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Biotransformation and Pharmacokinetics of 4-(3,4-Dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside, an Antioxidant Isolated from *Origanum vulgare*

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4-(3,4-Dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside (OV-16) is a polyphenolic glycoside isolated from oregano (*Origanum vulgare* L.), which is a popular Chinese herb and a common spice in Western diet. To understand the biotransformation and pharmacokinetics of OV-16, rats were orally administered OV-16 and oregano decoction. Blood samples were withdrawn at specific time points. The presence of OV-16 and its metabolites protocatechuic acid (PCA) and *p*-hydroxybenzyl alcohol (HBA) in serum were determined by HPLC method, whereas their conjugated metabolites were assayed indirectly through hydrolysis with β-glucuronidase and sulfatase. Our results showed that when OV-16 was orally administered, free forms of OV-16, PCA, and HBA were not present in blood and the major metabolites were the glucuronides/sulfates of PCA and HBA sulfate. The serum metabolites of OV-16 exhibited free radical scavenging activity. When oregano decoction was given, the glucuronides and sulfates of PCA were the major metabolites in blood.

KEYWORDS: ***Origanum vulgare*; oregano; biotransformation; glucuronide; sulfate; pharmacokinetics**

INTRODUCTION

Oregano (*Origanum vulgare* L.), a widely used Chinese herb and a common spice in Western diet, was reported to possess antithrombin (*1*), anti-*Helicobacter pylori* (*2*), antibiotic (*3–5*), antihyperglycemia (*6*), and antioxidation effects (*4, 7–13*). 4-(3,4-Dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside (OV-16 (see Abbreviations Used), structure shown in **Figure 1**), a major constituent of oregano, might contribute to the proprietorial antioxidation activity (*13*). Although promising antioxidation capacity of oregano had been reported, the results were essentially obtained from in vitro studies (*13–15*). Whether the in vitro activity of oregano could predict the in vivo effect remained an important unanswered question before understanding the biotransformation and pharmacokinetics of the constituents in oregano.

Due to the relatively high polarity, most polyphenolic glycosides are not easy to permeate the membrane of intestine and not feasibly absorbed per se. When OV-16 is taken orally, it is assumed to be absorbed after being hydrolyzed to less polar aglycone by intestinal enzymes and microflora. Moreover, on the basis of the findings on the metabolic fate of polyphenols in a recent decade, the aglycone of polyphenol would be rapidly and extensively metabolized to their conjugated metabolites by phase II enzymes in gut and/or liver (*16*). Thus the absorbed aglycone of OV-16 would be transformed to its glucuronides and sulfates, which would be then hydrolyzed by esterase to form the glucuronides and sulfates of protocatechuic acid (PCA, structure shown in **Figure 1**) and *p*-hydroxybenzyl alcohol (HBA, structure shown in **Figure 1**). Because the metabolic fate of OV-16 has not been reported in the literature, this study investigated the biotransformation and pharmacokinetics of OV-16 and oregano decoction in rats. Furthermore, to explore the in vivo antioxidation activity of OV-16, the serum metabolite of OV-16 was prepared and the scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated.

MATERIALS AND METHODS

Chemicals. OV-16 was isolated and identified by Dr. Yun-Lian Lin, one of the authors at the National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC (*14*). PCA, HBA, gallic acid, DPPH, β-glucuronidase (type B-1 from bovine liver, containing 1,240,000 units/g of β-glucuronidase) and sulfatase (type H-1 from *Helix pomatia*, containing 14,000 units/g of sulfatase and 498,800 units/g of β-glucuronidase) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl acetate, acetonitrile, methyl alcohol, sodium acetate, L(+)-ascorbic acid, *o*-phosphoric acid (85%), Cremophor

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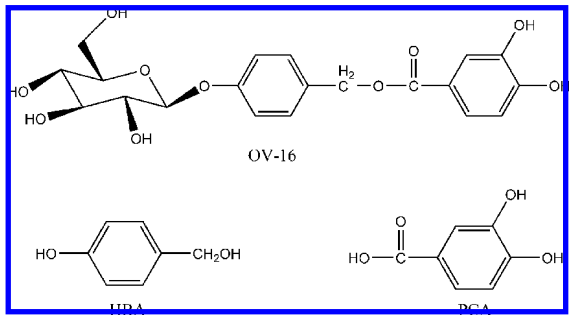

Figure 1. Chemical structures of OV-16, HBA, and PCA.

