrosol = 66 Ci/mol) (*13*). Water used in all experiments was obtained from a Milli-Q water purification system (Millipore).

Animal Experiments. The experimental procedure is fully described in Tuck et al. (*12*). Briefly, rats were dosed with radiolabeled hydroxytyrosol contained within either olive oil (orally administered) or aqueous solutions (intravenously administered); $n = 5$ for each experiment. Urine samples were collected (when possible) at 1, 2, 3, 4, 8, and 24 h time intervals after dosing.

Apparatus. High-performance liquid chromatography (HPLC) analysis was performed on a Hewlett-Packard 1100 series system consisting of an 1100 series isocratic pump, an 1100 series autosampler, and an 1100 series variable-wavelength detector and with an analytical DuPont phenyl Zorbax (250 × 4.6 mm i.d.) column, mobile phase [99.5% H_2O (containing 0.2% acetic acid)/0.5% MeOH, 1 mL/min]. The compounds were detected at 281 nm. HPLC radiometric analysis was performed with a Radiomatic 150TR flow scintillation analyzer (scintillant flow = 2.5 mL/min and HPLC flow = 1 mL/min). Samples were freeze-dried, reconstituted in a minimal amount of water, and purified by reversed-phased preparative HPLC using an analytical DuPont phenyl Zorbax (250 × 4.6 mm i.d.) column.

Analysis of HPLC extracts was carried out using an Applied PE Biosystems API 2000 instrument in the negative ion mode. Sample solutions, typically in methanol/water (1:1) 1.0 μg/mL, were infused at 20−50 μL/min. The system operating parameters were as follows: turbo spray tip voltage, −4000 V; orifice plate voltage, −36 V; RNG voltage, −330 V; and collision energy, 17 eV.

NMR spectra were measured with a Varian spectrometer with an operating frequency of 600 MHz with deuterated water as the NMR solvent, unless otherwise stated. ^{1}H resonances are quoted in parts per million downfield from the ^{1}H resonance of tetramethylsilane.

Enzyme Hydrolysis. Aliquots (50 μL) of urine, from an appropriate period after dosing, were diluted with mobile phase (500 μL). The pH was adjusted to 5.7 using 0.5 M sodium hydroxide solution, and either β-glucuronidase (50 μL) or sulfatase (30 μL) was added. The samples were incubated at 37 °C for 1 h and then analyzed by HPLC radiometric detection.

^{1}H NMR Analysis of M3 and M5. *^{1}H NMR spectrum of M3, monoglucuronide conjugate of hydroxytyrosol:* δ 6.94 (d, 1H, $J = 8.4$ Hz, H5), 7.03 (dd, 1H, $J = 2.4$ and 8.4 Hz, H6), 7.21 (d, 1H, $J = 2.4$ Hz, H2).

^{1}H NMR spectrum of M5, 3-hydroxy-4-methoxyphenylacetic acid (homovanillic acid): δ 3.44 (s, 2H), 3.84 (s, 3H), 6.75 (dd, 1H, $J =$ 1.8 and 7.8 Hz, H6), 6.81 (d, 1H, $J = 7.8$ Hz, H5), 6.93 (d, 1H, $J =$ 1.8 Hz, H2). A ROESY spectrum showed that H2 was adjacent to the methoxy group.

^{1}H NMR literature values of hydroxytyrosol (13): δ 2.70 (2H, t, J = 6.8 Hz, H1), 3.75 (2H, t, $J = 6.8$ Hz, H2), 6.70 (1H, dm, $J = 8.0$ and 2.0 Hz, H6), 6.80 (1H, d, $J = 2.0$ Hz, H2), 6.84 (1H, d, $J = 8.0$ Hz, H5), (300 MHz, $CDCl_3$).

DPPH Scavenging Test. Test compounds were added to a 50% ethanolic solution of DPPH radical (100 μM). The reaction mixtures were then incubated with shaking at 25 °C for 30 min. The absorbance of the remaining DPPH was determined at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH, expressed as a percentage of the absorbance of a control DPPH solution without test compounds. All assay conditions were optimized with respect to time, protein concentration, and substrate concentrations to ensure linearity. All experiments were carried out in triplicate at five different concentrations. Results were expressed as a percentage activity, and mean scavenging concentrations (SC_{50}) and 95% confidence intervals were calculated by regression analysis using JMP software (SAS Institute).

