0090-9556/01/2911-1492–1498$3.00
DRUG METABOLISM AND DISPOSITION
Vol. 29, No. 11
383/941704
DMD 29:1492–1498, 2001
Printed in U.S.A.

PHARMACOKINETICS AND METABOLISM OF HYDROXYTYROSOL, A NATURAL ANTIOXIDANT FROM OLIVE OIL

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(Received March 5, 2001; accepted August 14, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

3,4-Dihydroxyphenylethanol (DOPET) is the major *o*-diphenol detectable in extra virgin olive oil, either in free or esterified form. Despite its relevant biological effects, mainly related to its antioxidant properties, little data have been reported so far on its toxicity and metabolism. The aim of the present work is to evaluate DOPET toxicity and to investigate its molecular pharmacokinetics by using the ^{14}C-labeled diphenol. When orally administered to rats, the molecule does not show appreciable toxicity up to 2 g/kg b.wt. To identify and quantify its metabolites, [^{14}C]DOPET has been synthesized and intravenously injected in rats. The pharmacokinetic analysis indicates a fast and extensive uptake of the molecule by the organs and tissues investigated, with a preferential renal uptake. Moreover, 90% of the administered radioactivity is excreted in urine collected up to 5 h after injection, and about 5% is detectable in feces and gastrointestinal content. The characterization of the labeled metabolites, extracted from the organs and urine, has been performed by high-pressure liquid chromatography analysis. In all the investigated tissues, DOPET is enzymatically converted in four oxidized and/or methylated derivatives. Moreover, a significant fraction of total radioactivity is associated with the sulfo-conjugated forms, which also represent the major urinary excretion products. On the basis of the reported results, an intracellular metabolic pathway of exogenously administered DOPET, implying the involvement of catechol-*O*-methyltransferase, alcohol dehydrogenase, aldehyde dehydrogenase, and phenolsulfotransferase, has been proposed.

Several epidemiological studies suggest that olive oil significantly contributes to the well known effects of the Mediterranean diet in lowering the incidence of degenerative pathologies, including coronary heart disease and cancer (Keys, 1970; Martin-Moreno et al., 1994; Trichopoulou et al., 1995; Willett et al., 1995; Lipworth et al., 1997; Willett, 1997; Braga et al., 1998). In this respect, converging evidence indicates that the protective effects of olive oil could be ascribed not only to its high oleic acid content but also to the antioxidant properties of its polyphenols, absent in seed oil (Bravo, 1998; Visioli and Galli, 1998). Their concentration differs greatly among olive oils, depending on various conditions, such as the cultivar, the soil composition, the climate, the degree of ripeness of the olives, and the extraction procedures (Montedoro and Servili, 1992a). In this respect, virgin olive oils can be classified on the basis of their polyphenol content, ranging from 100 mg up to 1000 mg/kg (Montedoro et al., 1992b,c).

The 3,4-dihydroxyphenylethanol (hydroxytyrosol; DOPET[1]) is the major component of olive oil phenolic fraction, either as simple phenol or esterified with elenolic acid to form oleuropein aglycone (Capasso et al., 1996). This hydrosoluble and liposoluble molecule is an efficient scavenger of peroxyl radicals (Aeschbach et al., 1994; Visioli et al., 1998), and it greatly contributes to determine the shelf life of the oil, preventing its auto-oxidation (Papadopoulos and Boskou, 1991; Baldioli et al., 1996). The biological activities of DOPET have been explored by several groups, as recently reviewed by Manna et al. (1999a). DOPET in vitro prevents LDL oxidation (Grignaffini et al., 1994; Salami et al., 1995), platelet aggregation (Petroni et al., 1995), and inhibits 5- and 12-lipoxygenases (Kohyama et al., 1997; de la Puerta et al., 1999). It also exerts an inhibitory effect on peroxynitrite dependent DNA base modifications and tyrosine nitration (Deiana et al., 1999). Experiments from our laboratory demonstrated that DOPET effectively counteracts the cytotoxic effects of reactive oxygen species in various human cellular systems (Manna et al., 1997, 1999b). Preincubation of intestinal Caco-2 cells with DOPET prevents the damages of oxidative stress, such as lipid peroxidation and alterations of cell permeability and viability (Manna et al., 1997). Similarly, DOPET exerts a protective effect against the H_20_2 induced oxidative hemolysis and malondialdehyde formation in the red blood cells (Manna et al., 1999b). Moreover, we have recently demonstrated

This work was supported in part by a research grant from the International Olive Oil Council.

[1] Abbreviations used are: DOPET, 3,4-dihydroxyphenylethanol; LDL, low-density lipoprotein; MOPET, 4-hydroxy-3-methoxyphenylethanol (homovanillic alcohol); HVA, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid); DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; HPLC, high performance liquid chromatography; TCA, trichloroacetic acid; COMT, catechol-*O*-methyltransferase.

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that the diphenol exerts an antiproliferative effect, inducing apoptosis in HL-60 cells and in resting and activated peripheral blood lymphocytes (Della Ragione et al., 2000). Finally, it has been recently shown that the molecule permeates cell membranes of human intestinal cells via a passive diffusion mechanism (Manna et al., 2000).

