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Technical Notes

Determination of Hydroxytyrosol in Plasma by HPLC

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Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol), a phenolic compound present in extravirgin olive oil, has been reported to contribute to the prevention of cardiovascular disease. The present study describes an accurate and reproducible reversed-phase HPLC method to measure hydroxytyrosol in plasma. This compound was extracted from acidified plasma by solid-phase extraction using an Oasis HLB copolymer. The plasma sample was rinsed with water and methanol in water (5:95; v/v). Hydroxytyrosol was eluted with methanol, which was subsequently evaporated under a nitrogen stream. Analysis by HPLC with diode array-UV detection was carried out using a C_{18} column and a gradient elution with acidified water and methanol/acetonitrile (50:50; v/v). The method was validated by the analyses of plasma samples spiked with pure hydroxytyrosol, obtaining a linear correlation (0.9986) and precision with a coefficient of variation ranging from 0.79 to 6.66%. The recovery was ∼100%, and the limit of detection was 37 ng/mL. The oral administration of hydroxytyrosol to rats and its subsequent detection in plasma showed that the method is suitable for pharmacokinetic studies.

The healthy effects of the Mediterranean diet have been attributed, at least in part, to the intake of fat with a high content of monounsaturated fatty acids and a lower consumption of saturated fatty acids.[1,2] Olive oil is the main source of fat containing the higher monounsaturated/saturated ratio in the Mediterranean diet,[3,4] and it is believed to contribute to the prevention of cardiovascular disease[3,5] and cancer.[6,7] Its beneficial effects may also be due to the presence of antioxidants in the nonsaponifiable fraction[8−11] such as hydroxytyrosol (2-(3,4-dihydroxyphenyl)-ethanol) (Figure 1) and its secoiridoid derivatives.[12]

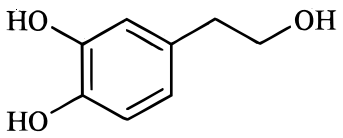

Figure 1. Chemical structure of hydroxytyrosol.

Experiments in vitro have shown that hydroxytyrosol has many effects that could be beneficial in vivo, including antioxidant activity,[13] protection of lipoproteins against oxidative and free-radical damage,[10,14−16] and inhibition of platelet aggregation and eicosanoid formation.[17]

Notwithstanding the effects described in vitro, the mechanisms of hydroxytyrosol absorption, bioavailability, and tissue distribution in humans are still undefined. This lack of information is due not only to the difficulty of developing sensitive methods for measuring hydroxytyrosol in plasma and body fluids but also to the absence of a commercially available pure standard.

Here we develop a simple, accurate, and sensitive method for identifying and quantifying hydroxytyrosol in human plasma. We present a rapid procedure for sample preparation, avoiding successive plasma manipulations by means of an SPE cartridge, followed by HPLC analysis with photodiode array detection. The

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(1) Mensink, R. P.; Katan, M. B. *Arterioscler. Thromb.* **1992**, *12*, 911−919.
(2) Nestle, M. *Am. J. Clin. Nutr.* **1995**, *61*, 1313S−1320S.
(3) Willett, W. C.; Sacks, S.; Trichopoulou, A.; Drescher, G.; Ferro-Luzzi, A.; Helsing, E.; Trichopoulos, D. *Am. J. Clin. Nutr.* **1995**, *61*, 1402S−1406S.
(4) Serra-Majem, Ll.; Ribas, L.; Ramon, J. M. *Br. J. Nutr.* **1999**, *81* (2), S105−S112.
(5) Katan, M. B.; Zock, P. L.; Mensink, R. P. *Am. J. Clin. Nutr.* **1995**, *61*, 1368S−1373S.
(6) Trichopoulou, A. *Cancer Causes Control* **1995**, *6*, 475−476.
(7) Lipworth, L.; Martinez, M. E.; Angell, J.; Hsien, C. C.; Trichopoulos, D. *Prev. Med.* **1997**, *26*, 181−190.
(8) Scaccini, C.; Nardini, M.; D′Aquino, M.; Gentili, V.; Felice, M. D.; Tomassi, G. *J. Lipid Res.* **1992**, *33*, 627−633.
(9) Wiseman, S. A.; Mathod, J. N. N. J.; de Fouw, N. J.; Tijburg, L. B. M. *Atherosclerosis* **1996**, *12*, 15−33.
(10) Visioli, F.; Vinceri, F. F.; Galli, C. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60−64.
(11) De la Puerta, R.; Ruíz-Gutierrez, V.; Hoult, J. R. S. *Biochem. Pharmacol.* **1999**, 445−449.
(12) Montedoro, G. F.; Servilli, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. *J. Agric. Food Chem.* **1993**, *41*, 2228−2234.
(13) Chimi, H.; Cillard, J.; Cillard, P.; Rahmani, M. *J. Am. Oil Chem. Soc.* **1991**, *68* (5), 307−312.
(14) Grignaffini, P.; Roma, P.; Galli, C.; Catapano, A. L. *Lancet* **1994**, *343*, 1296−1297.
(15) Visioli, F.; Bellomo, G.; Montedoro, G. F.; Galli, C. *Atherosclerosis* **1995**, *117*, 25−32.
(16) Manna, C.; Galleti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. *J. Nutr.* **1997**, *127*, 286−292.
(17) Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli, C. *Thromb. Res.* **1995**, *78* (2), 151−160.

