Determination of Synthetic Hydroxytyrosol in Rat Plasma by GC-MS

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2-(3,4-Dihydroxyphenyl)ethanol (DPE), the major phenolic compound in olive oil, may contribute the antioxidative activities and other beneficial effects to olive oil. However, the lack of commercial available DPE and procedures sensitive enough to quantitatively determine DPE in body fluids have limited the bioavailability and metabolism studies on this phenolic compound. In the present study, DPE was synthesized with high yield and high purity and administered orally to rats. DPE concentration in rat plasma, after absorption, was measured using a sensitive GC-MS-SIM method. The results indicated that the highest level of DPE in plasma was detected at 5−10 min after administration. During this period, the concentration of DPE fluctuated widely with the individual.

Keywords: *Hydroxytyrosol; dihydroxyphenylethanol (DPE); plasma; GC-MS; metabolism*

INTRODUCTION

2-(3,4-Dihydroxyphenyl)ethanol (DPE), also known as hydroxytyrosol, is the major natural phenolic compound present in olive fruits (*Olea europea* L.) and olive oil (Montedoro, 1972) and has demonstrated strong antioxidative activities not only against oil rancidity (Papadopoulos and Boskou, 1991) but also against intracellular oxidative stress in cell culture model systems (Manna et al., 1997). Studies of the low incidence of cardiovascular disease in Mediterranean countries, where olive oil is the major dietary fat, indicated that DPE may reduce the risk of coronary heart disease and atheroscelosis (Grignaffini et al., 1994; Salami et al., 1995). Although some explanations on the beneficial effects of DPE have been put forward, such as by inhibiting arachidonic acid lipoxygenase (Petroni et al., 1997) or by inhibiting platelet aggregation (Petroni et al., 1994, 1995), in vivo studies on its bioavailability and metabolism are rare despite of their obvious importance. Wiseman et al. (1996) noted that DPE can increase the resistance of oxidation of low density lipoproteins in experimental animal but failed to provide the metabolic data on DPE. This lack of information is due to the lack of commercially available authentic DPE of high purity and sensitive method to determine DPE in body fluids.

In a previous study (Kohyama et al., 1997), it was found that DPE was a specific inhibitor of lipoxygenase activities of both 12-lipoxygenase and 5-lipoxygenase and suggested that DPE may penetrate into cell membrane easily since DPE can inhibit the production of leukotriene B_4 (LTB_4) effectively from endogenous arachidonic acid. Because the bioavailability and metabolic kinetics of authentic DPE in vivo are very critical to understand its action mode and to develop it into a health-care product, the following studies were carried out, gram scale DPE of high purity was synthesized and purified by an improved procedure, and the metabolic kinetics of DPE in rat plasma after oral administration was determined by a sensitive GC-MS quantitative method.

MATERIALS AND METHODS

Reagents. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylsilyldiazomethane ($TMSCHN_2$) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nonadecanoic acid (C19:0 fatty acid) was obtained from GL Sciences Inc. (Tokyo, Japan) and dissolved in *N*,*N*-dimethylformamide (DMF) at 1 mg/mL as an internal standard (I.S.). 3,4-Dihydroxyphenylacetic acid was obtained from Sigma (St. Louis, MO). Water used in all experiment was obtained through a Milli-Q water purification system (Millipore, U.S.A.). All other chemicals were analytical grade and used without further purification.

Apparatus. Mass spectra of the compounds were obtained on a JMS-SX-102A mass spectrometer (JEOL, Japan) by fast atom bombardment (FAB) ionization. NMR spectra for ^1H at 300.13 MHz were recorded on a DRX-300 spectrometer (Bruker, Germany) at room temperature in deuterated acetone. The GC-MS apparatus (Model QP-5000, Shimadzu, Tokyo, Japan) was equipped with an AOC-17 auto injector and a HP-5 (Hewlett-Packard Co., USA) ultra performance capillary column (12 m × 0.2 mm i.d.) coated with a 0.33 μm film thickness of 5% diphenyl and 95% dimethylpolysiloxane. The conditions for GC-MS analysis were as follows. An initial temperature of 100 °C was maintained for 1 min and then was increased to 190 °C at a rate of 10 °C/min and then to 250 °C at a rate of 30 °C/min. The final oven temperature 250 °C was maintained for 2 min. Temperatures at the injection port and detector were 250 °C. Ultrapure helium was used as carrier gas, with pressure on the head of the column of 100 kPa.