Despite the variety of interesting DOPET biological effects, little data have been reported on its bioavailability and metabolism. Preliminary reports indicate that orally administered DOPET can be absorbed both in rats (Bai et al., 1998) and in humans (Visioli et al., 2000), whereas no information is available on its toxicity, organ distribution, and metabolism. The studies reported in this article represent the first systematic analysis of these aspects: the synthesis of the labeled DOPET allowed us to carry out a molecular pharmacokinetic analysis and to elucidate its metabolic pathways.

Materials and Methods

Chemicals. [^{14}C]DOPET (2.25 mCi/mmol) was custom synthesized by PerkinElmer Life Sciences (Cologno Monzese, Italy). The radiochemical purity of [^{14}C]DOPET was 98.9%, according to thin layer chromatography on Silica Gel GF using benzene/dioxane/acetic acid (90:25:4) as eluent.

Ready organic, Ready gel, and BTS-450 (a quaternary ammonium hydroxide in toluene) were obtained from Beckman Coulter, Inc. (Fullerton, CA). Type L-II β-glucuronidase, type VI sulfatase, homovanillic alcohol (3-hydroxy-4-methoxyphenylethanol; MOPET), homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid; HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), and horse alcohol dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO).

Syntehtic Procedures. *Preparation and purification of DOPET*. The chemical synthesis of DOPET was performed according to Baraldi et al. (1983), with some modifications. A solution of 3,4-dihydroxyphenylacetic acid (3.5 g, 0.018 mol) in anhydrous tetrahydrofuran (50 ml) was added dropwise to an ice-cooled stirred suspension of $LiAlH_4$ (2 g, 0.054 mol) and Pt on activated carbon in anhydrous tetrahydrofuran (150 ml). The resulting mixture was heated 6 h at reflux temperature, cooled, and the excess of hydride was destroyed by addition of 150 ml of 1 N HCl. To the final mixture, 100 ml of ethyl acetate were added. The organic layer was separated, and the aqueous phase was extracted with ethyl acetate (3 $\times$ 100 ml). The combined organic phases were dried with sodium sulfate and evaporated "in vacuum", and the residue was purified on a silica gel column (petroleum ether/ethyl acetate, 1:1 v/v) to give a colorless oil (2.4 g). The yield was about 70%. DOPET was identified by ^{1}H and ^{13}C NMR. ^{1}H NMR in CD_3OD: 6.75 (1H, d; J = 8.1 Hz), 6.73 (1H, d; J = 2.0 Hz), 6.59 (1H, dd; J + 8.1, J = 2.0 Hz), 3.75 (2H, t; J = 7.5 Hz). ^{13}C NMR: 146.0 (s), 144.4 (s), 131.7 (s), 121.2 (d), 117.0 (d), 116.2 (s), 64.5 (2C, t), 39.5 (2C, t). Infrared (film); 3400, 1600 cm^{-1}. The infrared spectra were taken on a PerkinElmer 1760-X IFT spectrophotometer in film (PerkinElmer Optoelectronics, Santa Clara, CA). The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Fourier-transform Bruker spectrometer AMX 500 equipped with a Bruker X-32 computer (Bruker, Newark, DE), using the UXNMR software package. To prevent oxidation, the compound was routinely stored under vacuum.

DOPAL synthesis. 3,4-Dihydroxyphenylacetaldehyde (DOPAL) was obtained by enzymatic synthesis as follows: DOPET (100 μM) was incubated at 25°C in 0.1 M sodium phosphate buffer, pH 7.4, in the presence of 2.5 mM NAD^+ and alcohol dehydrogenase (0.2 U/ml) (Mardh and Vallee, 1986). Absorbance at 340 nm was then monitored spectrophotometrically. Aldehyde formation was followed by HPLC analysis as indicated below. To prevent oxidation, the compound was routinely stored under vacuum.

Animal Studies. *Animals*. Young adult Sprague-Dawley rats were routinely used for the in vivo experiments. The animal treatments were carried out by RBM-Laboratories & Clinics Group (Colleretto Giacosa, Italy).

Toxicity test. Six male and six female rats, about 3-months old and weighing 210 to 262 g, were used for the experiment. They were acclimatized at least 5 days before starting the test and fasted about 16 h before the experiment. A single dose of 2 g/kg b.wt. DOPET was administered by gavage. Three hours after treatment diet was available "ad libitum". During the study period, rats were housed under controlled environmental conditions. Animals were maintained and handled according to the Directive 86/609/EEC, enforced by the Italian D.L. No. 116 January 1992. The rats were observed and weighed daily, after administration of DOPET, until day 14. At the end of the test, rats were sacrificed, and gross pathological changes in main organs were evaluated. Toxicity was determined from the death/survival ratio of treated animals.

Pharmacokinetics of [^{14}C]DOPET. Twelve young adult male rats, weighing 204.5 $\pm$ 6.4 g, were used for this investigation. During the study period, the rats were housed under controlled environmental conditions. A single intravenous dose of [^{14}C]DOPET (1.5 mg/kg–87.5 μCi/kg) was administered to animals, which had been fasted about 16 h before treatment. To prevent DOPET oxidation, the radioactive molecule was kept lyophilized under vacuum, and the solution was prepared just before the injection. Immediately after administration, the animals were placed in individual plastic metabolic cages. At the selected time-intervals (5, 10, 30, 60, 120, and 300 min after intravenous administration), two rats were anesthetized with diethyl ether and killed by exsanguination from the abdominal aorta. Blood (5 ml) was collected and frozen at −80°C. The other whole blood (5 ml) was centrifuged for 10 min at 5000g. The plasma and the cell fraction were separated and then both preserved at −80°C. Brain, heart, kidney, liver, lung, skeletal muscle, and gastrointestinal content were promptly frozen at −80°C. This last sample was obtained by dissecting stomach and intestine and scraping away the content with a spatula. Feces and urine were collected from animals sacrificed after 300 min from [^{14}C]DOPET administration.

