Urinary Excretion of Olive Oil Phenols and Their Metabolites in Humans

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We have recently demonstrated, in humans, the bioavailability of hydroxytyrosol (3,4-dihydroxyphenylethanol; HT), one of the major antioxidant components of virgin olive oil. In particular, we reported that this compound is present in lipoproteins involved in atherosclerotic processes and is excreted in the urine mainly as glucuronide-conjugate. The aim of the present study was to elucidate, in humans, the metabolic fate of HT after ingestion of virgin olive oil. After administration of virgin olive oil, 24-hour urine collections of healthy volunteers were prepared for gas chromatography-mass spectrometry analyses in order to identify and quantify HT and its metabolites homovanillic alcohol (HVA1c) and homovanillic acid (HVA). The results indicate that this compound undergoes the action of catechol-*O*-methyl transferase (COMT), enzymes involved in the catecholamine catabolism, resulting in an enhanced excretion of HVA1c. We also found a significant increase of HVA, indicating an oxidation of the ethanolic residue of HT and/or of HVA1c in humans. The excretion of both metabolites significantly correlated with the dose of administered HT.

THE BENEFICIAL EFFECTS of Mediterranean dietary habits on human health may in part result from their high proportion of antioxidant compounds.[1] In particular, olive oil, the principal fat component of this diet, contains a series of phenolic "minor components," which are also responsible for its typical taste.[2] In addition, these compounds, namely, hydroxytyrosol (3,4 dihydroxyphenylethanol; HT) and oleuropein aglycon (OEA), which share a catecholic (ortho-diphenolic) structure, exhibit a series of in vitro biological activities such as protection of low-density lipoprotein (LDL) against peroxyl radical- or copper-induced oxidation, inhibition of platelet aggregation, and potentiation of the nitric oxide-mediated macrophagic immune response.[3] Recently, we demonstrated that, after olive oil consumption, 4-hydroxyphenylethanol and HT are dose-dependently absorbed in humans and are excreted in the urine, mostly as glucuronide conjugates.[4] Furthermore, an increase in the administered concentration of phenols led to an increase in the proportion of glucuronide conjugation.[4] Finally, olive oil phenols have been found in human LDL and high-density lipoproteins shortly after the intake of olive oil.[5]

It is noteworthy that HT exists in the brain as an endogenous catabolite of catecholic neurotransmitters such as dopamine and norepinephrine,[6] but its presence in urine has never, until now, been described. On the other hand, the formation of homovanillic alcohol (HVAlc), ie, the *O*-methylated derivative of HT, was reported by Manna et al[7] in human Caco-2 cells incubated with HT. Therefore, this report prompted us to reanalyze the urinary samples described by Visioli et al[4] for the presence of catechol-*O*-methyl transferase (COMT) derivatives and/or additional HT metabolites.

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MATERIALS AND METHODS

β-glucuronidase and arylsulfatase, homovanillic acid (HVA), HVAlc, and α-naphthol, used as internal standard, were purchased from Sigma-Aldrich (Milan, Italy), while HT was a generous gift of Prof G.F. Montedoro, University of Perugia, Italy. OEA were obtained by enzymatic hydrolysis of oleuropein glycoside, as previously described.[8] All reagents and solvents were of analytical grade (Merck, Milan, Italy).

The group of Prof F.F. Vincieri, University of Florence, prepared 4 olive oil samples (hereinafter referred to as A, B, C, and D, respectively) by adding different amounts of an olive oil phenolic extract to a phenol-poor oil.[4] The characterization of the phenolic fraction was carried out by gas chromatography- and liquid chromatography-mass spectrometry, as previously described,[9] in order to quantify simple (ie, HT) and complex (ie, OEA, phenols, HVA, and HVAlc) components.

