Human, Rat, and Mouse Metabolism of Resveratrol

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Received September 3, 2002; accepted September 9, 2002

Purpose. Resveratrol, a phenolic phytoalexin occurring in grapes, wine, peanuts, and cranberries, has been reported to have anticarcinogenic, antioxidative, phytoestrogenic, and cardioprotective activities. Because little is known about the metabolism of this potentially important compound, the *in vitro* and *in vivo* metabolism of *trans*-resveratrol were investigated.

Methods. The *in vitro* experiments included incubation with human liver microsomes, human hepatocytes, and rat hepatocytes and the *in vivo* studies included oral or intraperitoneal administration of resveratrol to rats and mice. Methanol extracts of rat urine, mouse serum, human hepatocytes, rat hepatocytes, and human liver microsomes were analyzed for resveratrol metabolites using reversed-phase high-performance liquid chromatography with on-line ultraviolet-photodiode array detection and mass spectrometric detection (LC-DAD-MS and LC-UV-MS-MS). UV-photodiode array analysis facilitated the identification of *cis*- and *trans*-isomers of resveratrol and its metabolites. Negative ion electrospray mass spectrometric analysis provided molecular weight confirmation of resveratrol metabolites and tandem mass spectrometry allowed structural information to be obtained.

Results. No resveratrol metabolites were detected in the microsomal incubations, and no phase I metabolites, such as oxidations, reductions, or hydrolyzes, were observed in any samples. However, abundant *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate were identified in rat urine, mouse serum, and incubations with rat and human hepatocytes. Incubation with β-glucuronidase and sulfatase to release free resveratrol was used to confirm the structures of these conjugates. Only trace amounts of *cis*-resveratrol were detected, indicating that isomerization was not an important factor in the metabolism and elimination of resveratrol.

Conclusion. Our results indicate that *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate are the most abundant metabolites of resveratrol. Virtually no unconjugated resveratrol was detected in urine or serum samples, which might have implications regarding the significance of *in vitro* studies that used only unconjugated resveratrol.

KEY WORDS: *trans*-resveratrol; metabolism; LC-MS-MS; glucuronides; sulfates.

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INTRODUCTION

Cancer chemoprevention may be regarded as the ingestion of nontoxic quantities of dietary or pharmaceutical agents that are capable of preventing, inhibiting or reversing the process of carcinogenesis (1,2). One dietary compound under investigation as a chemoprevention agent is *trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Fig. 1) (3,4), which is a naturally occurring phytoalexin produced by plants in response to fungal infection or abiotic stresses, such as heavy metal ions or ultraviolet light (UV) (5). Resveratrol has attracted considerable attention because of its presence in dietary sources, such as grapes, wine, peanuts, and cranberries (6). In addition to cancer chemoprevention (3,4), other properties of resveratrol include antioxidative (7–9), antiplatelet (10–12), antifungal (13), phytoestrogenic (14,15), and cardioprotective activities (10,16,17).

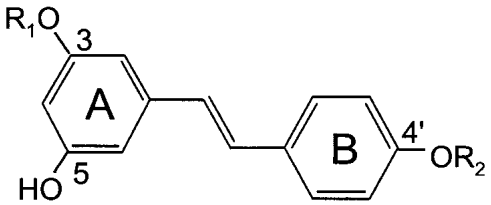
The efficacy of orally administrated resveratrol will depend on its absorption, metabolism, and tissue distribution. Although many studies have implicated a role of resveratrol in disease prevention, only a few studies have addressed the bioavailability and metabolism of resveratrol (18–23). However, none of these has provided a conclusive metabolic profile for resveratrol including its metabolite structures.

Human liver-derived experimental systems have been used extensively for the evaluation of drug metabolism, including the use of intact cell systems, such as hepatocytes, and cell-free systems, such as microsomes, or recombinant enzymes, like specific cytochrome P-450 isozymes. Intact hepatocytes with full complements of enzymes and cofactors at physiologic levels and natural orientations should be more representative of the liver than cell-free systems with disrupted membranes, incomplete cofactors, and enzymes (24). *In vivo* studies provide an overall indication of drug metabolism resulting from all organ systems. In the present study, cryopreserved human and rat hepatocytes were compared with human liver microsomes in the evaluation of resveratrol metabolism (24,25). In addition, resveratrol metabolites were identified in rat urine and mouse serum. All metabolites were characterized using high-performance liquid chromatography (HPLC) with diode array detector (DAD) UV detection, connected on-line with electrospray mass spectrometry or tandem mass spectrometry (LC-DAD-MS and LC-UV-MS-MS).