Preparation of biological samples for radioactivity measurement. Biological samples were prepared according to Galletti et al. (1985). Tissues were homogenized in water 1:3 (w/v), and aliquots corresponding to 100 mg of tissue were incubated with 1 ml of BTS-450 at 40°C in a water bath, until complete solubilization (4–5 h). The samples were decolorized with a few drops of 30% H_2O_2, and glacial acetic acid (70 μl) was added to eliminate chemiluminescence (Galletti et al., 1985). Ready organic (10 ml) was then added.

For treatment of feces, water (100 μl) was added to 20 mg of sample. After 1 h at room temperature, the sample was incubated at 40°C with 1 ml of BTS-450 for 1 to 2 h; isopropanol (500 μl) was added, followed by 200 μl of 30% hydrogen peroxide. After 10 min at room temperature, the feces were incubated at 40°C for 2 h. The sample was diluted with 5 ml of water, and Ready gel (10 ml), containing 7 ml/l glacial acetic acid, was added.

The liquid samples were treated according to Skierkowski (1990). BTS-450/isopropanol (750 μl; 1:2 v/v) was added to 250 μl of sample. After 1 h of incubation at 40°C, 30% hydrogen peroxide (500 μl) was added dropwise. Following 15 min at room temperature, samples were incubated 30 min at 40°C. Ready gel (10 ml), containing 7 ml/liter glacial acetic acid, was added. For urine analysis, Ready gel (10 ml) was directly added to 10 μl of sample.

Radioactivity was measured in a Beckman LS 7800 β-counter equipped with an automatic quench correction system. The standardization was performed by using samples containing [^{14}C]DOPET (1 $\times$ 10^5 dpm) and increasing aliquots of solubilized liver tissues as a quencher.

HPLC Analysis. Thirty percent TCA was added (1:1 v/v) to aliquots of tissue samples, prepared as above reported. After centrifugation at 13,000g for 15 min, the pellet was washed once with 10% TCA, and the resulting supernatants, accounting for 100% of total tissue radioactivity, were combined and used for HPLC analysis. HPLC separation of DOPET and its metabolites was performed by reversed-phase chromatography on 150- $\times$ 4.6-mm C_{18} 5-μm column (Kromasil), using a Beckman Apparatus (Gold-126) equipped with an UV detector fixed at 278 nm. The column was eluted at a flow rate of 1.0 ml/min with acetic acid 0.2%, pH 3.1, (A)/methanol (B) as the mobile phase; the gradient was changed as follow: 95% A/5% B for 2 min, 75% A/25% B in 22 min, 0% A/100% B in 5 min, 0% A/100% B for 5 min, and 95% A/5% B in 5 min. Labeled species were identified on the basis of the retention times of authentic standard references (DOPET, MOPET, HVA, DOPAC, and DOPAL).

Enzymatic Hydrolysis and Identification of Conjugated Forms. To verify the presence of radioactivity associated with conjugated forms of DOPET and/or its metabolites, plasma and urine samples were treated with both β-glucuronidase and sulfatase.

Glucuronides. Plasma and urine aliquots were acidified to pH 5.0 with 0.1 volume of 0.58 mol/l acetic acid solution (Manach et al., 1999) and incubated at 37°C for different times in the presence of 8 $\times$ 10^5 U/l β-glucuronidase from

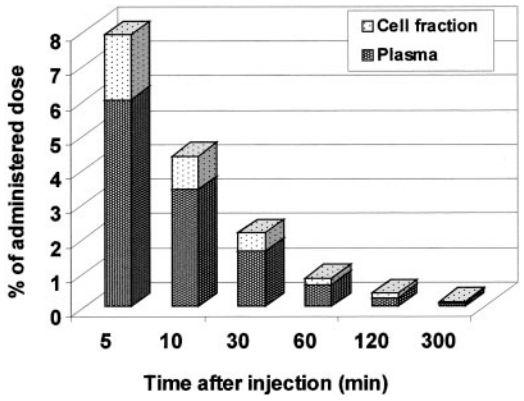

FIG. 1. *Kinetic analysis of blood radioactivity in rats injected with [^{14}C]DOPET.*

The rats were injected intravenously with 0.3 mg of [^{14}C]DOPET (38×10^6 dpm). At the indicated time-intervals, two rats were killed, blood samples were drawn, and both the pellet and the supernatant were analyzed for radioactivity, as reported under *Materials and Methods*. The results are expressed as a percentage of the injected dose in the total blood volume.