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performance of the method was assessed by oral administration of hydroxytyrosol to Wistar rats and its measurement in plasma.

EXPERIMENTAL SECTION

Instrument. HPLC analyses were performed on a 1100 Hewlett-Packard (HP) (Palo Alto, CA) gradient liquid chromatograph equipped with a Rheodyne injection valve with a 20-μL fixed loop, a Lichrospher 100 RP-18 reversed-phase column (250 mm × 4 mm, 5 μm) from Merck (Darmstadt, Germany), preceded by a precolumn of the same material as the stationary phase, and a HP 1100 diode array UV−visible detector. An HP Chemstation software system controlled all the equipment and carried out the data processing.

Chemicals and Reagents. A chemically pure standard of hydroxytyrosol was synthesized by $LiAlH_4$ reduction of 3,4-dihydroxy-1-benzeneacetic acid as described by Baraldi et al.[18] The internal standard, catechol, was obtained from Merck (Darmstadt, Germany). Acetonitrile, methanol, and acetic acid, all HPLC grade, were purchased from Romil Ltd. (Cambridge, England). All other chemicals were analytical grade and were used without further purification. Water used in all experiments was passed through a Milli-Q (Millipore, Milan, Italy) water purification system (18 mΩ). A stock solution of catechol (1.02 mg/mL) in water was used as internal standard.

Preparation of Plasma Samples. Blood samples were collected in tubes containing EDTA-K_3 and centrifuged at 2700 rpm (Beckman, model TJ-6 centrifuge, rotor TH-4 with buckets) for 15 min, and the plasma was immediately separated from cells. A standard solution (10 μg/mL) of catechol and 10 μL of phosphoric acid (85% v/v) were added to 1-mL aliquots of plasma and mixed in a vortex for 1 min.

Solid-Phase Extraction (SPE) of Plasma Samples. Solid-phase purification of hydroxytyrosol from plasma samples was carried out on an Oasis HLB extraction cartridge (30 mg, 1 mL; WAT 094226) purchased from Waters (Milford, MA). The cartridge was placed in a device fitted with a small vacuum pump and a waste receiver. The pressure was carefully monitored during the critical cartridge conditioning, equilibration, and sample loading steps, thus preventing changes in the separation efficiency due to variations in the flow rate. The cartridge was conditioned at a flow rate of 4−5 mL/min, and the analyte was loaded, washed, and eluted at 1.5−2 mL/min. Prior to use, the cartridge was conditioned with 1 mL of methanol followed by equilibration with 1 mL of water. A 1-mL aliquot of spiked acidified plasma was slowly loaded into the cartridge, followed by 0.5 mL of water, and then 1 mL of 5% (v/v) methanol in water. The vacuum was released to replace the waste receiver with a conical glass tube containing 10 μL of 1% ascorbic acid solution. The SPE cartridge was eluted under vacuum with 2 mL of methanol. The conical tube containing the eluted fraction was capped with Parafilm and stored at 4 °C until HPLC analysis. Just before the HPLC analysis, the eluted fraction was evaporated under a nitrogen stream to a final volume of 0.2 mL.

HPLC Procedure. A 20-μL aliquot of the evaporated sample was injected into a Lichrospher 100 RP-18 (Merck) reversed-phase column (250 mm × 4 mm) with a 5-μm particle operating at 30 °C. The mobile phase included solvent A, consisting of 3% acetic acid in 97% deionized water, and solvent B, containing equal volumes of acetonitrile and methanol. The flow rate was 1 mL/min. Separation was performed using the gradient elution program: 0 min, 95% A and 5% B; 25.0 min, 70% A and 30% B; 27.5 min, 60% A and 40% B; 30.0 min, 30% A and 70% B; 40 min, 0% A and 100% B; 45 min, 95% A and 5% B. There was a 5-min delay prior to the injection of the next sample to ensure reequilibration of the column.