EL, and 1,2-propanediol were all reagent grade. Milli-Q plus water (Millipore, Bedford, MA) was used for all processes.

HPLC Instrumentation and Conditions. The high-performance liquid chromatography apparatus (Shimadzu, Japan) was assembled by a LC-10AT pump, a SPD-10AV UV–visible spectrophotometric detector, and a SIL-10AD autosampler. The RP-18 column (Apollo, 5 μm, 250 × 4.6 mm) fitted with a prefilter was used. The mobile phase was constituted of acetonitrile and 0.1% *o*-phosphoric acid at the ratios of 4:96 (v/v) and 10:90 (v/v) for the analysis of serum and oregano decoction, respectively. The flow rate was 1.0 mL/min, and the detection wavelength was set at 220 nm. The chromatography data processing system was supplied by the Scientific Information Service Corp. Inc. (Taipei, Taiwan, ROC).

Preparation and Quantitation of Oregano Decoction. Oregano was purchased from a Chinese drug store in Taichung, Taiwan. A 100 g amount of oregano was soaked in 4 L of distilled water for a half-hour and heated to boiling on a gas stove. The mild boiling was held to concentrate the volume until less than 100 mL. The residue was removed, and the filtrate was added with water to make 100 mL. For diminishing the bias, the extraction was performed in triplicate. After combining the three extracts and boiling with faint fire until the volume was less than 50 mL, the extract was added with water to make 50 mL and stored at –20 °C for later use.

For the quantitation of OV-16, PCA, and HBA in oregano decoction, 200 μL of the decoction was added to 800 μL of methanol. After diluting the supernatant with 20-fold methanol, an equal volume of internal standard solution (10 μg/mL methyl paraben in methanol) was added, and 20 μL was subject to HPLC analysis.

Animals and Drug Administration. All animal experiments adhered to "The Guidebook for the Care and Use of Laboratory Animals (2002)" (published by the Chinese Society of Animal Science, Taiwan, ROC). Male Sprague-Dawley rats were supplied by the National Laboratory Animal Center (Taipei, Taiwan) and kept in the Animal Center of China Medical University (Taichung, Taiwan). Animals were fasted for 12 h before the experiment. OV-16 solution was freshly prepared by dissolving in a vehicle composed of equal volume of Cremophor EL and 1,2-propanediol to afford a concentration of 20 mg/mL. The frozen oregano decoction was thawed and warmed to room temperature in water bath before administration. OV-16 solution and oregano decoction were orally given via gastric gavage to 6 and 8 rats, respectively, at equivalent dose of 100 mg/kg of OV-16.

Serum Sample Collection. After oral administration, blood samples were withdrawn via cardiac puncture at 5, 10, 15, 30, 60, 120, 240, and 480 min. The sera were collected by centrifuging at 10000*g* for 15 min to obtain the supernatants and stored at –30 °C until analysis.

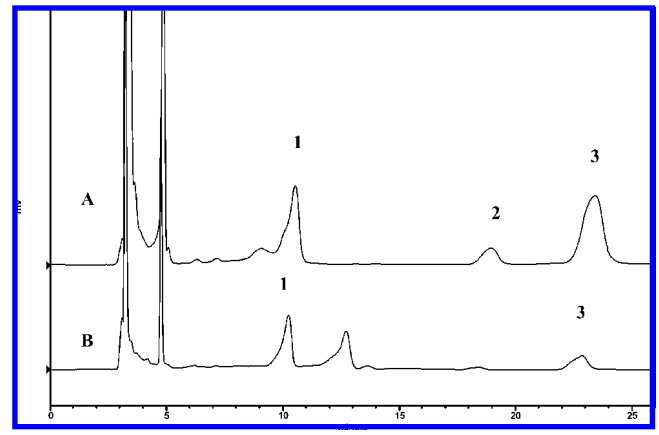

Figure 2. Chromatograms of rat serum samples hydrolyzed with sulfatase after intake of (**A**) OV-16 solution and (**B**) oregano decoction: (**1**) gallic acid; (**2**) HBA; (**3**) PCA.