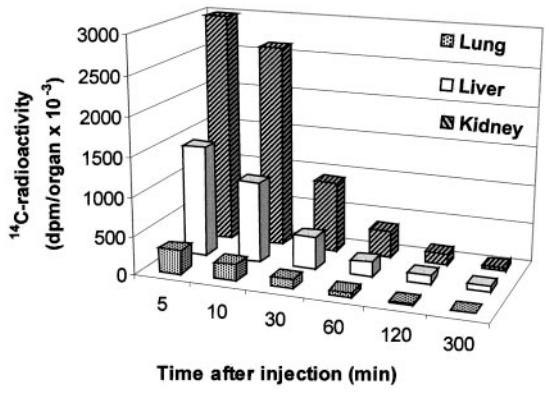

FIG. 2. *Radioactivity distribution in lung, liver, and kidney of rats after intravenous injection of [^{14}C]DOPET.*

The animals were treated as described in the legend to Fig. 1 and killed at different time intervals after the injection. The organs were processed and analyzed for radioactivity, as described under *Materials and Methods*. The values represent the average of data obtained from two animals.

Patella vulgata in which the contaminating sulfatase activity is inhibited by 0.1 M phosphate.

Sulfates. Plasma and urine aliquots were incubated at 37°C for different times, in 5 mM Tris-HCl, pH 7.4, containing 1×10^5 U/l sulfatase from *Aerobacter aerogenes*, devoid of any detectable β-glucuronidase activity. At the end of the incubation, the samples were treated with TCA and analyzed by HPLC, as reported above.

In Vitro Experiments with Human Blood. Whole blood and plasma from rats and humans were used to analyze in vitro metabolism of [^{14}C]DOPET. Samples (200 μl) were incubated at 37°C with 50,000 dpm of [^{14}C]DOPET. After 30 min, 30% TCA was added (1:1 v/v) to the samples. After centrifugation at 13,000*g* for 15 min, the pellet was washed once with 10% TCA, and the resulting supernatants, accounting for 100% of total tissue radioactivity, were combined and used for HPLC analysis.

Results and Discussion

Toxicity Test. A preliminary study on in vitro toxicity was performed using cultured intestinal Caco-2 cells. These cells undergo spontaneous differentiation to form a polarized monolayer closely resembling, both morphologically and functionally, the human small intestinal epithelium (Hidalgo et al., 1989). No decrease in cell viability, measured by the neutral red assay (Manna et al., 1997), has been observed up to 900 μM DOPET (data not reported).

To evaluate DOPET acute toxicity, a single dose of the compound (2 g/kg b.wt.) was orally administered to Sprague-Dawley rats, as indicated under *Materials and Methods*. The animals were observed and weighted daily for 2 weeks. During the study period, no death occurred in the treated animals; the only clinical sign observed in males and females was piloerection, which started 2 h after gavage and disappeared within 48 h from treatment. Body weight did not vary after drug administration, and the autoptic analysis failed to show appreciable macroscopic alterations of internal organs. The absence of adverse effects at concentrations as high as 2 g/kg b.wt. does not allow the calculation of the LD_{50} value.

Radioactivity Tissue Distribution. The pharmacokinetic analysis was carried out in rats intravenously injected with [^{14}C]DOPET (1.5 mg/kg b.wt., 38×10^6 dpm). As shown in Fig. 1, less than 8% of the administered radioactivity is still present in the blood stream 5 min after injection (6% associated with plasma and 1.9% with cell fraction). The time course analysis shows a further gradual decrease of blood radioactivity; only 0.1% of the administered dose is detectable 5 h after the treatment (Fig. 1). Furthermore, DOPET is rapidly metabolized both by blood cells and by different rat tissues, and its radioactive metabolites are already detectable in the blood stream 5 min after the injection, as it will be discussed under *DOPET Metabolism*. The rapid decrease of radioactivity in blood stream prevented an accurate evaluation of the blood half-life of DOPET, which is probably within 1 ÷ 2-min range. These data further support our previous finding on the absence of biological barriers hampering DOPET distribution within the cell (Manna et al., 2000). It should be noted, however, that its absorption and tissue distribution could be different when the natural occurring esterified precursors of DOPET, oleuropein and its aglycone, are administered. As a matter of fact, Coni et al. (2000) report a significant protective effect of oleuropein-rich diets on LDL oxidation in rabbit.

The ^{14}C radioactivity associated with liver, kidney, and lung at different time intervals from the injection is reported in Fig. 2. The time course analysis indicates that the highest level of radioactivity is detected 5 min after injection, followed by a rapid decrease. A similar pattern is observable in the other investigated tissues. Table 1 shows tissue distribution, expressed either as the percentage of the administered dose or as specific radioactivity (disintegrations per minute per gram of wet tissue), at different times after administration. The ^{14}C radioactivity appears uniformly distributed in all organs and tissues investigated. At 5 min, the percentage of the administered dose associated with the various organs parallels the organ weight expressed as the percentage of body weight, with the notable exception of the kidney (8.1% of administered dose versus 0.8% of body weight). The preferential renal uptake of the molecule is also inferred by kidney-specific radioactivity, which, at all investigated times, is about 10 times higher when compared with other organs. Finally, it is worth noting that DOPET is able to cross the blood-brain barrier, even tough its brain uptake is lower when compared with other organs.

Renal and Intestinal Excretion. Ninety percent of the administered radioactivity is detected in urine collected up to 5 h from the injection, indicating that renal excretion represents the preferential route for the disposition of DOPET and/or its metabolites. Moreover, 9% of administered radioactivity is detectable in the gastrointestinal content 5 min after injection (Fig. 3). This value remains almost constant during the other time intervals, decreasing to 2.5% at 5 h. Finally, 3.2% of the injected dose is recovered into the feces 300 min after the administration.