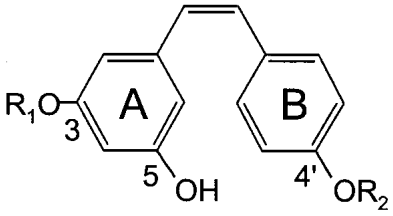
MATERIALS AND METHODS

Materials

trans-Resveratrol, NADPH, β-glucuronidase (type B-10 from boving liver), and sulfatase (type IV: from *Aerobacter aerogenes*, partially purified) were purchased from Sigma Chemical (St. Louis, MO, USA). Formic acid (88%) was obtained from J. T. Baker (Phillipsburg, NJ, USA) and was diluted to the desired concentration using deionized distilled water. HPLC-grade methanol, water, dimethyl sulfoxide (DMSO), and acetonitrile were purchased from Fisher (Fair Lawn, NJ, USA). Naringenin was purchased from Indofine Chemical (Somerville, NJ, USA). Human liver microsomes, cryopreserved human and rat hepatocytes, Krebs-Henseleit



(a) R_1 = H, R_2 = H
(b) R_1 = glucuronic acid, R_2 = H
(c) R_1 = H, R_2 = glucuronic acid
(d) R_1 = SO_3H, R_2 = H
(e) R_1 = H, R_2 = SO_3H



(f) R_1 = H, R_2 = H
(g) R_1 = glucuronic acid, R_2 = H

Fig. 1. Structures of resveratrol and resveratrol conjugates. (a) *trans*-resveratrol; (b) *trans*-resveratrol-3-*O*-glucuronide; (c) *trans*-resveratrol-4′-*O*-glucuronide; (d) *trans*-resveratrol-3-sulfate; (e) *trans*-resveratrol-4′-sulfate; (f) *cis*-resveratrol; and (g) *cis*-resveratrol-3-*O*-glucuronide.

buffer, and minimum essential media (MEM) were obtained from In Vitro Technologies (Baltimore, MD, USA).

To convert *trans*-resveratrol into *cis*-resveratrol, 0.7 mL of a 0.01 mg/mL *trans*-resveratrol solution in methanol was exposed to UV light (λ = 254 nm) for 10 min.

Methods

Incubation of Resveratrol in Human Liver Microsomes

Human liver microsomes (1 mg protein/mL) were incubated in 50 mM phosphate buffer (pH 7.4) with 5 mM resveratrol in the presence of 1 mM NADPH for 0, 15, 30, and 60 min. After incubation, two volumes of acetonitrile were added to the reaction mixture, vortexed for 30 s, and then centrifuged for 3 min at 10,000 g. The supernatant was collected and evaporated to dryness, and the residue was redissolved in 200 μL of methanol for analysis.

Incubation of Resveratrol with Hepatocytes

A cryopreserved sample of human hepatocytes was immersed in a 37°C water bath and shaken gently for 80–90 s The cell suspension was transferred to a 50-mL centrifuge tube that had been precooled in ice. Minimum essential medium (12 mL) was added to the cell suspension at the rate of 1 mL per 15 s. After each addition of 1 mL of medium, the tube was shaken gently to prevent the cells from settling. Next, the tube was centrifuged at 50 g at 4°C for 5 min. The supernatant was removed by aspiration, and the cell pellet was resuspended in 5 mL of Krebs-Henseleit buffer to provide a cell density of approximately 1×10^6 viable hepatocytes/mL. A 1-mL aliquot of the human hepatocytes suspension was transferred to a well of a 12-well plate, and 0.1 mL of 0.1 mM *trans*-resveratrol in 10% DMSO was added. The 12-well plate was incubated at 37°C for 4 h. During the incubation, 0.35-mL aliquots of the cell suspension (one aliquot per well) containing *trans*-resveratrol metabolites were removed at 1, 2, and 4 h and transferred to Eppendorf tubes. After the addition of 0.35 mL of cold methanol for deactivation, each tube was vortex-mixed for 30 s A control experiment was performed using the same procedures except that the hepatocytes were deactivated by the addition of cold methanol before incubation with resveratrol. After centrifuging at 10,000 g for 3 min, the supernatant was transferred to another 1.5-mL Eppendorf tube and evaporated to dryness *in vacuo*. The residue was redissolved in 0.2 mL of methanol and centrifuged at 10,000 g for 3 min. The supernatant was collected and mixed with 0.4 mL of water. Rat hepatocyte samples were prepared in the same manner as the human hepatocyte samples.