RESULTS

Labeled hydroxytyrosol was dosed to rats either orally as an oil solution or intravenously (iv) as a saline solution. Postdosing, urine samples were collected and analyzed by HPLC radiometric detection. Representative radiometric chromatograms from the oral oil dosing and iv dosing are shown in **Figure 2**. The relative percentages of hydroxytyrosol and its metabolites (M1−M5) excreted in urine over 24 h after oral and iv dosing of radiolabeled hydroxytyrosol could be determined from the radiometric chromatograms. Results are shown in **Table 1**.

The metabolites were purified by HPLC and subjected to mass spectrometry. The mass spectra obtained for M1, M3, hydroxytyrosol, and M5 are reproduced in **Figure 3**. The ROESY spectrum for M5 is shown in **Figure 4**.

The radical scavenging abilities of homovanillic acid, homovanillic alcohol, hydroxytyrosol, M1, M3, and M5 were

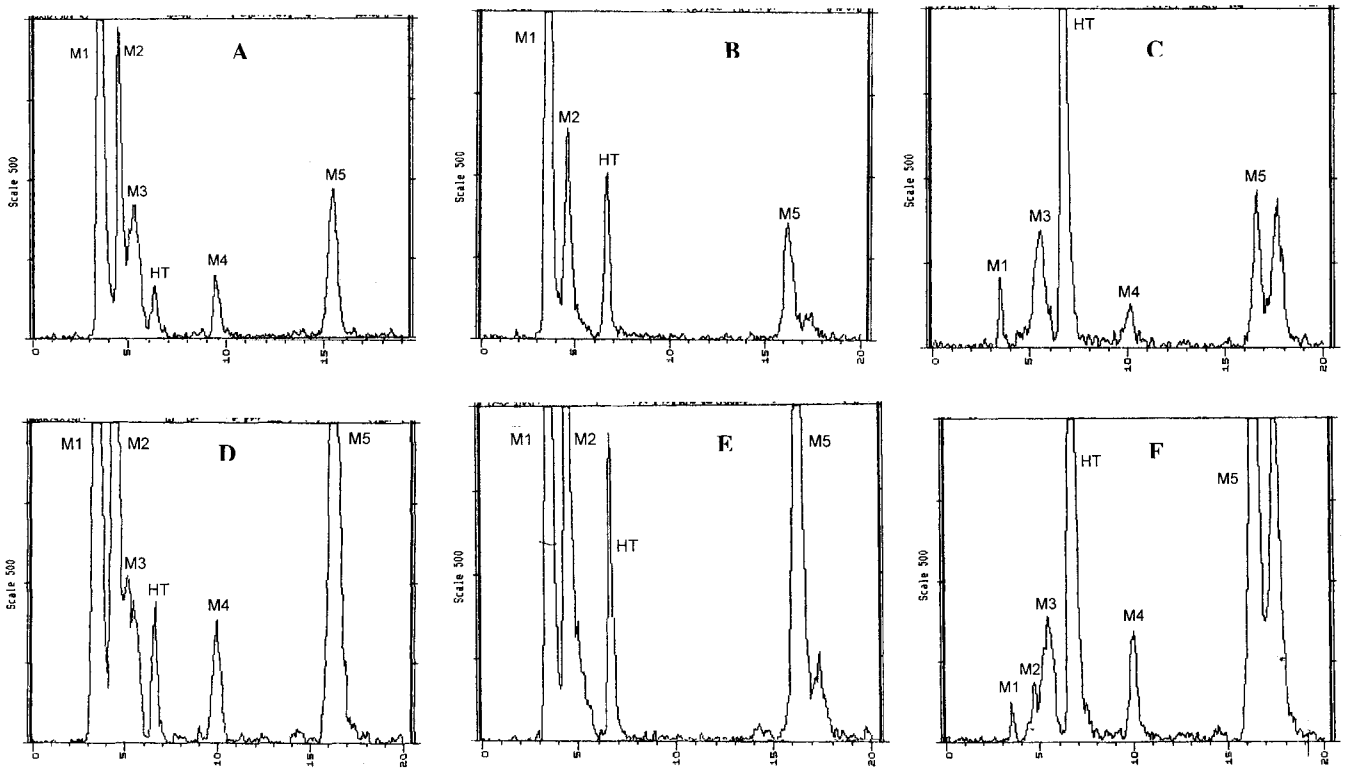

Figure 2. (A) Typical radiometric chromatogram of a urine sample (3 h sample) after oral dosing with tritium-labeled hydroxytyrosol dispersed in an olive oil solution. (B) Radiometric chromatogram after treatment of sample A with β-glucuronidase. (C) Radiometric chromatogram after treatment of sample A with sulfatase. (D) Typical radiometric chromatogram of a urine sample (2 h sample) after i.v. dosing with tritium-labeled hydroxytyrosol in a saline solution. (E) Radiometric chromatogram after treatment of sample D with β-glucuronidase. (F) Radiometric chromatogram after treatment of sample D with sulfatase.

Table 1. Cumulative Percentage of Total Radiolabeled Hydroxytyrosol and Its Metabolites (M1–M5) Eliminated in Urine within 24 h by Rats after Oral and Intravenous Dosing of Radiolabeled Hydroxytyrosol[a]