These results suggest the occurrence of a basolateral-apical intestinal transport of the injected [^{14}C]DOPET and/or its metabolites, which is in agreement with our previous data. In fact, Manna et al.

TABLE 1

Radioactivity distribution in rat tissues after injection of [^{14}C]DOPET

The animals were treated as described in the legend to Fig. 1. At the indicated times, they were killed, and the organs were processed as described under *Materials and Methods*. Results are expressed both as percentages of injected radioactivity and as disintegrations per minute per gram of wet tissue. The values represent the average of data obtained from two animals.

	% of Body Weight	5 Min After Injection		60 Min After Injection		120 Min After Injection	
		% of Administered Dose	dpm/g	% of Administered Dose	dpm/g	% of Administered Dose	dpm/g
Skeletal Muscle	56.27 ± 0.014[a]	61.00 ± 5.00	195,500 ± 11,500	5.4 ± 0.8	18,250 ± 2,250	2.1 ± 0.1	6,875 ± 75
Kidney	0.80 ± 0.067	8.10 ± 0.90	2,014,665 ± 125,705	0.95 ± 0.15	217,290 ± 22,350	0.365 ± 0.005	89,975 ± 8,185
Liver	3.19 ± 0.15	3.80 ± 0.39	217,955 ± 6,835	0.485 ± 0.025	28,710 ± 790	0.29 ± 0.02	16,162 ± 2,047
Lung	0.53 ± 0.05	0.81 ± 0.14	269,360 ± 46,400	0.125 ± 0.015	47,435 ± 3,665	0.05 ± 0.01	16,925 ± 3,535
Heart	0.39 ± 0.03	0.34 ± 0.01	150,385 ± 15,895	0.045 ± 0.005	19,440 ± 850	0.019 ± 0.002	8,905 ± 635
Brain	0.89 ± 0.04	0.31 ± 0.01	64,070 ± 1,000	0.07 ± 0.01	14,285 ± 1,195	0.02 ± 0.002	4,315 ± 315

[a] The percentage of body weight for skeletal muscle was determined according to Mordenti (1986).

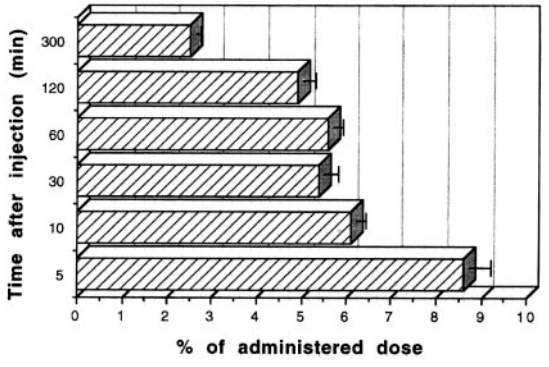

FIG. 3. *Radioactivity in gastrointestinal content after intravenous injection of [^{14}C]DOPET.*

The experimental conditions are those reported in the legend to Fig. 1. At the indicated time intervals, the animals were killed, and the radioactivity associated with gastrointestinal content was evaluated as reported under *Materials and Methods*. Values represent the average of data obtained from two animals.

(2000) demonstrated that [^{14}C]DOPET transport through a monolayer of differentiated intestinal cells occurs via a passive diffusion mechanism and that it is bi-directional.

DOPET Metabolism. Little data are available in the literature on pharmacokinetics and metabolism of exogenously administered DOPET or oleuropein. A preliminary study from our laboratory, using labeled DOPET in cultured intestinal cells, indicated that the only labeled metabolite detectable in the culture medium is MOPET, the methylation being supposedly catalyzed by catechol-*O*-methyltransferase (COMT) (Manna et al., 2000).

To identify [^{14}C]DOPET metabolites, the hydrosoluble labeled products were extracted from the various organs and characterized by reversed phase HPLC. Figure 4 compares the HPLC profile of a standard mixture of DOPET structurally related compounds (Fig. 4A), with a typical chromatographic separation of the ^{14}C molecular species present in rat liver, 5 min after injection (Fig. 4B). A similar chromatographic pattern is observable in the plasma and in the other analyzed organs. The quantitative results, reported in Table 2, indicate a rapid metabolic transformation of the injected phenol into four major metabolites, including the methylated derivative (MOPET). Enzymatic methylation is presumably operative in the brain, where MOPET represents 41.9% of the detected, labeled species. This reflects the key role of COMT in central nervous system (Guldeberg and Marsden, 1975), although a transport of MOPET from other tissues cannot be ruled out. Moreover, the occurrence, in all analyzed organs, of both labeled DOPAL and DOPAC implies a sequential oxidation of DOPET ethanol side chain catalyzed by alcohol and aldehyde dehydrogenase, respectively. Labeled HVA, the product of both methylation and oxidation, has also been identified.