Preparation of Rat Urine Samples

Resveratrol (20 mg/kg, dissolved in 6.5% ethanol in neobee oil) was administered intraperitoneally (IP) to three Sprague–Dawley female rats (75 days old, average weight = 228 g), and urine was collected in metabolic cages for 2 h. Also, control urine samples from two rats receiving no resveratrol were obtained. A 100-μL aliquot of each urine sample was mixed with 250 μL of acetonitrile and centrifuged at 10,000 g for 3 min at 4°C. The supernatant was collected for analysis. The research using rats and mouse adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

Preparation of Mouse Serum Samples

Resveratrol was dissolved in a minimum volume of ethanol and then mixed with corn oil as a vehicle for drug delivery. The final concentration of ethanol in the corn oil was 5.4%. In one experiment, resveratrol at 20 mg/kg was administrated via IP injection to 12 Balb/c female mice (4 weeks old, average body weight 19.5 g). In another experiment, resveratrol at 20 mg/kg was administered via gavage to 12 Balb/c female mice (4 weeks old, average weight 19.3 g). Control mice that did not receive resveratrol were used for both the IP injection and gavage experiments.

Blood samples (100–400 μL) were obtained by heart puncture at 0.25, 0.5, 1, 2, 4, and 6 h, after treatment with resveratrol. After clotting, the blood samples were centrifuged at 100 g for 20 min, and serum was collected from each sample and stored at –70°C until analysis. A 25-μL aliquot of each mouse serum sample was mixed with 75 μL of acetonitrile and centrifuged at 10,000 g at 4°C for 3 min. Supernatants were collected for analysis.

The third experiment was conducted using gavage and the same procedures except for the following modifications. The dose of resveratrol was increased from 20 mg/kg to 60 mg/kg. Also, blood samples were obtained at 0.25, 0.5, 1, 2, and 3 h after administration of resveratrol.

Enzymatic Hydrolysis of Resveratrol Glucuronide and Resveratrol Sulfate

To confirm the presence of sulfate and glucuronide conjugates of resveratrol, enzymatic cleavage was performed ac-

cording to the method of Kuhnle *et al.* (20) and Sfakianos *et al.* (26) with the following modifications. For resveratrol sulfate, aliquots of the extracts were evaporated to dryness and reconstituted in 150 μL of 50 mM phosphate buffer (pH 7.4) containing 0.5 U (30 μL) of aryl sulfatase. After incubating at 37°C for 2.5 and 18.5 h, respectively, 100 μL of cold methanol was added into each sample vial to stop the reaction. For resveratrol glucuronide, aliquots of extracts were incubated with β-glucuronidase (final concentration = 8,000 U/mL) in 50 mM phosphate buffer (pH 5.5, 150 μL) for 1 h. The reaction was terminated by adding 150 μL of cold methanol. After centrifugation at 10,000 g for 3 min at 4°C, supernatants were collected, evaporated to dryness *in vacuo*, and reconstituted in 50 μL of 50% methanol. Control incubations were performed without addition of enzyme. All sample preparations were performed in dim light to minimize photochemical isomerization of *trans*-resveratrol to the *cis*-form.

Synthesis of Resveratrol Monosulfate Isomers

To determine the attachment site of sulfate in resveratrol sulfate, resveratrol monosulfate isomers were synthesized. Resveratrol sulfate was formed by standard reaction conditions with one equivalent of sulfur trioxide-pyridine complex in dry pyridine (27). The yield of monosulfates was approximately 30%, and the remainder was unreacted resveratrol. The ratio of 3- and 4′-monosulfates was determined to be 7:3, and the mixture was separated by preparative HPLC. Structures of the isomers were confirmed by nuclear magnetic resonance measurements. Spectra were recorded on a Bruker (Billerica, MA) 300 MHz instrument, and the following data were obtained.