	percentage of compound eliminated						
	1 h	2 h	3 h	4 h	8 h	24 h	*t* test
oral hydroxytyrosol	1.19 ± 0.45	2.57 ± 0.33	3.41 ± 0.38	3.69 ± 0.42	4.10 ± 0.37	4.10 ± 0.37	$P < 0.05$
iv hydroxytyrosol	1.68 ± 0.22	2.21 ± 0.17	2.30 ± 0.16	2.33 ± 0.18	2.35 ± 18	2.35 ± 0.18	
oral M1	11.52 ± 4.92	26.44 ± 3.36	35.19 ± 4.49	38.17 ± 4.33	43.58 ± 4.04	48.42 ± 3.37	$P < 0.05$
iv M1	18.03 ± 3.22	27.36 ± 0.76	29.55 ± 0.76	30.87 ± 0.72	31.82 ± 0.81	34.24 ± 0.52	
oral M3	1.66 ± 0.67	4.69 ± 0.48	6.77 ± 0.61	7.58 ± 0.60	8.97 ± 0.45	9.53 ± 0.31	$P < 0.01$
iv M3	2.09 ± 0.40	3.28 ± 0.19	3.45 ± 0.23	3.57 ± 0.23	3.58 ± 0.22	3.58 ± 0.22	
oral M5	2.31 ± 1.00	5.52 ± 0.77	7.97 ± 0.94	8.69 ± 0.93	10.26 ± 0.89	10.26 ± 0.89	$P < 0.01$
iv M5	10.04 ± 2.39	16.69 ± 0.66	17.67 ± 0.74	18.33 ± 0.65	18.69 ± 0.57	18.69 ± 0.57	
oral other metabolites	3.27 ± 2.27	9.02 ± 1.81	12.84 ± 2.73	14.59 ± 3.21	18.44 ± 3.10	20.27 ± 2.87	$P < 0.05$
iv other metabolites	13.29 ± 5.28	24.04 ± 1.55	26.32 ± 1.38	27.77 ± 0.92	28.80 ± 1.24	30.87 ± 0.81	

[a] Values are reported as means ± SEM, $n = 5$.

determined with DPPH. These results were expressed as a percentage activity and mean scavenging concentrations (SC_{50}) with 95% confidence intervals (**Table 2**).

DISCUSSION

Tritiated hydroxytyrosol was synthesized according to a previously published procedure. The tritium label was incorporated at all unsubstituted positions on the aromatic ring. The stability of the tritium label of hydroxytyrosol was investigated in aqueous solutions (pH 7) and in urine. In both cases no exchange of the label was observed by radiometric HPLC after 24 h (*13*).