The experimental protocol was fully described by Visioli et al.[4] Briefly, 6 fasting healthy male volunteers were given 50 mL of oil samples, accompanied by 40 g of bread. This treatment was repeated 4 times, after a 1-month period of washout, so that, overall, each subject received the 4 different oil samples. Urine was collected for 24 hours and its volume was recorded. For the quantification of total HVAlc and HVA, 1 mL of urine was added with 1 μg/mL of α-naphthol as internal standard, and the enzymatic reaction with both β-glucuronidase and arylsulfatase (320 U/mL; acetate buffer at pH 5) was performed for 17 hours at room temperature. At the end of the reaction, samples were acidified with HCl (0.3 mol/L):acetonitrile (1/1; vol:vol) and extracted twice with 3 vol of ethyl acetate; for the quantitative analyses, the organic phase was evaporated to dryness under nitrogen flow and an aliquot was derivatized as previously described .[4] Gas chromatography-mass spectrometry analyses were performed on a BP1 fused silica column (SGE s.r.l., Italy) connected with a GCQ (Thermoquest, San Jose, CA) mass spectrometry. The trymethylsilyl ether derivatives of HVAlc and HVA showed individual peaks with retention times of 9.1 and 9.46 minutes, respectively. Parent ions at m/z 312 (100%, HVAlc) and 326 (100%, HVA) were selected for selected IOM monitoring (SIM) analyses. Calibration curves were prepared using human urine samples (1 mL) spiked with α-naphthol (1 μg/ml) and increasing amounts of authentic HVAlc and HVA in the 0.5 to 10 μg/mL range. A good correlation ($r^2 > 0.98$) was observed between intraday and interday analyses. Correlations between HT in olive oil administered and excreted were obtained by linear regression. Data were compared using the paired two-tailed *t* test, as each subject ingested all 4 oils over the experimental period; this allowed for a statistical comparison of HT metabolism within the same subject. In turn, the levels of metabolites derived from oil A, which contained the lowest level of HT, were compared with metabolite levels from the other oils to verify that

Table 1. Doses of OEA, Which Contains HT as Part of its Moiety, and HT Administered to Human Volunteers

Oil	OEA (mg/50 mL)	HT Equivalent (mg/50 mL)	Free HT (mg/50 mL)	Total HT Administered (mg/50 mL)
A	12.6	5.1	1.9	7.0
B	23.7	9.7	3.7	13.3
C	33.7	13.7	5.5	19.2
D	39.5	16.1	7.1	23.2

higher doses of HT resulted in higher concentrations of HVAlc and HVA. A P value less than .05 was considered statistically significant.

RESULTS AND DISCUSSION

The composition of the phenolic fraction of the oil samples, given to the volunteers, is reported in Table 1. The lowest administered dose (oil A) was 7 mg of total HT/50 mL oil, whereas oil D provided about 23 mg/50 mL oil. The major portion of HT was found as OEA and the ratio between OEA and HT was about 7/1 in all oil samples.

In humans, as previously described in Caco-2 cells, HT undergoes the action of COMT, resulting in a significant linear regression between the amount of ingested HT and the urinary excretion of HVAlc of each volunteer (test f = 38, *df* 1,21; R = 0.90, $P < .01$; Fig 1A).

HVAlc contributes to 22.0% ± 2.03% (mean ± SD, n = 24) of the total excretion of HT and its metabolites (Table 2). This linear increase in HVAlc urinary concentrations suggests that the enzymes involved in the methylation and glucuronidation of HT are below saturation in this experimental setup.

In the same urine samples, we have identified and quantified HVA, the oxidation product of the alcoholic residue of HT, also generated by the catabolism of catecholamines; the metabolic pathway leading to the formation of HVA may either precede or follow the action of COMT on HT. We found a significant linear regression (test f= 13.03, *df* 1,22; R = 0.80; $P < .01$; Fig 1B) between the administered HT and HVA in the urine samples of each volunteer, despite a rather high basal urinary concentrations of HVA (Table 2). This finding suggests that the oxidation of the ethanolic residue of HT or HVAlc occurs in human.

The amount of HT excreted in the urine of each volunteer shows a significant linear regression (test f = 22.8, *df* 1,23; R = 0.85; $P < .01$) with the total amount of HT provided with the