Quantitation of PCA and HBA in Serum. A 100 μL aliquot of serum was added to 100 μL of acetate buffer (pH 5.0), 50 μL of ascorbic acid (200 mg/mL), and 20 μL of 0.1 N HCl and then partitioned with 270 μL of ethyl acetate containing 0.5 μg/mL of gallic acid as internal standard. After centrifuging at 10000*g* for 15 min, the supernatant was blown with nitrogen gas to dryness and reconstituted with an appropriate volume of methanol for HPLC analysis.

Quantitation of the Sulfates/Glucuronides of PCA and HBA in Serum. To 100 μL of serum, 100 μL of sulfatase solution (containing 10 units of sulfatase and 356 units of β-glucuronidase in pH 5.0 acetate buffer) was added and incubated at 37 °C for 120 min for optimum hydrolysis, which had been determined by a time study. The quantitation was performed as the procedures described above.

Quantitation of the Glucuronides of PCA and HBA in Serum. To 100 μL of serum, 100 μL of β-glucuronidase solution (containing 10 units of β-glucuronidase in pH 5.0 acetate buffer) was added and incubated at 37 °C for 240 min for optimum hydrolysis, which had been determined by a time study. The quantitation was performed as given in the procedures described above.

Calculation of Pharmacokinetic Parameters. Pharmacokinetic parameters were calculated from each individual set of data by the software WinNonlin (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The peak serum concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained from experimental observation. The areas under the curves (AUC_s) from time zero to time last were calculated by the trapezoidal rule.

Preparation of Serum Metabolite of OV-16. Animals were fasted for 12 h before the experiment. OV-16 solution was orally given via gastric gavage at a dose of 100 mg/kg. Referring to the mean T_{max} of OV-16 metabolites obtained in the pharmacokinetic study, rat blood was withdrawn via cardiac puncture. The serum was deproteinized with 2-fold methanol. After centrifuging at 10000*g* for 15 min, the supernatant was concentrated in a rotatory evaporator under vacuum to dryness and an appropriate amount of methanol was added to dissolve the residue and then stored at –30 °C for later use.

Determination of Radical Scavenging Capability against DPPH (*17*). The serum metabolite of OV-16 was added to a methanol solution of DPPH (0.5 mM). After incubation for 30 min at room temperature, the absorbance of the remaining DPPH was determined at 540 nm. The analysis was carried out in triplicate.

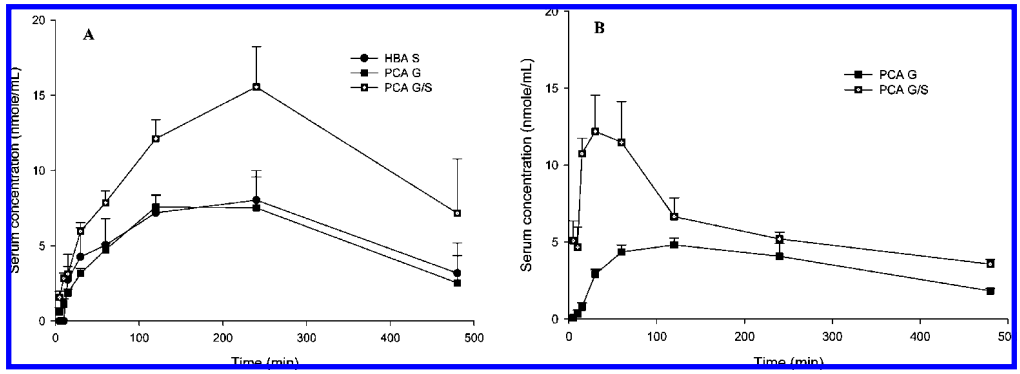

Figure 3. Mean (±SE) serum concentration−time profiles of PCA glucuronides/sulfates (G/S), PCA glucuronides (G), and HBA sulfate (S) after oral administration of (**A**) OV-16 solution (100 mg/kg, $n = 6$) and (**B**) oregano decoction (equivalent to 100 mg/kg of OV-16, $n = 8$) to rats.