The amount of hydroxytyrosol eliminated in urine when orally dosed was greater than when dosed iv (see **Table 1**). The amounts of M1 and M3, later identified as a sulfate and a glucuronide conjugate, excreted in urine were also significantly higher when hydroxytyrosol was orally dosed than when dosed iv ($P < 0.05$). The amount of M5, later identified as homovanillic acid, excreted in urine was greater when hydroxytyrosol was iv dosed ($P < 0.05$).

Tentative Identification of M1. On the basis of specific enzyme-mediated hydrolysis, M1 was tentatively identified as a sulfate conjugate of hydroxytyrosol. The chromatographic peak of M2 also decreased after treatment of the urine samples with sulfatase, and it was assumed that M2 possessed a sulfate group, although further structural information could not be obtained.

MS/MS analysis confirmed that M1 could be a monosulfate conjugate of hydroxytyrosol. It had a negative molecular ion at

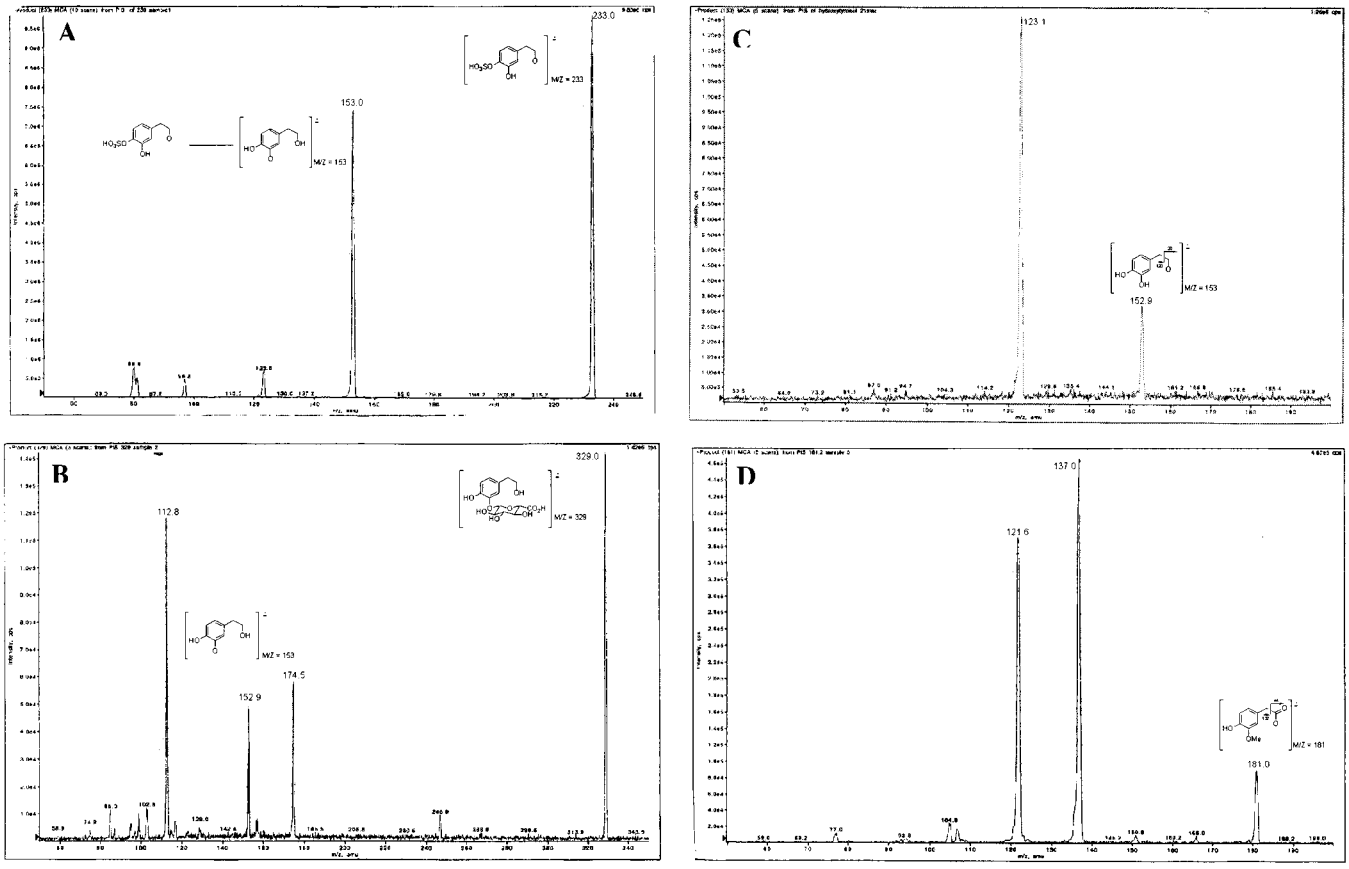

Figure 3. MS/MS negative fragment ion spectrum of M1 (A), M3 (B), hydroxytyrosol (C), and M5 (D).

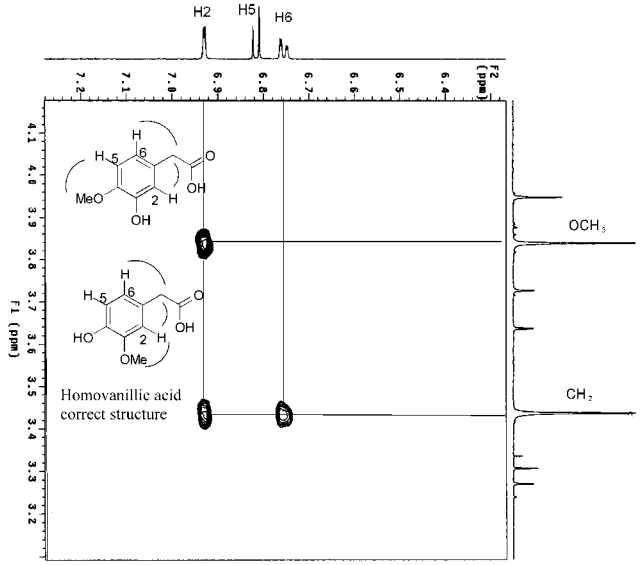

Figure 4. ROSEY spectrum of M5.

m/*z* 233, and the fragment at *m*/*z* 153 was presumably due to the loss of the sulfate group (**Figure 3**). A proton NMR of M1 could not be obtained as M1 could not be sufficiently purified from other polar compounds present in urine.

Tentative Identification of M3. On the basis of specific enzyme-mediated hydrolysis, M3 was tentatively identified as a glucuronide conjugate of hydroxytyrosol. Under these conditions the peak due to metabolite M4 also disappeared after treatment of the urine samples with β-glucuronidase with the M5 peak increasing in size.