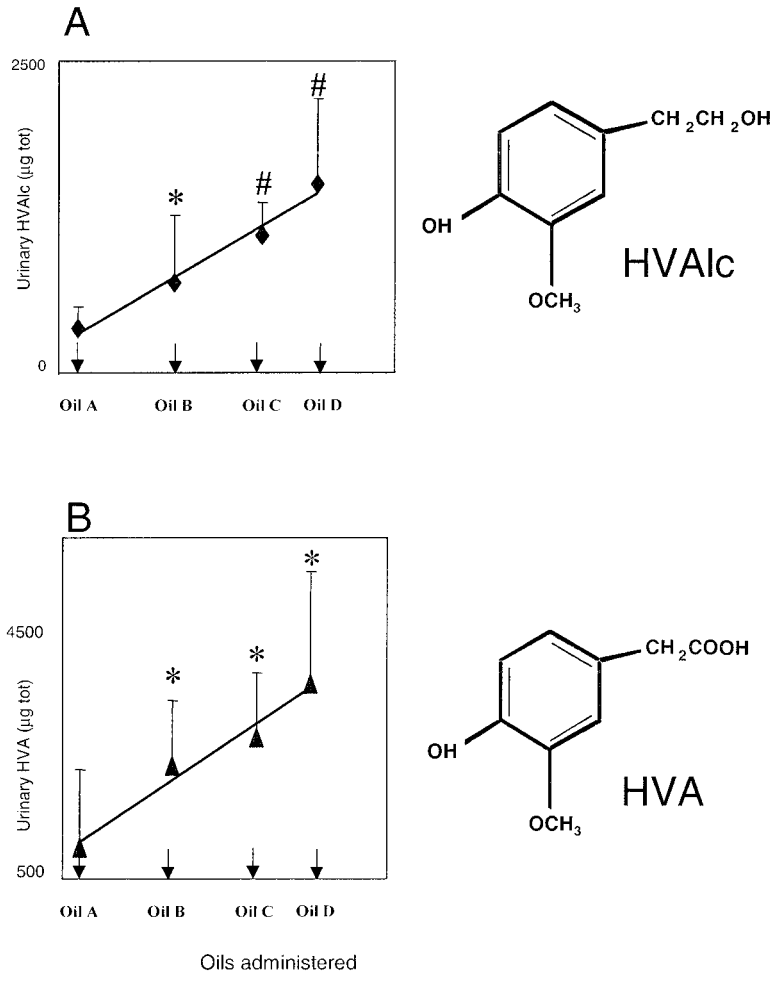

Fig 1. (A) Correlation between the amount of ingested HT and the urinary excretion of HVAlc (chemical structure shown on the right). (B) Correlation between the amount of ingested HT and the urinary excretion of HVA (chemical structure shown on the right). Data are means ± SD. *$P < .05$ and #$P < .005$ compared to metabolites derived from the lowest level of HT administered (7 mg, oil A).

Table 2. Urinary HT Metabolites (HVAlc and HVA) in Human Volunteers After Ingestion of Four Different Oil Samples

Oil	HT Excretion Over Basal Levels (μg/24 h)	% of Total Metabolites	HVAlc Excretion Over Basal Levels (μg/24 h)	% of Total Metabolites	HVA Excretion Over Basal Levels (μg/24 h)	% of Total Metabolites	Total Excretion (μg/24 h)	Total Absorption (% of given dose)
A	267	16.8	367	22.0	1,027	61.8	1,661	23.7
B	1,328	29.8	749	16.8	2,380	53.4	4,457	33.5
C	1,112	21.8	1,137	22.3	2,837	55.8	5,086	26.5
D	1,653	23.7	1,567	22.4	3,759	53.9	6,979	30.1
Urinary excretion (mean $\pm$ SD)		23 $\pm$ 5.37		21 $\pm$ 2.72		56 $\pm$ 3.86		28 $\pm$ 4.30

NOTE. All 6 subjects, at completion of the study, received the 4 oil samples.

oil samples, ie, as a free catechol or as part of the OEA moiety.[4] In terms of percentage of total urinary HT and its metabolites, HT was found to be 8.0% $\pm$ 4.6% (mean $\pm$ SD, n = 24) (Table 2). Whether OEA in the administered oils may contribute to the excretion of HT and its metabolites will be the subject of further investigations.

In conclusion, we describe novel pathways of HT metabolism in humans after olive oil consumption. In particular, we report for the first time the urinary excretion of HVAlc, in large excess over its basal excretion (57 $\pm$ 3 μg excreted in 24 hours, mean $\pm$ SD, n = 6). We also describe the substrate-induced enhancement of HVA formation, also a product of catecholamines metabolism, in addition to its basal urinary excretion (1,660 $\pm$ 350 μg excreted in 24 hours, mean $\pm$ SD, n = 6). Indeed, assuming that the proportion of excretion is identical for dietary and experimental (see below) oils, the results reported here suggest that HT increases the basal excretion of HVA even at low doses, eg, 7 mg as in the case of oil A.

A major limitation of this study is that it employed oil samples artificially enriched with a phenolic extract, and thus extrapolation of these results to a typical Mediterranean diet pattern should be exerted with care. However, the correlation between the metabolites and the total amount of HT provided with the oil samples, which is maintained over the range of doses we adopted, ie, from 7.00 to 23.15 mg, suggests that the proportion of the different metabolites is also retained at much lower intakes. Future investigations will adopt commercially available virgin olive oils, thus allowing to further elucidate the in vivo kinetics of olive oil phenolics in habitual consumption quantities.

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