Table 1. Pharmacokinetic Parameters of HBA Sulfate (S), PCA Glucuronides (G), and PCA Glucuronides/Sulfates (G/S) after Oral Administration of OV-16 (100 mg/kg) and Oregano Decoction (Equivalent to 100 mg/kg of OV-16) to Rats[a]

	OV-16 ($n = 6$)			oregano decoction ($n = 8$)	
parameter	HBA S	PCA G	PCA G/S	PCA G	PCA G/S
T_{max} (min)	155.0 ± 39.8	180.0 ± 26.8	200.0 ± 25.3	101.3 ± 23.3[b]	27.5 ± 5.5[c]
C_{max} (nmol/mL)	8.8 ± 1.5	9.7 ± 1.1	16.4 ± 2.2	5.9 ± 0.6[b]	15.6 ± 2.3
AUC_{0-480} (nmol · min/mL)	2272.8 ± 813.8	2282.7 ± 665.4	4985.7 ± 1113.9	1639.6 ± 147.2	2911.5 ± 311.1[b]
MRT (min)	178.7 ± 27.2	176.4 ± 27.9	213.3 ± 19.2	207.9 ± 5.7	193.9 ± 12.8

[a] Data expressed as mean ± SE. [b] $P < 0.05$. [c] $P < 0.001$ compared with administration of OV-16.

RESULTS

HPLC analysis showed that OV-16 and PCA were present in the oregano decoction, but no HBA was detected. The calibration curves for the quantitation of OV-16 and PCA in oregano decoction over the concentration ranges of 10.0–250.0 and 2.2–31.3 μg/mL, respectively, were with excellent linearities ($r > 0.99$). The precision evaluation showed that all coefficients of variation were below 8.8% for OV-16 and 10.6% for PCA; the accuracy analysis showed that the relative errors to the true concentration of analyte were below 5.7% for OV-16 and below 18.8% for PCA. The quantitation results showed that the concentrations of OV-16 and PCA in the oregano decoction were 7.1 and 1.9 mg/mL, respectively.

For serum analysis, our preliminary study indicated that the free forms of OV-16, PCA, and HBA were not present in all serum specimens following intake of OV-16 and oregano decoction. Gallic acid was a satisfactory internal standard for the quantitation of PCA and HBA; typical HPLC chromatograms of rat serum specimens hydrolyzed by sulfatase are shown in **Figure 2**. Good linearities ($r > 0.99$) were obtained for PCA and HBA in the concentration range of 0.5–20.0 μg/mL in serum. The precision evaluation showed that all coefficients of variation were below 15.1%, and the accuracy analysis showed that the relative errors to the true concentrations were below 9.5%. The recoveries of PCA from serum were 98.2, 109.5, and 95.9% for 10.0, 2.5, and 1.0 μg/mL, and those of HBA were 104.7, 107.2, and 81.4% for 10.0, 2.5, and 1.0 μg/mL, respectively. The limits of detection of PCA and HBA were 0.1 and 0.3 μg/mL, respectively.

When OV-16 and oregano decoction were orally administered, the mean serum concentration−time profiles of conjugates of PCA and HBA are shown in **Figure 3**. The pharmacokinetic parameters were listed in **Table 1**. Following administration of OV-16, glucuronides and sulfates of PCA as well as HBA sulfate were found in serum, whereas HBA glucuronide was not detected. When oregano decoction was given, the sulfates and glucuronides of PCA were found in serum, whereas the conjugates of HBA were not detected. The marked gap between the profiles of PCA glucuronides/sulfates and PCA glucuronides during the early phase following oregano intake indicated that PCA sulfates were the predominant metabolites. The AUC of PCA glucuronides/sulfates after OV-16 intake was significantly higher than oregano, whereas their C_{max} were comparable. The T_{max} of PCA glucuronides/sulfates after administration of oregano was much shorter than that of OV-16.