Preparation and Purification of DPE. To a stirred solution of 3,4-dihydroxyphenylacetic acid (11.9 mmol) in methanol (15 mL)−ether (50 mL) was added $TMSCHN_2$ (15.5 mmol, 2 mol/L) in hexane at room temperature. The reaction mixture was stirred for a further 0.5 h at room temperature. After the reaction solution was concentrated to dryness, the residue was suspended in ice−water (ca. 50 mL), $NaBH_4$ (100 mmol) was added, and the reaction was continued with strong stirring for 1.5 h at 0 °C. Then the solution was acidified with 2 mol/L HCl while cooling and extracted with ethyl acetate. The upper organic phase was separated and dried over Na_2SO_4. After filtration and evaporation, the residue was purified

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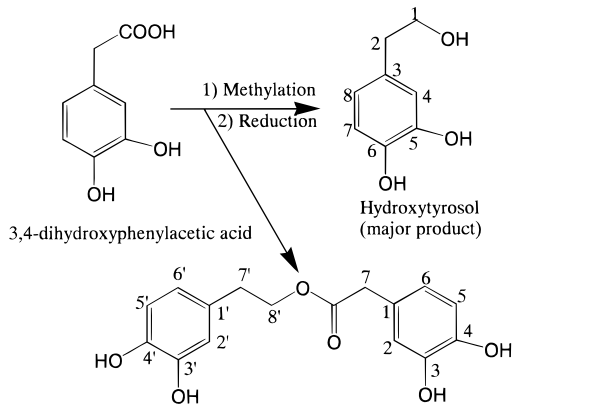

Figure 1. Hydroxytyrosol synthesis and byproduct formation through dihydroxyphenylacetic acid.

by flash chromatography on silica gel with $CHCl_3$:MeOH (7:1) as eluting solvent to yield DPE as a colorless oil. The yield was about 90%.

Animal Experiment. Thirty-three healthy male Wistar rats, weighing 170−190 g, were randomly selected to be in 11 groups, fasted overnight before experiment, and orally administered of DPE (10 mg/mL in 0.5% tragacanth solution) at a single dose of 1 mL, except for three rats as blank control. At each timepoint of 2, 5, 10, 20, 30, 60, 180, 360, 1440, and 2880 min after oral administration, each group (three rats) was sacrificed and blood samples were collected and heparinized. Rat plasma obtained was centrifuged and stored at −20 °C until analysis. During the experiment, the rats were not fed with any other diets other than those of 1440 and 2880 min, *i.e.* six rats.

Extraction of DPE from Rat Plasma. To a tube containing 1 mL of plasma were added 10 μg of I.S. (as DMF solution) and 1 mL of 0.3 mol/L HCl−acetonitrile (1:1, v/v). The solution was vortex-mixed for 15 s and incubated for 5 min at room temperature. Followed by extraction with 3 mL of ethyl acetate, the content in tube was centrifuged at 1000*g* for 6 min. This extraction was repeated twice, and organic layers were combined and dried over Na_2SO_4. After filtration and evaporation, the residue was pertrimethylsilylated for 0.5 h by adding 75 μL of DMF and 75 μL of BSTFA. One microliter of the reaction solution was submitted to GC-MS analysis.

Validation of GC-MS Quantitative Analysis. DPE was dissolved in DMF solution at various concentrations (each containing 5 μg I.S.) and 75 μL of DMF and 75 μL of BSTFA were added for trimethylsilyl derivation. The reaction was carried out at room temperature for 0.5 h, and 1 μL of the reaction solution was injected for GC-MS analysis. Selected ion monitoring (SIM) was used for quantitative analysis. Recovery of DPE from rat plasma was carried out as follows: known amount (0.01, 0.1, and 1 μg) of DPE was added to a pool rat plasma, respectively, after treated with HCl−acetonitrile, and extracted with ethyl acetate; it was quantified as described as above.