The chromatograms were obtained according to the retention time with detection at 280 nm, at which the absorbance of hydroxytyrosol presents a maximum. Peak identification of this compound was carried out by comparison of the retention time and its UV spectra (from 200 to 400 nm) with those of a standard.

Method Validation. The solid-phase extraction of hydroxytyrosol from human plasma followed by HPLC analysis was validated according to *The United States Pharmacopoeia*.[20]

Samples. A pool of human plasma samples obtained from fasted healthy individuals by venipuncture (Blood Bank, Hospital Clínic i Provincial de Barcelona, Spain) was prepared. Aliquots of the pooled plasma were stored at −20 °C until the analyses were performed, and the samples were prepared as indicated in preparation of plasma samples.

Linearity. A stock solution of hydroxytyrosol was obtained by dissolving the pure standard in water; the solution was stored at 4 °C and used within 4 weeks. Spiked plasma samples containing increasing concentrations of hydroxytyrosol: 0.5, 0.75, 1, 2.5, 5, 7.5, and 10 μg/mL and a fixed concentration of catechol as internal standard (IS) (10 μg/mL) were analyzed according to the procedure described above. Linearity was checked by performing linear regression analysis of the peak area ratio of hydroxytyrosol/catechol against hydroxytyrosol concentration.

Precision. The precision of the analytical method was determined by assaying a sufficient number of plasma samples (n = 4−6) at seven different concentrations of hydroxytyrosol ranging from 0.5 to 10 μg/mL and was expressed as relative standard deviation (coefficient of variation). Peak areas were considered for the calculation of the concentration and establish the precision.

Accuracy. One milliliter of plasma and 1 mL of water as control were spiked with hydroxytyrosol at concentrations of 1 and 10 μg/mL together with 10 μg/mL of catechol as internal standard. The samples were purified by SPE and analyzed by HPLC as described. Accuracy was calculated by comparing the values for the plasma samples and control; results are expressed as percentages of analyte recovered.

Sensitivity. The limit of detection (LD) and the limit of quantification (LQ) were calculated by measuring the analytical background response, running six blanks of plasma using the maximum sensitivity allowed by the system. The signal-to-noise ratio was used to determine the LD, and it was estimated as the concentration of hydroxytyrosol in plasma samples that generated a peak with an area at least 3 times higher than the baseline noise. LQ was considered to be 10 times the standard deviation of the six blank samples analyzed using the maximum sensitivity allowed by the system. The LQ was subsequently validated by the analysis of six plasma samples known to be near the LQ.

(18) Baraldi, P. G.; Simoni, D.; Manfredini, S.; Menziani, E. *Liebigs Ann. Chem.* **1983**, 684−686.

(19) Bai, C.; Yan, X.; Takenaka, M.; Sekiya, K.; Nagata, T. *J. Agric. Food Chem.* **1998**, 46, 3998−4001.

(20) *U.S. Pharmacopoeia*, USP XXIII **1995,** *1225*, 1982−1984.

Animal Studies. Male Wistar rats (200−250 g) were housed three per cage, in a temperature-controlled room, with a light−dark cycle of 12 h and free access to water and a commercial rat chow. Animal treatment was in full accordance with the European Community Guidelines for the care and management of laboratory animals. Hydroxytyrosol dissolved in water was administered orally to overnight-fasted rats at a dose of 20 mg/kg, except for the control group, which was given only water. Animals were anesthetized with ether and blood was collected by cardiac puncture and transferred to a tube containing EDTA-K_3 as anticoagulant, at 5 and 10 min after the administration. Immediately after obtaining the plasma by centrifugation, the analyte was purified using the SPE procedure described above.

RESULTS AND DISCUSSION

Optimization of Solid-Phase Extraction. Methanol was the solvent of choice since hydroxytyrosol is usually obtained by extraction with this solvent from extravirgin olive oil.[21,22] Furthermore, it proved to be an efficient solvent, breaking the hydrophobic bond, eluting hydroxytyrosol, and rendering excellent recoveries.

Sample acidification was taken into account in order to disrupt hydroxytyrosol−protein binding and enhance recovery. Therefore, in initial attempts, extractions were carried out by mixing the plasma sample with 10 and 20 μL/mL phosphoric acid and without acidification. The addition of 10 μL/mL phosphoric acid was selected because it yielded the best recovery.

