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Metabolic Transformation of Sesamol and ex Vivo Effect on 2,2′-Azo-bis(2-amidinopropane)-dihydrochloride-Induced Hemolysis

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Sesamol (3,4-methylenedioxyphenol), a phenolic constituent in roasted sesame, was reported to exhibit various beneficial activities. To understand the metabolic transformation of sesamol in vivo, rats were given sesamol intravenously and orally. The blood samples were withdrawn via cardiopuncture at specific time points. The serum samples were assayed by high-performance liquid chromatography method before and after hydrolysis with sulfatase and β-glucuronidase. Our results indicated that following either intravenous or oral administration, sesamol declined rapidly and the sulfate/glucuronide of sesamol emerged instantaneously. The peak serum concentration and systemic exposure of sesamol were markedly lower than sesamol sulfate/glucuronide. Ex vivo evaluation revealed that sesamol exerted profoundly higher capability against 2,2′-azo-bis(2-amidinopropane)dihydrochloride-induced hemolysis than the serum metabolites. In conclusion, sulfate and glucuronide of sesamol were the principle metabolites of sesamol in the bloodstream of rats. The conjugated metabolites of sesamol warrant more bioactivity investigations to understand the in vivo effect of sesamol.

KEYWORDS: Sesamol; pharmacokinetics; metabolism; AAPH

INTRODUCTION

Sesamol (3,4-methylenedioxyphenol) is a constituent of roasted sesame, seeds of *Sesamum indicum* L., an important oilseed crop. Several beneficial effects of sesamol, including antioxidation (*1*−*3*), chemoprevention (*4*), antimutagenic (*5*), antihepatotoxic activities (*6*), and induction of the apoptosis of cancer and cardiovascular cells (*7*) have been reported by previous in vitro studies. On the other hand, only one in vivo study has reported the attenuation of the diabetes-associated cognitive deficit in rats by sesamol (*8*).

Sesamol has a phenolic and benzodioxole group in its molecular structure as shown in **Figure 1**. Despite various beneficial effects of sesamol reported in the literature, no reports are available with regard to the metabolic transformation and pharmacokinetics of sesamol till now. In recent decades, pharmacokinetic studies of flavonoids have confirmed that polyphenols were vulnerably metabolized by conjugation reactions with the free forms rarely found in circulation (*9, 10*). What indeed is the biological fate of sesamol and can the in vitro bioactivities of sesamol be counted on to infer the effects in animals? To shed light on these questions, this study attempts to investigate the metabolic transformation and pharmacokinetics of sesamol in rats. As the extension of our pharmacokinetic results and to mimic in vivo conditions, the serum metabolites of sesamol were prepared from rats, and an ex vivo effect on 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH)-induced hemolysis was in turn evaluated and compared with that of sesamol.

MATERIALS AND METHODS

Chemicals and Reagents. Sesamol, sulfatase (type H-1, *Helix pomatia*, containing 14000 units/g of sulfatase and 498800 units/g of β-glucuronidase), and β-glucuronidase (type B-1, from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). 6,7-Dimethoxycoumarin (98%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and ethyl acetate were LC grade and purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Hydrochloric acid was a product of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-(+)-Ascorbic acid was purchased from Riedel-deHean AG (Seelze, Germany). PEG 400 (polyethylene glycol 400) was obtained from Merck-KgaA (Darmstadt, Germany). Milli-Q plus water (Millipore, United States) was used for all preparations.

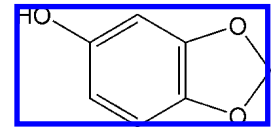

Figure 1. Chemical structure of sesamol.

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Instrumentation and High-Performance Liquid Chromatography (HPLC) Conditions. The HPLC apparatus included one pump (LC-10ATVP, Shimadzu, Japan) and an UV spectrophotometric detector (SPD-10AVP, Shimadzu). The RP-18 column (Cosmosil, 150 mm × 4.6 mm) was equipped with a prefilter. The mobile phase consisted of acetonitrile and 0.1% *ortho*-phosphoric acid (22:78 v/v). The flow rate was 1.0 mL/min, and the detection wavelength was set at 290 nm.