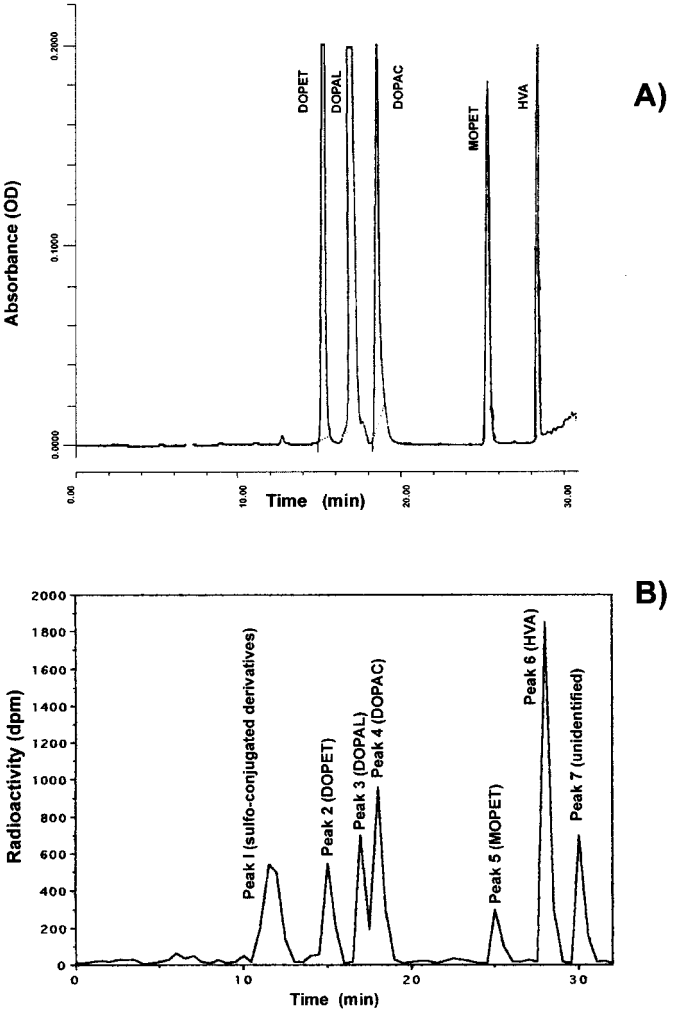

FIG. 4. *HPLC analysis of DOPET and its metabolites.*

A, HPLC separation of a standard mixture of DOPET and its structurally related compounds. HPLC analysis was run as described under *Materials and Methods*. Retention times: DOPET, 15.26 min; DOPAL, 17.02 min; DOPAC, 18.56 min; MOPET, 25.43 min; HVA, 28.48 min. B, a typical profile of the chromatographic separation of ^{14}C molecular species detectable in rat liver after intravenous injection of [^{14}C]DOPET. Rats were treated as reported in the legend to Fig. 1, and liver extract for HPLC analysis was prepared as reported under *Materials and Methods*. Labeled compounds were identified on the basis of their retention times.

To identify the component(s) of peak 1 (Fig. 4B), which is expected to contain DOPET glucuronide and/or sulfate conjugates, samples of

TABLE 2

Labeled metabolites detected in rat organs after intravenous injection of [^{14}C]DOPET

The animals were treated as reported in the legend to Fig. 1. Plasma and organs from rats sacrificed 5 min after injection were processed and analyzed by HPLC, as described under *Materials and Methods*. DOPET derivatives were identified on the basis of their retention times.

Peak[a]	Labeled Metabolites	Radioactivity[b]					
		Plasma	Brain	Heart	Kidney	Liver	Lung
		%					
1	Sulfo-conjugated derivates	43.3	13.4	25.2	44.1	17.9	39.5
2	DOPET	8.6	14.1	9.5	7.4	9.7	13.7
3	DOPAL	10	17.9	6.3	14.7	11.6	8.8
4	DOPAC	12.3	6.8	29.6	10.5	16.4	11.9
5	MOPET	11.4	41.9	14.6	1.8	5.2	3.1
6	HVA	12.5	5.9	11.2	18.9	28.1	20.9
7		1.9		3.6	2.6	11.1	2.1

[a] Peak number refers to the HPLC profile reported in Fig. 4.
[b] 100 = total radioactivity present in the tissue.

plasma were incubated either with β-glucuronidase from *P. vulgata* (containing sulfatase activity inhibited by 0.1 M phosphate) or with sulfatase from *A. aerogenes* (devoid of any detectable β-glucuronidase activity).

The HPLC analysis of sulfatase-treated plasma samples shows the quantitative disappearance of radioactivity associated with peak 1 and the parallel increase of DOPET and its metabolites, mainly the methylated derivative MOPET. Conversely, the treatment with β-glucuronidase does not result in any appreciable modification of HPLC profile. These data allow to identify sulfo-conjugated derivatives of DOPET and its metabolites as the only components of peak 1 and confirm the well established role of sulfation in the metabolism of catechols. These conjugated metabolites represent the major labeled molecular species excreted in urine (Fig. 5). It is worth noting that Visioli et al. (2000) reported that in humans, after a dietary intake of olive oil, this phenol is dose dependently absorbed and excreted in urine, presumably as glucuronide conjugates. The absence of these derivatives in rat samples, after intravenous administration of DOPET, is in agreement with literature data stating that glucuronidation of orally administered epicatechin in rat occurs at the level of intestinal mucosa, whereas its sulfation mainly occurs in liver (Piskula and Terao, 1998).

The DOPET metabolites observed in rat plasma are presumably formed intracellularly. To verify this hypothesis, [^{14}C]DOPET was incubated in vitro in rat and human plasma and in total blood. Figure 6 shows the HPLC analysis of labeled molecular species formed in the rat plasma. Under these experimental conditions, DOPET remains largely unmodified; the presence of the 6% DOPAL can be ascribed to trace amounts of alcohol dehydrogenase in the plasma. Conversely, when [^{14}C]DOPET is incubated with the whole blood, it is rapidly converted into the same metabolites detected in the in vivo experiments. The large amount of sulfo-conjugated derivatives reflects the high-sulfotransferase activity of platelets (Anderson et al., 1991). Superimposable results were obtained with total human blood, suggesting that a similar metabolic pathway is probably operative in humans.