The optimal amount of water and the percentage of methanol in water that was sufficient to wash the sample without eluting hydroxytyrosol were carefully evaluated due to the high polarity of this compound. An Oasis cartridge was loaded with a known amount of hydroxytyrosol dissolved in water, and the washout fractions were monitored by injecting them in the HPLC. The optimal volume of water for the first rinsing step was studied over a range from 0.5 to 2 mL and found to be 0.5 mL. For the second washing step, different amounts of methanol in water were assessed: 2.5, 5, and 7.5%. A concentration of 5% proved to be the optimal solution to eliminate interferences of plasma without eluting the analyte. No hydroxytyrosol was detected in the optimal washout fractions, and it was completely recovered when 2 mL of 100% methanol was loaded into the cartridge. One milliliter of methanol was sufficient to elute the analyte; however, an excess of 1 mL is advised.

The concentration of the eluted fraction under a nitrogen stream to a final volume of 0.2 mL enhanced the sensitivity of the analytical method allowing the quantification of very low amounts of this compound. However, volumes lower than 0.2 mL were prone to erratic recoveries. During concentration, methanol was evaporated; thus, the 0.2 mL corresponded to an aqueous solution of hydroxytyrosol that, when injected into the HPLC, gave a chromatogram (Figure 2) with excellent peak shapes.

Selection of the Internal Standard. Catechol, an alcohol with structural similarity to hydroxytyrosol, which eluted at 9.05 min and was not present in plasma samples, was used as IS. To confirm that catechol was retained by the Oasis cartridge, 1 mL of plasma and 1 mL of water as control were spiked with 10 μL/mL catechol.

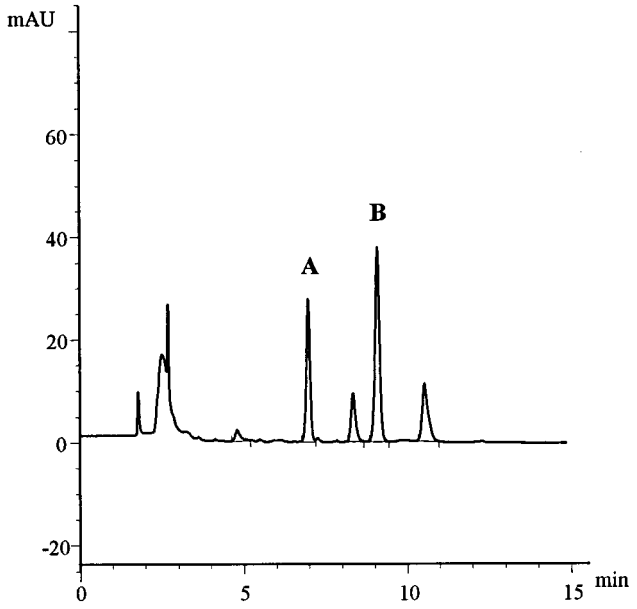

Figure 2. HPLC chromatogram at 280 nm of hydroxytyrosol in human plasma after isolation. Peak A, hydroxytyrosol; peak B, catechol (internal standard).

The samples were extracted as described in the Experimental Section, and the recovery was ∼98%.

Stability of Hydroxytyrosol. In the course of prior experiments (data not shown), a decrease in hydroxytyrosol stability was observed. Although this lack of stability was not noticed in the SPE extraction from plasma, it was thought advisable to add an antioxidant. The addition of 10 μL of 1% ascorbic acid and storage of the final methanolic extract in a tube capped with Parafilm at 4 °C, followed by evaporation under nitrogen stream just before the HPLC analysis, stabilized the hydroxytyrosol for at least one week. These conditions allowed a large number of samples to be extracted and stored for later HPLC analysis.

Method Validation. The analytical performance parameters assessed for the overall assay were linearity, precision, accuracy, sensitivity, and selectivity.

Linearity. The response of hydroxytyrosol was checked in the range of application of the analytical method by linear regression analysis by the least-squares method of peak area ratio of hydroxytyrosol/catechol against different hydroxytyrosol concentrations. The response was linear in the range of concentrations evaluated from 0.5 to 10 μg/mL, giving an equation of $y = 0.062x + 0.001$ ($n = 31$) and a regression coefficient of 0.9986.