RESULTS AND DISCUSSION

Improvement of DPE Synthesis by Inhibiting Byproduct Formation. Synthesis of DPE from 3,4-dihydroxyphenylacetic acid, a commercially available compound, was not so successful, according to the method of Bianco et al. (1988). DPE, after purified, was identified by ^{1}H NMR (300 MHz, CD_3COCD_3), compared with that of Montedoro et al. (1993), as δ 2.52 (2H, *t*, 2-H), 3.54 (2H, *t*, $J_{1,2}$ = 7.0 Hz, 1-H), 6.41 (1H, *dd*, $J_{4,8}$ = 2.0 Hz, $J_{7,8}$ = 7.0 Hz, 8-H), 6.57 (1H, *d*, 4-H), 6.59 (1H, *d*, 7-H) (Figure 1). But the yield of DPE was only about 50%, with the formation of a byproduct shown in the ^{1}H NMR spectrum, which was consist about 15% of the total product. This byproduct had a similar R_f with DPE in silica gel TLC and could not be easily removed from DPE. After further isolation and purification, this byproduct was found to be (3,4-dihydroxyphenylacetic acid ester of 2-(3,4-dihydroxyphenyl)ethanol from FAB-MS spectra (*m/z* 305 $[M + H]^+$, 327 $[M + Na]^+$, 397 [M + glycerol + H]$^+$), and was finally certified by ^{1}H NMR spectrometry (300 MHz, CD_3COCD_3), as δ 2.61 (2H, *t*, $J_{7',8'}$ = 7.2 Hz, 7′-H), 3.30 (2H, *s*, 7-H), 4.03 (2H, *t*, 8′-H), 6.40 (1H, *dd*, $J_{2,6}$ = 2.0 Hz, $J_{5,6}$ = 8.0 Hz, 6-H), 6.45 (1H, *dd*, $J_{2',6'}$ = 2.0 Hz, $J_{5',6'}$ = 8.0 Hz, 6′-H), 6.70 (4H, *m*, 2-H, 5-H, 2′-H, 5′-H).

This unexpected result was caused by weak basic reaction condition, high reaction temperature, and long reaction time. A simple and efficient synthesis method was developed by using a quick methylation reagent, $TMSCHN_2$ (Hashimoto et al., 1981), and the reaction products were reduced by $NaBH_4$ in an ice−water solution for 1.5 h with strong stirring. After purification by flash chromatography on silica gel, the yield of DPE may exceed 90%, and no byproduct interfered. The purity of DPE was about 98% estimated from ^{1}H NMR.

Quantitative Analysis of DPE by GC-MS. Although HPLC and GLC have been used extensively to determine DPE in olive oil (Akasbi et al., 1993; Janer del Valle et al., 1980; Solinas et al., 1982; Tsimidou et al., 1992), these methods cannot be applied to plasma samples because components in plasma remain very complex even after some necessary pretreatment. In our study, GC-MS was proved far more sensitive and selective since DPE had a high abundance of molecular ion (*m/z* 370.25; 30%). To reduce the endogenous interferences present in ethyl acetate extracts of plasma, SIM detection method was applied to raise the quantitative sensitivity by detecting the molecular ion at *m/z* 370.25 of pertrimethylsilylated DPE. Nonadecanoidic acid was used as an internal standard (I.S.) since both of them after TMS derivation had the same M^+ at *m/z* 370. The retention times for DPE and I.S. were 8.05 and 11.95 min, respectively.

The standard calibration curve was obtained by plotting the peak area ratio of DPE to I.S. with the known concentration in the stock standard solution, after the same derivation using BSTFA. The calibration regression line, expressed as correlation coefficients, was in very good linearity (r^2 = 0.9998) over concentration range from 0.01 to 1.3 and 1−5 μg. The detection limit was estimated as the peak height of DPE at least three times higher than the baseline noise range, as 0.01 μg.

Recovery of DPE from Rat Plasma. In developing a quantitative analysis method, the main problem was how to isolate DPE from plasma with good recovery and minimal interference, especially from proteins. Due to weak acidity of DPE, hydrophilic solvent containing hydrochloric acid was attempted to precipitate protein and recover DPE quantitatively. We tested four HCl concentrations (2, 1, 0.5, and 0.3 mol/L) in extraction and observed that the lower the pH, the faster the degradation rate of DPE, particularly when DPE level was lower than 0.1 μg. Thus 0.3 mol/L HCl was chosen for extraction. On the other hand, MeOH and CH_3CN were tested and found that CH_3CN yielded rather good recovery, but MeOH caused a severe loss of DPE (40−60%) during following concentration process.