Animals and Administration of Sesamol. Male Sprague−Dawley rats weighing 400−500 g were fasted for 12 h before sesamol administration and fasting continued for another 4 h. Sesamol was dissolved in water, filtered through a 0.2 μm filter, and given as an intravenous bolus at 10 mg/kg. On the other hand, two groups of rats were given single and seven oral doses of aqueous sesamol solution at 50 mg/kg, respectively. In addition, sesamol was dissolved in PEG 400, and a single dose at 50 mg/kg was given orally to another group of rats. Each treatment group included six rats. The oral administrations of sesamol were given via gastric gavage.

Blood Sample Collection. Blood samples (1 mL) were withdrawn via cardiopuncture at 5, 15, 30, 60, 120, 240, 480, 720, and 1440 min postadministration of sesamol. Blood samples were allowed to clot and then centrifuged at 10000*g* to obtain serum, which was stored at −30 °C for later analysis. The animal study adhered to "The Guide Book for the Care and Use of Laboratory Animals (2002)", published by the Chinese Society for the Laboratory Animal Science (Taiwan, Republic of China). The protocol was approved by the Committee of Animal Management, China Medical University (Taichung, Taiwan).

Quantitation of Sesamol and Its Conjugated Metabolites in Serum. For the determination of sesamol sulfate, 150 μL of serum was incubated with 50 μL of sulfatase (1000 units/mL in pH 5.0 acetate buffer) and 50 μL ascorbic acid (200 mg/mL) at 37 °C for 1 h. For the determination of sesamol glucuronide, 150 μL of serum was incubated with 50 μL of glucuronidase (1000 units/mL in pH 5.0 acetate buffer) and 50 μL of ascorbic acid (200 mg/mL) at 37 °C for 2 h. The reaction mixture was partitioned with 300 μL of ethyl acetate (containing 2.5 μg/mL 6,7-dimethoxycoumarin as internal standard) followed by the addition of 50 μL of 0.1 N HCl. The ethyl acetate layer was evaporated under N_2 gas to dryness and reconstituted with 50 μL of MeOH and then subjected to HPLC analysis. For the assay of the sesamol parent form, 150 μL of serum sample was subject to the same process except for the addition of 50 μL of enzyme-free buffer.

Preparation of Serum Metabolites of Sesamol and Blank Serum Controls. After fasting for 12 h, 10 rats were given an aqueous solution of sesamol (50 mg/kg) orally via gastric gavage. At 30 min after sesamol administration, about 10 mL of blood from each rat was withdrawn to obtain serum. A four-fold volume of methanol was mixed with serum and centrifuged at 10000*g* for 15 min to remove proteins. The supernatant was evaporated under vacuum to dryness and dissolved with a minimum amount of water. The aqueous solution was subjected to purification by solid phase extraction using Strata (Phenomenex, Torrance, CA) to remove sesamol free form. The eluent was analyzed with HPLC to confirm the absence of sesamol and then assayed for the concentration of sesamol sulfate/glucuronide following the method described previously. Then, the eluent was diluted with water to afford 190 and 95 μM sesamol sulfates/glucuronides solution. In addition, blank serum was collected from another 10 rats and processed in the same manner to prepare blank serum controls representing 1- and 0.5-fold of the serum matrix.

Effect of Sesamol and Serum Metabolites of Sesamol on AAPH-Induced Hemolysis. For erythrocytes preparation, the blood of rats was withdrawn via cardiac puncture and collected into heparinized tubes. The erythrocytes were separated from plasma and buffy coat, washed three times with phosphate-buffered saline (PBS), and then centrifuged at 1500*g* for 15 min to obtain a packed cell preparation, which was suspended in PBS to afford a 20% erythrocyte suspension.