Precision. The precision expressed as coefficient of variation (CV) ranged from 0.79 to 6.66%, at seven different concentrations ranging from 0.5 to 10 μg/mL, indicating that the analytical method is repeatable. The values obtained were as follows (CV, %): 6.66 (0.50 μg/mL), 3.63 (0.75 μg/mL), 1.60 (1.00 μg/mL), 3.01 (2.50 μg/mL), 3.57 (5.00 μg/mL), 0.79 (7.50 μg/mL), and 1.90 (10.00 μg/mL). The results were lower than those acceptable according to the CV established by Horwitz[23] for intralaboratory analysis.

Accuracy. The accuracy was determined for the overall assay by measuring the percentage of recovery after the addition of known amounts of standard to a control and to a pool of human

(21) Vázquez-Roncero, A.; Janer del Valle, C.; Janer del Valle, M. L. *Grasas Aceites* **1976**, *26*, 14−18.

(22) Gutfinger, T. *J. Am. Oil Chem. Soc.* **1981**, *58*, 96−968.

(23) Horwitz, W. *Anal. Chem.* **1982**, *54*, 67A−76A.

plasma, at two different concentrations. The mean recoveries at 1 and 10 μg/mL were 98.7 and 99.8%, respectively. The values indicate that hydroxytyrosol is quantitatively extracted using this method.

Sensitivity. The limit of detection was 37 ng/mL on the basis of a signal-to-noise ratio of 3. The limit of quantification was 96 ng/mL.

Selectivity. Hydroxytyrosol was quantified at 280 nm, its maximum absorbance to improve selectivity. Hydroxytyrosol was well resolved and free from interference peaks (Figure 2). The identity of the chromatographic peak was confirmed not only by its retention time but also by its spectrum (Figure 3).

Verification of the Method. Hydroxytyrosol was orally administered to rats at a dose of 20 mg/kg, to assess the validity of the method. Blood taken by cardiac puncture was analyzed as described in the Experimental Section, resulting in a hydroxytyrosol plasmatic concentration of 1.22 μg/mL at 5 min after administration, and 1.91 μg/mL at 10 min. The results showed that the method developed was reliable, reproducible, and easily applied to biological samples.

In contrast to the large number of methods published to determine hydroxytyrosol in olive oil,[24−27] there are few studies that analyze this compound in biological samples. The first procedure to determine hydroxytyrosol in rat plasma samples was reported by Bai et al.[19] This method included organic-phase extraction and derivatization prior to the analysis by gas chromatography. Solid-phase extraction of hydroxytyrosol with an Oasis cartridge was suitable for the determination of this compound since it avoided analyte losses and provided maximal sensitivity with minimal handling.

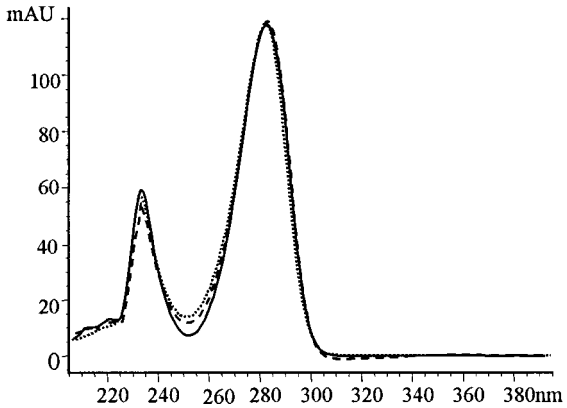

Figure 3. UV spectra of standard hydroxytyrosol (dotted line), hydroxytyrosol in human plasma after isolation (straight line), and hydroxytyrosol in rat plasma after isolation (dashed line), all obtained by diode array detection.

In conclusion, a simple, precise, sensitive, reproducible, and reliable method has been developed to study the levels of hydroxytyrosol in plasma. The handling of samples is easy and can be performed automatically. The oral administration of hydroxytyrosol to experimental animals and its detection in plasma has provided evidence of its absorption.

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(24) Montedoro, G. F.; Servilli, M.; Baldioli, M.; Miniati, E. *J. Agric. Food Chem*. **1992**, *40*, 1571−1576.

(25) Tsimidou, M.; Papadopoulos, G.; Boskou, D. *Food Chem*. **1992**, *44*, 53−60.

(26) Akasbi, M.; Shoeman D. W.; Csallany, S. *J. Am. Oil Chem. Soc*. **1993**, *70*, 367−370.

(27) Angerosa, F.; D'Alessandro, N.; Konstantinou, P.; Di Giacinto, L. *J. Agric. Food Chem*. **1995**, *43*, 1802−1806.