Table 2. Free Radical Scavenging Effect against DPPH[a]

	1-fold [b]	0.5-fold	0.25-fold	50 μM	20 μM
blank serum	5.2 ± 3.7	2.5 ± 0.3	1.7 ± 1.4		
OV-16 metabolites	13.9 ± 2.7[c]	7.2 ± 3.1	4.7 ± 1.2		
L-ascorbic acid				33.7 ± 1.0	9.8 ± 1.3

[a] The concentration of DPPH solution was 0.5 mM. The results are shown in % = [(OD in the absence of metabolites — OD in the presence of metabolite)/(OD in the absence of metabolite)] × 100. Data expressed as mean ± SD ($n = 3$). [b] Serum concentration. [c] $P < 0.05$ compared with the corresponding blank serum.

The radical scavenging effects of the serum metabolite of OV-16 at various concentrations against DPPH expressed as inhibition percentage are listed in **Table 2**. The results indicated that the metabolites of OV-16 at serum concentration exhibited excellent radical scavenging capability and are comparable to 20 μM ascorbic acid.

DISCUSSION

When OV-16 or oregano decoction was given to rats, the free forms of OV-16, PCA, and HBA were not present in the blood. The glucuronides/sulfates of PCA and sulfate of HBA were found following OV-16 intake, whereas only glucuronides/sulfates of PCA, but no conjugates of HBA, were found following oregano intake. On the basis of these results, the metabolic fate of OV-16 in rats is thus proposed as **Figure 4**.

For determining the conjugated metabolites of PCA and HBA, because the authentic standards were not available, we used β-glucuronidase and sulfatase to hydrolyze them to PCA and HBA. However, the commercial sulfatase contains not only

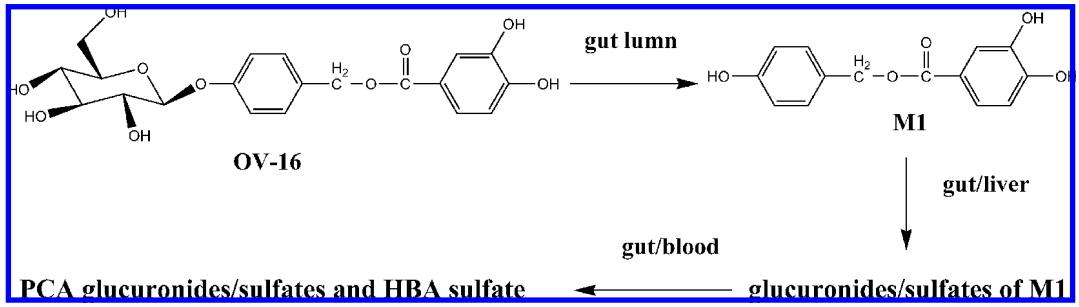

Figure 4. Proposed biotransformation fate of OV-16.

sulfatase but also a considerable amount of β-glucuronidase. Therefore, when sulfatase was used, both sulfates and glucuronides were hydrolyzed. Comparison of the serum levels between glucuronides/sulfates of PCA and glucuronides of PCA in most serum specimens indicated the presence of sulfates of PCA after intake of OV-16 and oregano.

Following administration of OV-16, HBA had not been detected after hydrolysis with β-glucuronidase; therefore, the HBA obtained after treatment with sulfatase/glucuronidase should be released from the sulfate of HBA. On the basis of the chemical structure of OV-16, equimolar PCA and HBA were expected to be produced from OV-16. However, the total AUC_{0-480} of PCA conjugates was 2.2-fold to that of HBA sulfate. The marked difference in systemic exposure between PCA conjugates and HBA conjugates might be resulted from their different pharmacokinetic properties. HBA sulfate might have larger volume of distribution or higher elimination rate than that of PCA conjugates.