AAPH and sesamol were dissolved in PBS (pH 7.4). A 100 μL amount of erythrocyte suspension was mixed with 200 μL of sesamol or serum metabolites, and 100 μL of AAPH was then added to the mixture. The reaction mixture was shaken gently while being incubated at 37 °C for 1, 3, 5, 7, and 9 h. After incubation, 400 μL of PBS was added to the reaction mixture, and it was centrifuged at 10000*g* for 2 min. The supernatant (700 μL) was diluted with 2.3 mL of PBS, and the absorbance at 540 nm was recorded using an enzyme-linked immunosorbent assay reader (*11*).

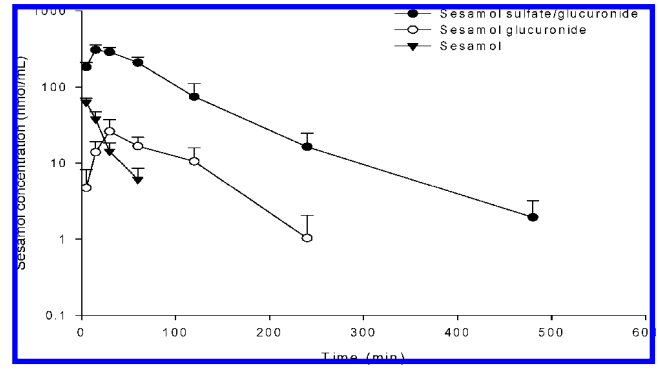

Figure 2. Mean (±SE) serum concentration−time profiles of sesamol (▼), sesamol sulfate/glucuronide (●), and sesamol glucuronide (○) after an intravenous bolus of sesamol aqueous solution (10 mg/kg) to six rats.

Data Analysis. The peak serum concentrations (C_{max}) were recorded as observed. WINNONLIN (version 1.1, SCI software, Statistical Consulting Inc., Apex, NC) was used for the computation of pharmacokinetic parameters. The parameters including the area under the serum concentration−time curve to the last point ($AUC_{0\text{-}t}$) and mean residence time (MRT) were calculated using a noncompartment model. The concentration of sesamol conjugates was calculated from the following equation:

$$\text{concn}_{\text{conjugates}} = \text{concn}_{\text{parent form+conjugates}} - \text{concn}_{\text{parent form}}$$

A paired Student's *t* test was used for statistical comparison of pharmacokinetic parameters between single-dose and multiple-dose treatments, taking $P < 0.05$ as significant.

RESULTS

The analytical method of sesamol in serum was developed and validated in this study. The coefficients of variation (CVs) of intraday and interday assay were less than 8% in the concentration range of 0.16−40.0 μg/mL, and the relative errors were below 14 and 20%, respectively. The lower limit of quantitation (LLOQ) was 0.16 μg/mL, and the limit of detection (LOD) was 0.09 μg/mL.

Figure 2 depicts the serum concentration−time profiles of sesamol and its conjugated metabolites after an intravenous bolus of aqueous sesamol solution (10 mg/kg). The parent form of sesamol declined rapidly and was only detected within 1 h after the bolus, whereas sesamol sulfate/glucuronide emerged instantaneously and remained present in the serum till 8 h. The concentrations of sesamol sulfate/glucuronide were higher than those of sesamol and sesamol glucuronide at all time points.

Figures 3 and **4** depict the serum concentration−time profiles of sesamol and its conjugated metabolites after 50 mg/kg of aqueous sesamol solution was orally administered at a single dose and seven doses, respectively. The sesamol parent form declined rapidly and was detectable only within 2 h after dosing, whereas sesamol sulfate/glucuronide emerged instantaneously and was detectable till 24 h. The concentrations of sesamol sulfate/glucuronide were higher than those of sesamol and sesamol glucuronide at all time points.

Figure 4 depicts the serum concentration−time profiles of sesamol sulfate/glucuronide and sesamol glucuronide after 50 mg/kg of sesamol in PEG 400 was orally administered to rats as a single dose. The parent form of sesamol was not detected. The concentrations of sesamol sulfate/glucuronide were higher than those of sesamol glucuronide at all time points.