MS/MS analysis confirmed that M3 could be a monoglucuronide conjugate of hydroxytyrosol. It possessed a negative molecular ion at *m*/*z* 329, and the fragment at *m*/*z* 153 resulted from loss of the glucuronide group (**Figure 3**). A proton NMR of M3 was obtained; however, the spectrum had impurities in the region of δ 3−5, and diagnostic resonances could not be determined. The aromatic region contained minimal impurities, and due to the shift of the aromatic resonances the position of the glucuronide group was determined to be in the 3-position.

Table 2. Scavenging Effects of Test Compounds on the DPPH Radical

test compound	SC_{50} (μM)	95% confidence intervals (μM)
hydroxytyrosol	11.0	10.5–11.6
homovanillic alcohol	11.4	10.0–13.0
homovanillic acid	14.8	13.1–16.7
M1	91.0	60.5–125.5
M3	2.3	1.8–2.7
M5	20.8	18.4–23.6

Identification of M5. On the basisof specific enzyme-mediated hydrolytic experiments, M5 was not a glucuronide or sulfate conjugate. From MS/MS analysis M5 was initially identified as 2-(3,4-dimethoxyphenyl)ethanol (**Figure 3**). This compound has a molecular weight of 182 and would produce a negative molecular ion at *m*/*z* 181; it would also potentially undergo cleavage to give the fragment at *m*/*z* 137. However, an authentic sample of this compound, analyzed under the same HPLC conditions as the urine samples, had a retention time of 45 min as compared to a retention time of 17 min for M5, and consequently the metabolite (M5) could not be 2-(3,4-dimethoxyphenyl)ethanol.