Resveratrol-3-sulfate: Amorphous white solid, ^{1}H-NMR (CD_3OD) d 7.38 (d, J = 8.6 Hz, H-2',6'), 7.05 (d, J = 16.5 Hz, *trans*-vinyl), 6.99 (dd, J = 2.2 Hz, H-2), 6.87 (d, J = 16.5 Hz, *trans*-vinyl), 6.78 (d, J = 8.6 Hz, H-3′,5′), 6.75 (dd, J = 2.1 Hz, H-6), 6.67 (dd, J = 2.1 Hz, H-4), HPLC retention time 13.1 min.

Resveratrol-4′-sulfate: Amorphous white solid, ^{1}H-NMR (CD_3OD) d 7.51 (d, J = 8.7 Hz, H-2',6'), 7.30 (d, J = 8.6 Hz, H-3′,5′), 7.05 (d, J = 16.3 Hz, *trans*-vinyl), 6.87 (d, J = 16.5 Hz, *trans*-vinyl), 6.50 (d, J = 2.1 Hz, H-2,6), 6.20 (dd, J = 2.1 Hz, H-4), HPLC retention time 11.6 min.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-DAD-MS analyses were performed using an Agilent (Palo Alto, CA, USA) 1100 HPLC system equipped with a photodiode array detector and interfaced to a model G1946A single quadruple electrospray mass spectrometer. UV spectra were recorded from 200–360 nm. A Micromass (Manchester, UK) Quattro II triple quadruple mass spectrometer equipped with a Waters (Milford, MA, USA) 2690 HPLC system and 2487 UV detector was used for LC-UV-MS-MS. UV chromatograms were recorded at 210 and 300 nm. HPLC separations were obtained at 25°C using a Waters XTerra MS C_{18} column (2.1 mm × 100 mm, 3.5-μm particle size).

Aliquots (10 μL) of urine or serum extracts were injected directly onto the HPLC column and eluted with a solvent system consisting of 0.1% (v/v) formic acid and acetonitrile. A 60-min linear gradient was used from 10–30% acetonitrile, and for some mouse serum samples a 30-min linear gradient was used from 10–80% acetonitrile. There was a 10-min re-equilibration period with the initial solvent mixture between analyses. The flow rate was 0.2 mL/min.

Negative ion electrospray mass spectra were obtained using the Agilent mass spectrometer with the electrospray capillary set at 4 kV. The flow rate of the nitrogen drying gas was 6.0 L/min at a temperature of 300°C. Mass spectra were recorded over the range of *m/z* 70–800. Alternatively, selected ion monitoring was used for greater sensitivity by recording signals for ions of *m/z* 227, 307, and 403 with a dwell time of 592 ms/ion. Negative ion electrospray tandem mass spectra were recorded using the Micromass triple quadruple instrument with the electrospray capillary set at 2.5 kV and a source block temperature of 100°C. Nitrogen was used as the drying and nebulizing gas at flow rates of approximately 8 L/min and 0.8 L/min, respectively. Argon at a pressure of 1.4×10^{-3} mbar was used as the collision gas for collision-induced dissociation (CID). UV spectra were monitored at 300 nm. Product ion scans were carried out using the deprotonated molecules of resveratrol and its metabolites as precursor ions. When greater sensitivity was required, multiple reaction monitoring was used as described in the Results and Discussion section.

For the quantitative analysis of *trans*-resveratrol, *trans*-resveratrol-3-sulfate, and *trans*-resveratrol-3-glucuronide, calibration curves were constructed by plotting the LC-MS-MS peak area ratio of *trans*-resveratrol or *trans*-resveratrol-3-sulfate to the internal standard naringenin (at 0.8 μM) against the analyte concentration. Note that no impurities and no *cis*-resveratrol were detected in the *trans*-resveratrol standard, which was used for all studies. The linear regression analyses of these standard curves showed a correlation coefficient of $r^2 = 0.999$. Because no resveratrol glucuronide standards were available, the concentration of *trans*-resveratrol-3-glucuronide was estimated using the *trans*-resveratrol standard curve.