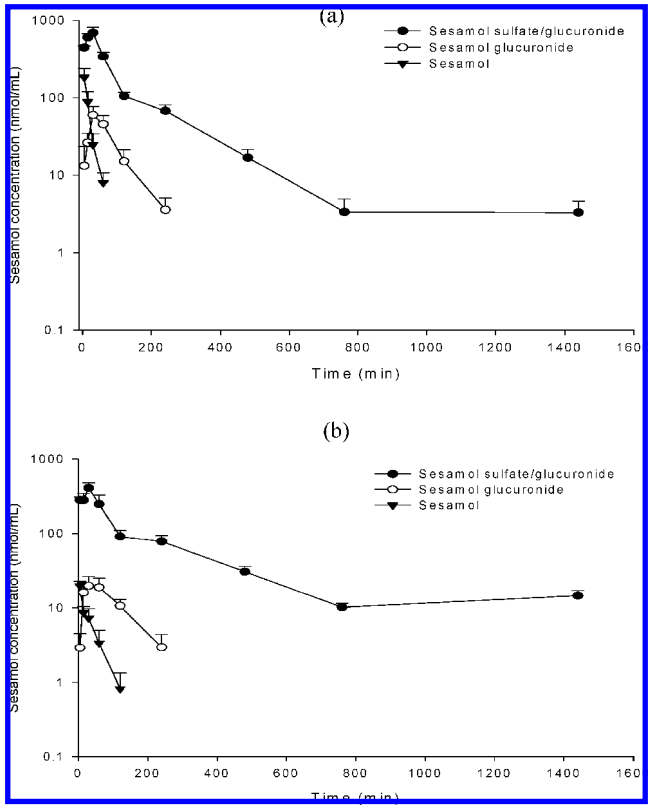

Figure 3. Mean (±SE) serum concentration−time profiles of sesamol (▼), sesamol sulfate/glucuronide (●), and sesamol glucuronide (○) after oral administration of single dose (**a**) and seven doses (**b**) of aqueous sesamol solution (50 mg/kg) to six rats.

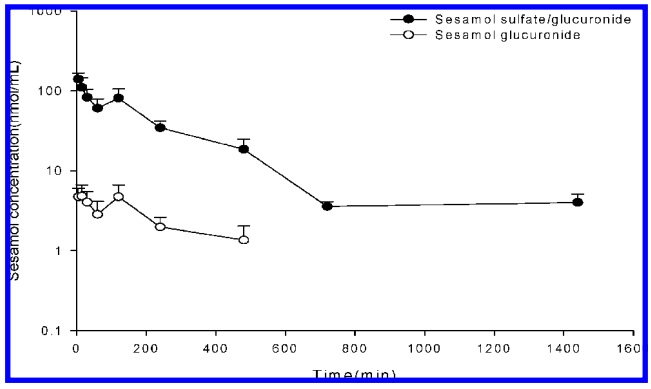

Figure 4. Mean (±SE) serum concentration−time profiles of sesamol sulfate/glucuronide (●) and sesamol glucuronide (○) after oral administration of sesamol in PEG 400 (50 mg/kg) to seven rats.

The pharmacokinetic parameters of sesamol and its conjugated metabolites following intravenous and oral administrations were listed in **Table 1**. After intravenous bolus of 10 mg/kg aqueous sesamol solution, the $AUC_{0\text{-}t}$ of sesamol sulfate/glucuronide was 14-fold of sesamol glucuronide and 20-fold of sesamol. After single-dose oral administration of 50 mg/kg sesamol in water, the C_{max} and $AUC_{0\text{-}t}$ of sesamol sulfate glucuronide were 11- and 13-fold of sesamol/glucuronide and 4- and 22-fold of sesamol, respectively. After seven-dose oral administration of 50 mg/kg sesamol aqueous solution, the C_{max} and $AUC_{0\text{-}t}$ of sesamol sulfate/glucuronide were 16- and 28-fold of sesamol glucuronide and 21- and 122-fold of sesamol, respectively. In contrast, when PEG 400 was the vehicle of sesamol and orally administered to rats at 50 mg/kg, no trace of sesamol was detected. The C_{max} and $AUC_{0\text{-}t}$ of sesamol sulfate/glucuronide were 21- and 24-fold of sesamol glucuronide, respectively.