As homovanillic acid and homovanillic alcohol were metabolites in humans (*10*), it was initially thought to be possible that M5 could be homovanillic acid or homovanillic alcohol.

From a comparison of HPLC retention times it was determined that homovanillic alcohol was not present in any of the urine samples; however, compound M5 had the same retention time as homovanillic acid. The fragmentation pattern and the molecular ion in the mass spectrum of M5 fitted that of homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid). However, mass spectrometry is incapable of distinguishing between the structural isomers 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and 3-hydroxy-4-methoxyphenylacetic acid.

There is some doubt as to whether homovanillic alcohol and homovanillic acid have been correctly identified as metabolites of hydroxytyrosol in previous studies. There are examples in the literature of methylation, sulfation, and glucuoronidation in the 3- and 4-positions of catechol compounds (*14*, *15*). The study in which homovanillic alcohol was first identified does not provide spectroscopic data to support this claim (*11*). The later report by Caruso et al. (*10*) describes the identification of homovanillic acid and homovanillic alcohol only by mass spectrometry, which is insufficient to distinguish between the structural isomers. It is likely that this metabolite (M5) will have the same structure in rats as in humas, and hence M5 was analyzed by proton spectroscopy. The ^{1}H NMR spectrum of M5 confirmed that it is either 3-hydroxy-4-methoxyphenylacetic acid or 4-hydroxy-3-methoxyphenylacetic acid. Conclusive structural information was obtained from the ROESY spectrum of the metabolite (M5) (**Figure 4**). It can be seen that the methoxy group at δ 3.85 is adjacent to H2 on the aromatic ring and to the methylene group at 1. If the compound was 3-hydroxy-4-methoxyphenylacetic acid, an interaction of the methoxy group with H5 would be observed. This is not the case, and the metabolite is conclusively identified as homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid). As the peak in the radiometric chromatograms due to metabolite M4 disappears after treatment of the samples with β-glucuronidase, M4 could thus possibly be a glucuronide conjugate of homovanillic acid.

Radical Scavenging Abilities with DPPH. There have been several studies that have investigated the radical scavenging abilities of hydroxytyrosol with DPPH (*16*−*18*). The study by Saija et al. (*16*) obtained an SC_{50} of 20.51 μM for hydroxytyrosol. Previous studies determined EC_{50} values (which is equivalent to the SC_{50} value) of 26.0 and 19 μM, respectively (*17*, *18*). The SC_{50} value for hydroxytyrosol in this study of 11.0 μM (10.5−11.61 μM, 95% confidence intervals) compares well with the earlier values.

The radical scavenging potencies of homovanillic acid and homovanillic alcohol with the radical DPPH showed them to be potent antioxidants, having SC_{50} values of 14.8 and 11.4 μM, respectively. The 3-*O*-glucuronide conjugate was more potent than hydroxytyrosol, with an SC_{50} of 2.3 μM in comparison to 11.0 μM, and the monosulfate conjugate was almost devoid of activity.

In conclusion, this study identifies a monosulfate conjugate, a 3-*O*-glucuronide conjugate of hydroxytyrosol, and homovanillic acid as metabolites of hydroxytyrosol after dosing of the labeled compound to rats. It was also determined that hydroxytyrosol is excreted unchanged in urine when it is dosed orally or intravenously. A fourth metabolite has been postulated to be an *O*-glucuronide conjugate of homovanillic acid. Hydroxytyrosol in rats is metabolized to at least two compounds that are capable of scavenging radicals, which may imply that the metabolites of hydroxytyrosol, in addition to the parent compound, are the source of the radical scavenging ability of olive oil-derived compounds in vivo.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; DDPH, 2,2-diphenyl-1-picrylhydrazyl radical; LDL, low-density lipoprotein; HPLC, high-performance liquid chromatography.

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