RESULTS AND DISCUSSION

Human Microsomes and Hepatocytes

After incubation of *trans*-resveratrol with human liver microsomes, intact resveratrol but no metabolites were detected using LC-DAD-MS. However, resveratrol incubated with human hepatocytes for 4 h showed several new peaks with abundant deprotonated molecules of *m/z* 403 and 307. Therefore, LC-UV-MS-MS with product ion scanning (Fig. 2) and multiple reaction monitoring (Fig. 3) were performed for greater sensitivity and selectivity to confirm these peaks as metabolites of resveratrol. *trans*-Resveratrol was detected at a retention time of 30.8 min, and the corresponding product ion CID mass spectrum is shown in Fig. 2A.

Resveratrol sulfate has been reported in studies with perfused rat intestine and human liver (19,21,22). In our studies, resveratrol sulfate was detected in incubations with some human hepatocytes, as well as with rat hepatocytes and in mouse serum. For example, in the LC-UV-MS-MS analysis of a mouse serum extract (Fig. 2B), the ion of *m/z* 307 fragmented to form a product ion of *m/z* 227 that corresponded to resveratrol itself after the loss of sulfate. Furthermore, only one resveratrol monosulfate and no disulfate metabolites were

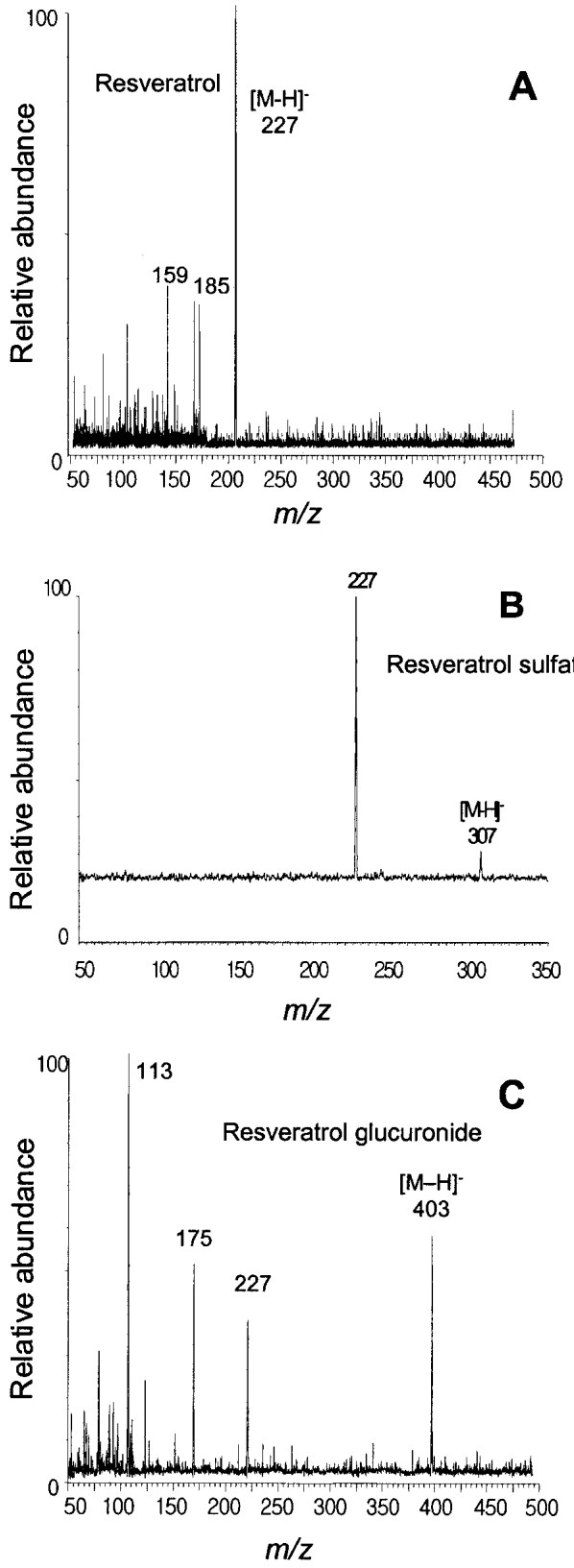


Fig. 2. LC-UV-MS-MS product ion mass spectra obtained using negative ion electrospray and CID during the analysis of resveratrol metabolites. (A) Resveratrol eluting at 30.8 min (extract of human hepatocyte incubation); (B) Resveratrol sulfate at 28.0 min retention time (mouse serum extract); (C) Resveratrol glucuronide at 18.1 min (human hepatocyte incubation)