Figure 5a,**b** showed the effects of sesamol and the serum metabolite of sesamol against the AAPH-induced hemolysis, respectively. The results indicated that sesamol inhibited the hemolysis at 0.2−122.0 μM in a dose-dependent manner, whereas the serum metabolites of sesamol were not effective at the concentrations of 190 and 95 μM sesamol sulfate/glucuronide.

DISCUSSION

Validation of the analytical method of sesamol in serum indicated that the precision and accuracy were satisfactory. Because the authentic compounds of sesamol sulfate and sesamol glucuronide were not available, their concentrations in serum were determined indirectly through hydrolysis with sulfatase and glucuronidase, respectively. Because of a considerable amount of glucuronidase in the sulfatase (type H-1) used in this study, treatment with this enzyme resulted in the hydrolysis of both sulfates and glucuronides. Through comparison between treatments with sulfatase and glucuronidase, the relative abundance of sesamol sulfate and sesamol glucuronide in serum could be estimated.

To observe the hepatic metabolism of sesamol, an intravenous bolus of sesamol was given to rats. Following intravenous bolus, the sesamol parent form declined rapidly and the systemic exposure of sesamol was only 5% of sesamol sulfate/glucuronide. Likewise, following single-dose oral administration of sesamol, the parent form declined rapidly and the systemic exposure of sesamol was only 5% of sesamol sulfate/glucuronide. These facts clearly indicated very rapid and extensive conjugation metabolism of sesamol by liver and/or intestine in rats. Comparison of the $AUC_{0\text{-}t}$ of sesamol and its conjugated metabolites following intravenous and oral administrations indicated that the systemic exposure of sesamol sulfate was greater than 13-fold of sesamol glucuronide and 20-fold of sesamol. It implied that sesamol sulfate was the major molecule in the circulation. The minor metabolite sesamol glucuronide had even greater systemic exposure than sesamol parent form. In comparison to a recent study (*12*) reporting the bioavailability and tissue distribution of sesamol in rats, a conflict of the amounts of glucuronide and sulfate in blood between two studies was found. We think that the major reason is that the previous study used whole blood sample for hydrolysis by sulfatase to result in higher glucuronide and lower sulfate than our results. This discrepancy can be accounted for by the presence of arylsulfatase and UDP-glucuronyltransferase in the platelets (*13, 14*), which had converted sesmol sulfate to glucuronide during the incubation with sulfatase. In contrast, our study used serum sample for hydrolysis, which preserved the virtual composition of metabolites in the blood at the sampling time.

The absolute bioavailability of sesamol was 41%, calculated from the ratio of $AUC_{0\text{-}t}$ of sesamol between oral administration and intravenous bolus with dose correction. This result is similar to that obtained by the previous study (35.5%) (*12*). If the total $AUC_{0\text{-}t}$ including sesamol and sesamol sulfate/glucuronide was compared between two administration routes, the absorption of sesamol was 46%.

When PEG 400 was the vehicle of sesamol and given orally at a single dose of 50 mg/kg, no trace of sesamol was detected in serum, which was not in good agreement with that following aqueous sesamol solution given orally at the same dose. Moreover, the C_{max} and $AUC_{0\text{-}t}$ of sesamol sulfate/glucuronide after giving sesamol in PEG 400 were significantly lower by