The concentration of DPE in DMF having 5 μg I.S. was calculated from standard calibration curve. The

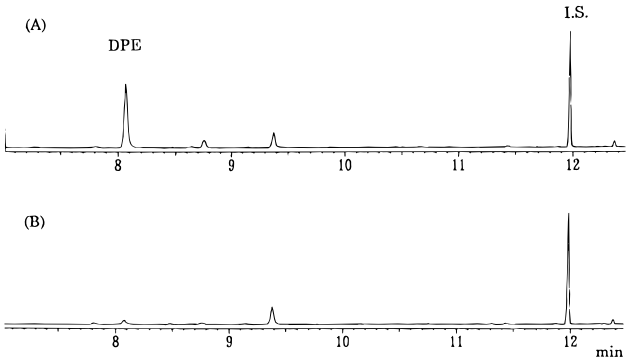

Figure 2. Typical chromatograms of DPE in plasma samples at 10 min (A) and 180 min (B) of oral administration.

Table 1. DPE Concentration in Plasma before and after Oral Administration

	DPE concentration in plasma (g/mL)				
time (min)	subject 1[a]	subject 2	subject 3	mean	SD
0	0	0	0	0	0
2	0	0.07	0.66	0.243	0.36
5	0.39	1.56	2.08	1.344	0.87
10	0.89	1.48	3.26	1.877	1.23
20	0.062	0.12	0.57	0.251	0.28
30	0.093	0.42	0.54	0.351	0.23
60	0.13	0.17	0.25	0.183	0.06
180	0	0.037	0.058	0.032	0.03
360	0	0	0.01	0.003	0.01
1440	0	0	0.01	0.003	0.01
2880	0	0	0	0	0

[a] Subject data was aligned from low to high values.

recovery of DPE in the above extraction for each concentration ranged from 97% to 104%, after addition of known amounts (0.01, 0.1, and 1 μg) of pure DPE to a rat plasma pool; each determination was in triplicate.

Metabolism Kinetics of DPE in Rat Plasma. Figure 2 shows typical chromatograms of DPE in rat plasma samples at 10 and 180 min respectively; two separate peaks were identical to the retention times of standard DPE (8.05 min of retention time) and I.S. (11.95 min). In Figure 2A, DPE peak was intensive and indicated that it was absorbed greatly into plasma at 10 min after administration. On the contrary, Figure 2B shows a little DPE peak, which indicated that DPE was almost eliminated and/or metabolized at 180 min after administration.

Table 1 shows the analytical results of DPE concentration in rat plasma before and after oral administration. DPE in rat plasma appeared at 2 min after administration (0−0.66 μg/mL) and rose quickly. The highest level in the plasma was detected at 5 min (0.39−2.08 μg/mL) to 10 min (0.89−3.26 μg/mL) after administration and then decreased gradually until 180 min (0−0.058 μg/mL). In the later samples such as at 360 min after administration, DPE was almost virtually disappeared. In the time range from 5 to 20 min, DPE concentration in rat plasma fluctuated widely with the individual, reflected as high SD value (0.87 μg/mL at 5 min and 1.23 μg/mL at 10 min).

To clearly illustrate the metabolic pattern of DPE in rat, Figure 3 was plotted with mean value of DPE concentration in plasma vs time course. This time−concentration profile can give an impression that DPE was absorbed into plasma very rapidly after oral administration, decreased rapidly to a certain level within 60 min, and then decreased rather slowly for 1−2 h until it totally disappeared. It was implied that DPE can have some time to exercise antioxidative effects on the blood. But as far as the concentration of DPE detected in the plasma was still very low compared to the total amount for administration, further research work is still needed to determine DPE concentration in digestive organs and to identify the metabolites of DPE after administration to fully clarify just how quickly DPE is metabolized.

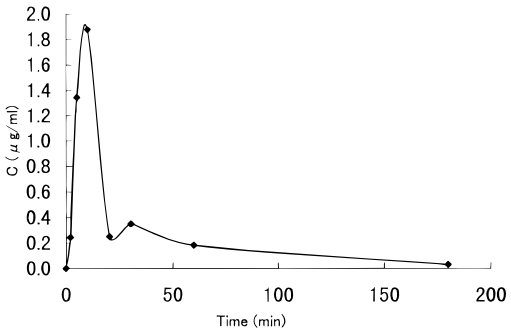

Figure 3. Time-average concentration profile of DPE in rat plasma.

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