The reported general pattern of the labeled derivatives is consistent with the metabolic pathway, indicated in Fig. 7; it is worth noting that the oxidized metabolites (i.e., HVA and DOPAC) are also the major molecular species deriving from dopamine metabolism through the action of monoamine oxidase and COMT in central nervous system. The back enzymatic conversion of DOPAC to DOPET by DOPAC reductase has been reported in rat brain (Xu and Sim, 1995). Moreover, Goldstein and Gerber (1963) demonstrated the conversion of [^{14}C]DOPAC to [^{14}C]MOPET in rat brain. More recently, Lamensdorf et al. (2000a,b) reported an increased level of DOPAL and

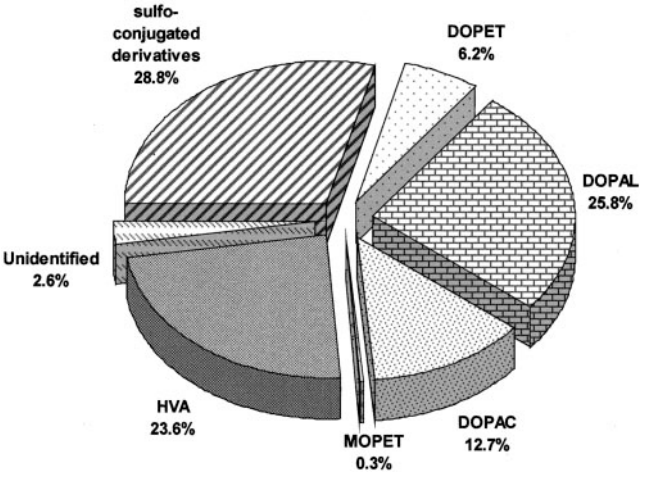

FIG. 5. *Labeled metabolites present in urine of rats treated with [^{14}C]DOPET.*

The rats, treated as reported in the legend to Fig. 1, were kept in metabolic cages. Urine collected from rats sacrificed after 5 h from injection were pooled and analyzed by HPLC, as reported under *Materials and Methods*. The results are expressed as the percentage of total radioactivity detected in urine.

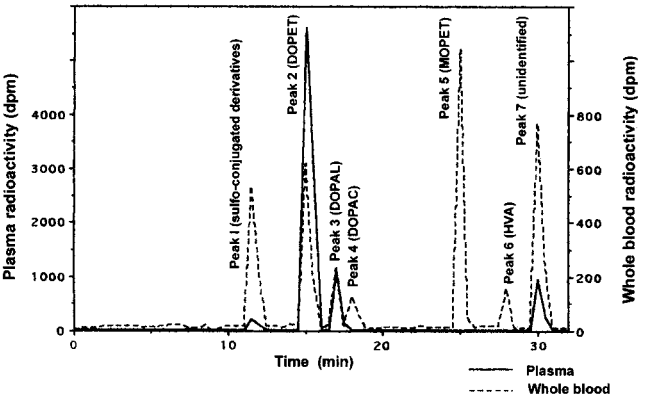

FIG. 6. *Labeled species detected in rat blood treated in vitro with [^{14}C]DOPET.*

HPLC profile of ^{14}C molecular species detectable in rat plasma and rat whole blood after incubation with [^{14}C]DOPET. Samples were treated as reported under *Materials and Methods*. Labeled compounds were identified on the basis of their retention times. At time 0, incubation of blood and plasma with [^{14}C]DOPET does not reveal any detectable transformation of the molecule (data not shown).

DOPET in catecholaminergic PC12 cells incubated in the presence of inhibitors of mitochondrial respiratory chain. The rapid metabolic conversion of DOPET raises the question whether its metabolites are