Table 1. Pharmacokinetic Parameters of the Free Form, Sulfate/Glucuronide (S/G), and Glucuronide (G) of Sesamol after an Intravenous Bolus (IV) of Sesamol (10 mg/kg) and Oral Administrations (PO) of Single and Seven Doses of Sesamol (50 mg/kg) to Six Rats in Each Group[a]

parameters	10 mg/kg, IV (1 dose, in water)	50 mg/kg, PO (1 dose, in water)	50 mg/kg, PO (7 doses, in water)	50 mg/kg, PO (1 dose, in PEG 400)
Free form				
T_{max}		6.7 ± 1.8	9.2 ± 4.6	ND
C_{max}	86.4 ± 13.0	185.4 ± 60.2	20.1 ± 2.98*	ND
AUC_{0-t}	1540.3 ± 317.1	3160.3 ± 1103.0	540.0 ± 180.7*	ND
MRT_{0-t}	13.9 ± 2.2	15.2 ± 1.4	23.1 ± 6.6	ND
S/G				
T_{max}	22.5 ± 9.2	20.8 ± 4.8	21.7 ± 5.8	24.3 ± 16.1
C_{max}	326.3 ± 49.3	717.0 ± 124.5	427.2 ± 74.7	162.8 ± 30.9***
AUC_{0-t}	30300.7 ± 6182.2	69213.5 ± 4918.6	65947.9 ± 9493.9	27945.4 ± 4216.4***
MRT_{0-t}	67.7 ± 12.0	147.7 ± 27.3	324.5 ± 39.0**	321.5 ± 54.5*
G				
T_{max}	47.5 ± 17.2	23.3 ± 4.8	42.5 ± 8.9	140.7 ± 65.2*
C_{max}	37.8 ± 8.4	68.2 ± 16.4	27.0 ± 8.3*	7.6 ± 1.6***
AUC_{0-t}	2233.3 ± 541.0	5235.8 ± 1776.2	2349.3 ± 461.2	1145.8 ± 322.0*
MRT_{0-t}	59.8 ± 15.8	66.8 ± 6.6	75.1 ± 9.8	175.3 ± 44.0*

[a] Data expressed as means ± SE. ND, not detected. *P < 0.05, **P < 0.01, and ***P < 0.001, as compared to single oral dose of 50 mg/kg. T_{max} (min), the time to reach peak serum concentration; C_{max} (nmol/mL), the peak serum concentration; AUC_{0-t} (nmol min mL^{-1}), area under serum concentration−time curve to the last point; and MRT_{0-t} (min), mean residence time.

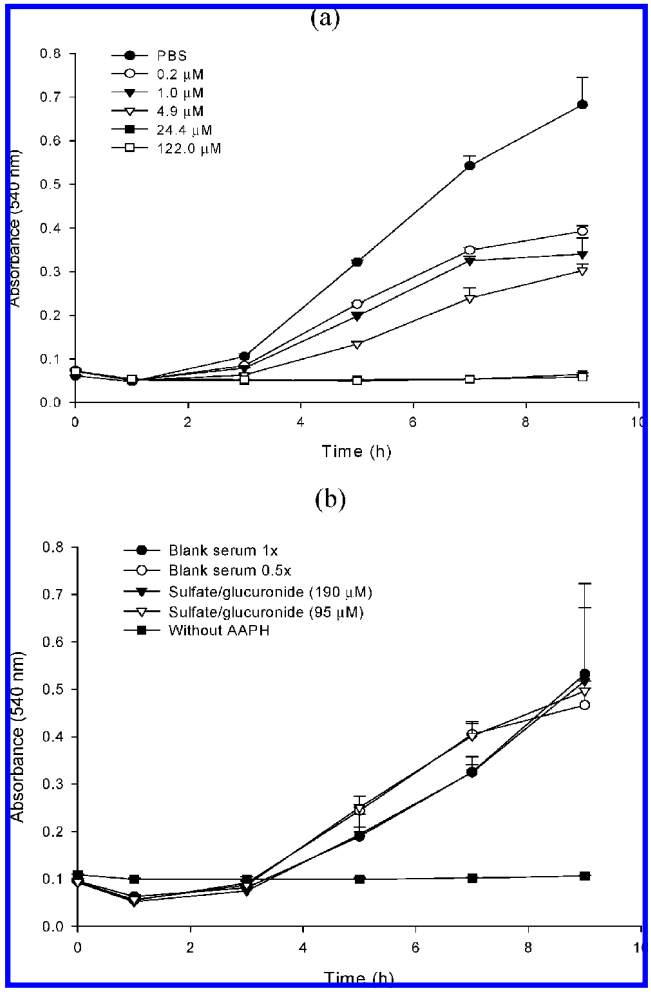

Figure 5. Effects of sesamol (**a**) and serum metabolites of sesamol (**b**) on AAPH induced-hemolysis.