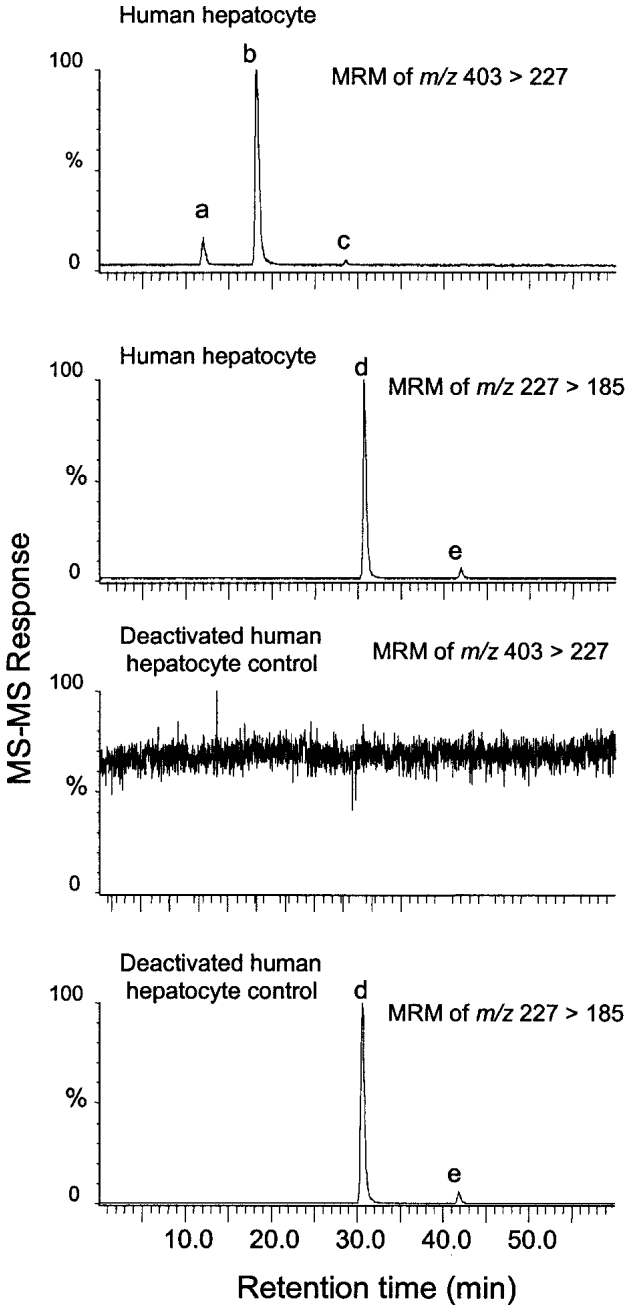


Fig. 3. Negative ion electrospray multiple reaction monitoring LC-UV-MS-MS analysis of resveratrol incubated for 4 h with human hepatocytes or deactivated human hepatocytes (control). Peaks were assigned to the following compounds. (a) *trans*-resveratrol-4′-*O*-glucuronide; (b) *trans*-resveratrol-3-*O*-glucuronide; (c) *cis*-resveratrol-3-*O*-glucuronide; (d) *trans*-resveratrol; (e) *cis*-resveratrol.

detected. This resveratrol sulfate isomer was synthesized and confirmed to be *trans*-resveratrol-3-sulfate.

LC-UV-MS-MS with multiple reaction monitoring of the transition of *m/z* 403 → 227 was performed and showed three resveratrol glucuronide peaks eluting at 12.1, 18.1, and 28.6 min (Fig. 3). LC-UV-MS-MS with MRM of an identical control containing deactivated human hepatocytes showed no signals for resveratrol glucuronide. Instead, only *trans*-resveratrol and a trace of the *cis*-isomer were detected at 30.8 and 42.0 min (Fig. 3), respectively. The ion of *m/z* 403 (Fig.