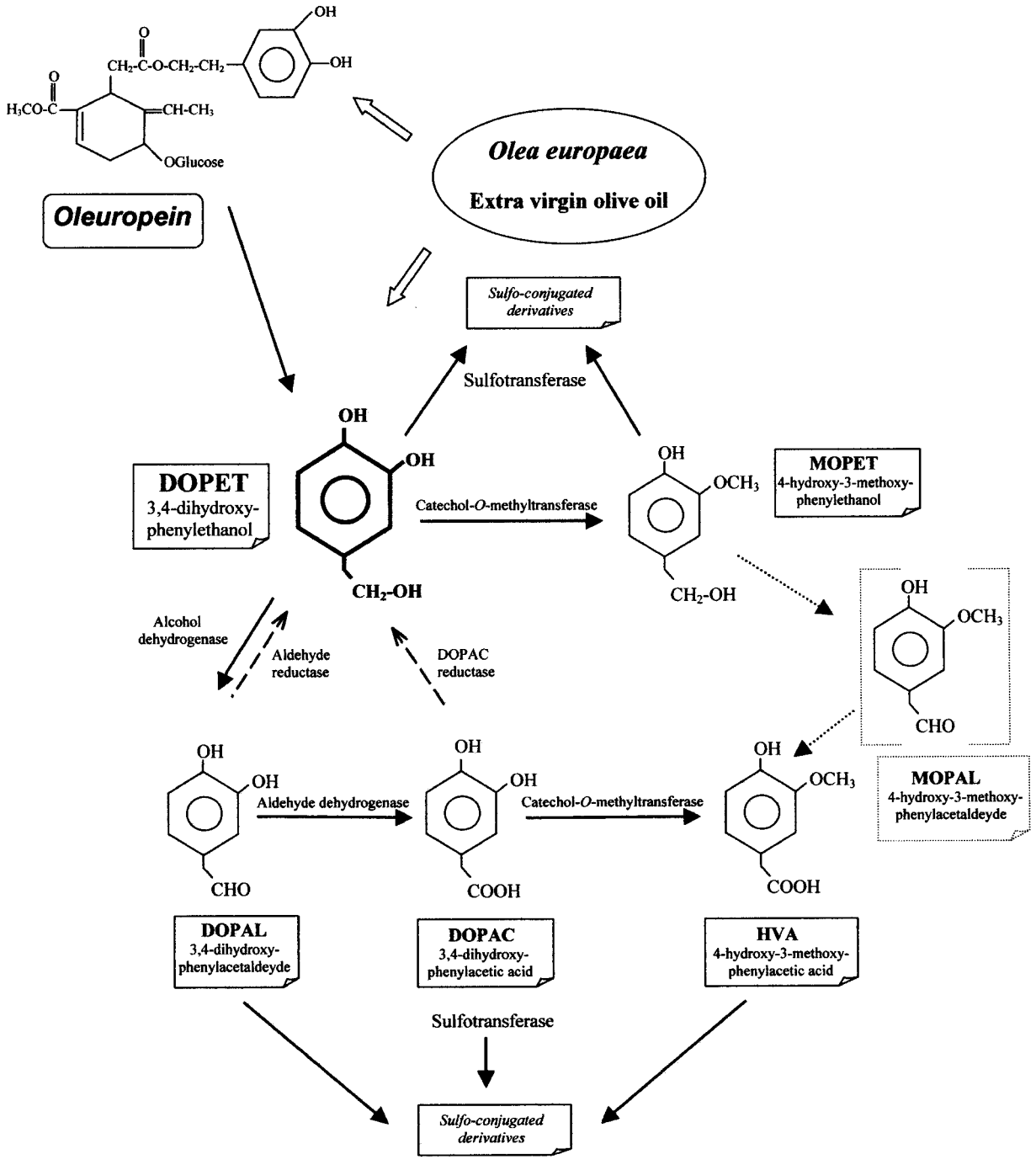

FIG. 7. *Metabolic pathways of exogenously administered DOPET in rats.*

The indicated compounds have been detected in rat organs, except 4-hydroxy-3-methoxyphenylacetaldehyde (MOPAL). The conversion of MOPET into HVA via MOPAL is presumptive.

still endowed with the variety of biological effects exerted by the molecule, which are presumably related to its antioxidant activity. Although no data are available on the biological activity of the methoxy derivatives, previous studies in different in vitro model systems (Kohyama et al., 1997; Manna et al., 1997) suggest that the antioxidant activity of DOPET is strictly related to the integrity of orthodiphenolic moiety. Methylation of the phenolic hydroxyl group should result in the loss of antioxidant activity. Moreover, we have recently demonstrated (Della Ragione et al., 2000) that DOPET induces apoptosis in white blood cells, as a consequence of a rapid cytochrome *c* release from intermembrane mitochondrial space. The presence of the orthodiphenol group is again a structural requirement for biological activity. In fact, tyrosol, the structural analog which presents only one hydroxyl group on the phenyl ring, did not induce the programmed cell death (Della Ragione et al., 2000). On the other hand, it is still questionable whether the molecular basis of all the biological effects of DOPET (and its metabolites) is strictly related to its antioxidant properties. In some instances, there has been reported a low pro-oxidant activity of the molecule (Aeschbach et al., 1994).

As already mentioned, olive oil is the principal fat component of the Mediterranean diet, which is associated with a low incidence of cancer at various anatomical sites. Moreover, there is clear evidence that

olive oil intake contributes directly to the reduced incidence of breast (Martin-Moreno et al., 1994; Trichopoulou et al., 1995; Willett, 1997), colorectal (Braga et al., 1998), and prostate (Willett, 1997) cancer. These observations, together with our recent data on the ability of DOPET to arrest cell proliferation and induce apoptosis in cultured human cells (Della Ragione et al., 2000), support the view that cancer prevention exerted by olive oil could be ascribed to its high content of DOPET and its precursor, oleuropein aglycone.

On the other hand, according to the "oxidation hypothesis" of atherosclerosis (Witztum, 1994), it is possible to speculate that the reported delayed progression of atheromatous plaque due to olive oil intake (Parthasarathy et al., 1990) can be, in part, attributed to the antioxidant properties of its phenolic component (Visioli and Galli, 2001). The inhibition of platelet aggregation (Petroni et al., 1995) and prevention of radical-induced LDL oxidation (Grignaffini et al., 1994; Salami et al., 1995) by DOPET and oleuropein may be critical in this respect.

In conclusion, literature data on biological effects of DOPET, together with our findings on the lack of toxicity and the fast uptake of the molecule, make this natural occurring phenol a good candidate for novel therapeutic strategies.

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