Following oregano administration, the gap between two profiles during the early phase revealed that PCA sulfates were at higher concentrations than PCA glucuronides. This could be accounted for by the different nature of sulfation and glucuronidation occurred on PCA. Sulfation was reported as a high affinity and low capacity reaction, whereas glucuronidation was a low affinity and high capacity reaction (*18*). When PCA in oregano was absorbed per se, it would proceed sulfation first. When the absorption of PCA increased, sulfation reaction was saturated and glucuronidation might then progress.

In this study, equivalent doses of OV-16 (100 mg/kg) were given as pure compound and decoction. The systemic exposure of PCA glucuronides/sulfates following OV-16 was significantly greater than that following oregano although the decoction contained PCA beside OV-16. In addition, HBA sulfate was present following OV-16 administration but not detected following oregano decoction. These facts indicated that OV-16 given as a pure compound appeared to show better bioavailability than oregano decoction. Because of the poor solubility of OV-16 in water (ca. 0.6 mg/mL), OV-16 solution was prepared using Cremophor EL and 1,2-propanediol in this study. In contrast, the oregano decoction was a concentrated aqueous extract in which OV-16 was essentially suspended in water. The better solubility of OV-16 in the surfactant vehicle than water can explain its higher bioavailability than oregano decoction. The T_{max} following oregano intake was much earlier than OV-16. This can be explained by the authentic presence of PCA in the decoction (*15*), which could be absorbed directly by the intestine (*19*). In contrast, OV-16, a polar glycoside, is not lipophilic enough to permeate through the cell membrane of enterocytes; therefore, it is indispensable to be hydrolyzed to less polar aglycone before absorption. The presystemic metabolism of OV-16 might explain the much delayed T_{max} of PCA glucuronides/sulfates following OV-16 administration than oregano decoction, which contained considerable amount of PCA.

OV-16, PCA, and HBA had been reported to show excellent antioxidation activities in in vitro studies (*14, 20–24*). However, our results indicated that the free forms of OV-16, PCA, and HBA were not present in the blood after intake of OV-16 and oregano decoction. In fact, PCA and HBA were extensively metabolized to their conjugated metabolites in vivo. Therefore, whether the serum metabolites of OV-16 containing the conjugates of PCA and HBA still retain antioxidation activity needs evaluation. Our results showed that metabolites of OV-16 at serum concentration exhibited comparable radical scavenging effect with that of 20 μM ascorbic acid, revealing excellent antioxidation capability. The antioxidation activity may be mainly contributed by the glucuronides/sulfates of PCA which still contain phenolic function.

There is growing evidence indicating that most polyphenols virtually existed in the bloodstream as phase II metabolites (*25–27*). Our result was in good agreement with previous findings on the metabolic fate of polyphenols. In general, phase II metabolites have rather different intrinsic characters from their parent forms. The glucuronides/sulfates of polyphenols are generally more polar, more water soluble than their aglycones, and mostly ionized into anions under physiological pH. Accordingly, the in vitro bioactivities of parent forms of most polyphenols are not appropriate to predict their in vivo effects. Recently, several phase II metabolites of polyphenols demonstrated various promising beneficial bioactivities (*28–31*). Therefore, the sulfates and glucuronides of polyphenols are not necessarily inactive metabolites.

In conclusion, oral intake of OV-16 presented glucuronides/sulfates of PCA and sulfate of HBA in the blood. These conjugated metabolites await more bioactivity studies to better understand the in vivo effects of OV-16 and oregano.

ABBREVIATIONS USED

OV-16, 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside; PCA, protocatechuic acid; HBA, *p*-hydroxybenzyl alcohol; HPLC, high-performance liquid chromatography; C_{max}, the peak serum concentration; T_{max}, the time to peak concentration; AUCs, the areas under the curves.

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