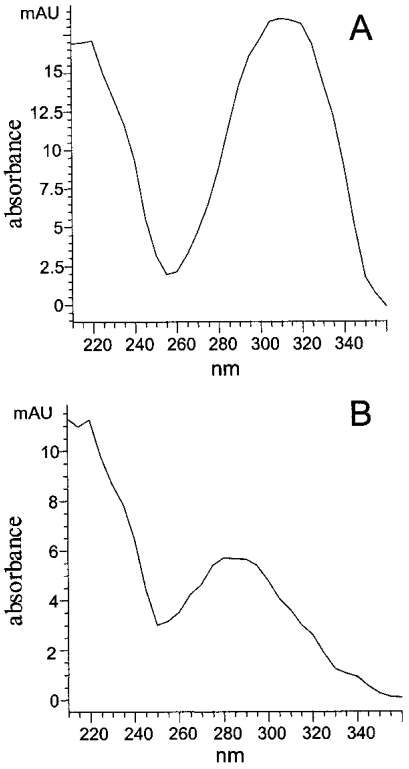


Fig. 4. UV spectra obtained during LC-DAD-MS of human hepatocytes for (A) *trans*-resveratrol conjugates, and (B) *cis*-resveratrol conjugates.

2C), which was detected at 18.1 min fragmented to form product ions of *m/z* 227 and *m/z* 175 that corresponded to resveratrol itself after the loss of glucuronic acid and dehydrated glucuronic acid, respectively. Also, a product ion of *m/z* 113 was observed, which can be considered characteristic of the presence of a glucuronic acid moiety. The ion of *m/z* 113 was formed by further dissociation of the fragment ion of *m/z* 175 and is common in the negative ion mass spectra of glucuronide metabolites (28,29). Therefore, the peak at 18.1 min was identified as resveratrol monoglucuronide. No peaks corresponding to a diglucuronide (mw = 580) were detected. For comparison, the negative ion MS-MS product ion spectrum of resveratrol (retention time 30.8 min) is shown in Fig. 2A. Note the absence of glucuronic acid ions of *m/z* 175 or 113 in the resveratrol tandem mass spectrum.

The use of a DAD in the LC-DAD-MS analysis allowed the confirmation of the identity of each chromatographic peak not only by its retention time but also by its spectrum. The UV/DAD spectra pattern obtained for *trans*-resveratrol and *cis*-resveratrol conjugates (Fig. 4) were consistent with those obtained for *trans*-resveratrol and *cis*-resveratrol standards that have been reported in other studies (30,31). It should be noted that *trans*-resveratrol produced the same UV/DAD spectrum whether conjugated at the 3- or 4′-position. Therefore, only one UV/DAD spectrum for these two glucuronides of *trans*-resveratrol is shown in Fig. 4. These spectra indicate that the conformations of the three resveratrol glucuronides detected at 12.1, 18.1, and 28.6 min in Fig. 3 are *trans*-, *trans*-, and *cis*-, respectively.

Because synthetic resveratrol-3-sulfate was retained longer than resveratrol-4′-sulfate during reversed-phase HPLC (13.1 and 11.6 min, respectively), it is probable that resveratrol-3-*O*-glucuronic acid will be retained longer than resveratrol-4′-*O*-glucuronide. The longer retention times of the conjugates at the C-3 position of the A ring are probably the result hydrogen bonding of the sulfate or glucuronic acid to the –OH group at C-5 of the A ring making the conjugate less polar than the corresponding conjugates at the C-4′ position. When the sulfate group or glucuronic acid is attached at the C-4′ of ring B, the two unconjugated phenolic OH groups on the A ring are fully exposed rendering the molecule more polar. As a result, resveratrol-4′-sulfate elutes earlier than resveratrol-3-sulfate. By analogy, *trans*-resveratrol-4′-*O*-glucuronide is probably the earlier eluting glucuronide peak at 12.1 min, and the glucuronide at 18.1 min is assigned as *trans*-resveratrol-3-*O*-glucuronide. The resveratrol glucuronide eluting at 28.6 min is assigned as *cis*-resveratrol-3-*O*-glucuronide by comparison of the retention times of *trans*-resveratrol, *cis*-resveratrol, and *trans*-resveratrol-3-*O*-glucuronide, respectively, and also considering steric effects. The structures of all of these compounds are shown in Fig. 1.

Because the amount of resveratrol sulfate was minor compared with the formation of the glucuronide in the incubations with human hepatocytes, resveratrol sulfate seems to be a minor human hepatic metabolite.