77 and 58%, respectively, than aqueous sesamol solution. The marked difference of sesamol pharmacokinetics in water and PEG 400 revealed the importance of vehicle in affecting the biological fate of sesamol.

To understand the pharmacokinetic behavior of sesamol after multiple-dose administration, seven doses were given to rats. In contrast to single-dose administration, unexpectedly, the C_{max} and AUC_{0-t} of sesamol were significantly lower by 89 and 83%, respectively. The systemic exposure of sesamol was only 0.8% of sesamol sulfate/glucuronide. In addition, the C_{max} of sesamol glucuronide was significantly lower by 60%. However, the systemic exposure of sesamol sulfate/glucuronide was not different between two dosage regimens. These facts implied that after multiple-dose administration, the extent of the major conjugation metabolism, that is, sulfation, was not profoundly altered, whereas glucuronidation, the minor pathway, was markedly inhibited. With regard to the marked decrease of sesamol parent form, we speculate that this phenomenon might be due to enhanced demethylenation through induction of cytochrome 2B4 after repetitive ingestions of sesamol (*15*). However, no attempt has been made in this study to determine and quantitate the metabolites formed via this pathway.

Numerous investigations have used erythrocytes as model systems for studying biomembrane oxidative damage (*16*−*19*). In many of these studies, free radical initiators such as AAPH have been used to generate free radicals in the aqueous phase that can attack the erythrocyte membrane and propagate lipid peroxidation, leading to hemolysis (*16, 18, 19*). Our results indicated that sesamol significantly inhibited the AAPH-induced hemolysis between 0.2 and 122.0 μM in a dose-dependent manner, indicating that pharmacological concentration of sesamol could exert promising antioxidation activity. However, the effective duration of sesamol in vivo might be very limited because of the very short mean residence time as shown in **Table 1**. Furthermore, as the extension of our pharmacokinetic results and to mimic in vivo conditions, we have prepared the serum metabolites of sesamol from rats and evaluated the in vitro effect on AAPH-induced hemolysis by comparison with corresponding blank serum controls. The results showed that the effects of the serum metabolites containing 190 and 95 μM sesamol sulfate/glucuronide on AAPH-induced hemolysis were not different from that of blank serum. On the basis of these ex vivo results, we can conclude that sesamol was the active form in serum, whereas sesamol sulfate/glucuronide were inactive metabolites.

The phenolic group of the molecule is generally responsible for the antioxidation activity of many natural products. On the other hand, benzodioxole derivatives are widely distributed in nature and have been shown to possess antitumor, antioxidation, and radioprotection activities (*20*−*22*). A previous study

reported that sesamol was the most effective constituent isolated from sesame cake extract based on in vitro studies on antioxidation (*23*). The presence of a phenolic group along with a methylenedioxy group may be responsible for the potent antioxidation activity of sesamol (*20*). In contrast, the serum metabolites of sesamol, although containing a considerably high concentration of sesamol sulfate/glucuronide, did not exhibit an antihemolysis effect, which could be accounted for by possessing no phenolic group.

In conclusion, sesamol was rapidly and extensively metabolized by conjugation reaction in rats, mainly sulfation. The antioxidation capability of serum metabolites of sesamol was profoundly less effective than sesamol. Therefore, caution should be taken in extrapolating in vitro findings of sesamol to the situation in vivo.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; C_{max}, the peak serum concentration; T_{max}, the time to peak concentration; $AUC_{0\text{-}t}$, area under serum concentration−time curve to the last point; MRT, mean residence time.

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