Table I. Summary of Resveratrol Metabolites Detected Using LC-UV-MS-MS

Experiments	Detected metabolites	Retention time
Human microsome control	None	—
Human microsome 1 h	None	—
Human hepatocytes control	None	—
Human hepatocytes 4 h	*trans*-resveratrol-4′-*O*-glucuronide	12.1 min
	trans-resveratrol-3-*O*-glucuronide	18.1 min
	cis-resveratrol-3-*O*-glucuronide	28.6 min
Rat urine control	None	—
Rat urine 2 h	*trans*-resveratrol-3-*O*-glucuronide	18.0 min
Rat hepatocytes control	None	—
Rat hepatocytes 4 h	*trans*-resveratrol-3-*O*-glucuronide	18.4 min
	trans-resveratrol-3-sulfate	32.9 min
Mouse serum control	None	—
Mouse serum gavage 15 min	*trans*-resveratrol-3-*O*-glucuronide	17.2 min
	trans-resveratrol-3-sulfate	30.1 min

Rat Urine and Hepatocytes

During LC-UV-MS-MS MRM analysis of rat urine samples, the transition *m/z* 403 → 227 was monitored for resveratrol glucuronide, *m/z* 307 → 227 for resveratrol sulfate, and *m/z* 227 → 185 for resveratrol. However, only one peak was detected at 18.0 min (data not shown) for the transition *m/z* 403 → 227. This metabolite was identified as *trans*-resveratrol-3-*O*-glucuronide and showed the same retention time as in the analysis of resveratrol metabolites from human hepatocytes. Analysis of rat hepatocytes was also performed using LC-UV-MS-MS with MRM. In this case, two major peaks were detected at 18.4 and 32.9 min (data not shown). Based on the mass chromatogram and by comparing the retention times, we have assigned these peaks as *trans*-resveratrol-3-*O*-glucuronide, and *trans*-resveratrol-3-sulfate, respectively. Resveratrol sulfate was the more abundant metabolite in rat hepatocytes. All of the resveratrol metabolites detected in these experiments are summarized in Table I.

Mouse Serum

Resveratrol was administered to mice at two different doses using IP injection or gavage, and serial blood samples were obtained various time points up to 4 h. Resveratrol glucuronide and resveratrol sulfate were detected as the only resveratrol metabolites in the mouse serum samples, and both of these metabolites were detected after IP or intragastric (IG) administration. The time courses for the appearance of resveratrol sulfate and resveratrol glucuronide in mouse serum following both routes of administration are shown in Fig. 5.

After the administration of 20 mg/kg, the maximum concentrations of both metabolites were observed in the serum samples obtained at the first time point of 15 min (Fig. 5A). In these samples, the concentration of resveratrol sulfate (13 μM) in the mouse serum was almost 3-fold greater than that of resveratrol glucuronide (5 μM). Only traces of unconjugated resveratrol were observed. Furthermore, no resveratrol or its metabolites were detected after 1 h (Fig. 5A).

To confirm the peak assigned to resveratrol sulfate and resveratrol glucuronide, aliquots of mouse serum samples were incubated with sulfatase and β-glucuronidase, respectively and then re-analyzed using LC-UV-MS-MS. As a result, the resveratrol sulfate peak disappeared whereas a resveratrol peak appeared in the sulfatase incubation (Fig. 6), and the resveratrol glucuronide peak disappeared whereas a resveratrol peak appeared in the β-glucuronidase incubation. The resveratrol peak intensity increased as the incubation time increased in both cases. Finally, these observations were confirmed using LC-DAD-MS with a UV photodiode array detector and mass spectrometric scanning in the range *m/z* 70–800. All of the UV/DAD spectra obtained were consistent with the data obtained in the studies of rat urine, rat hepatocytes, and human hepatocytes samples (Fig. 4). Furthermore, the mass spectra of these peaks were consistent with those observed in the other experiments. Resveratrol sulfate was not affected by the β-glucuronidase incubation and resveratrol glucuronide was not affected by sulfatase incubation and they were still observed eluting at approximately 30 and 17 min, respectively. Therefore, our data confirm the presence of resveratrol sulfate and resveratrol glucuronide in the mouse serum samples. Although the attachment site of glucuronide in resveratrol glucuronide is under investigation, it is reasonable to speculate that the conformations of major resveratrol metabolites in mouse serum are *trans*-resveratrol-3-sulfate and *trans*-resveratrol-3-*O*-glucuronide.

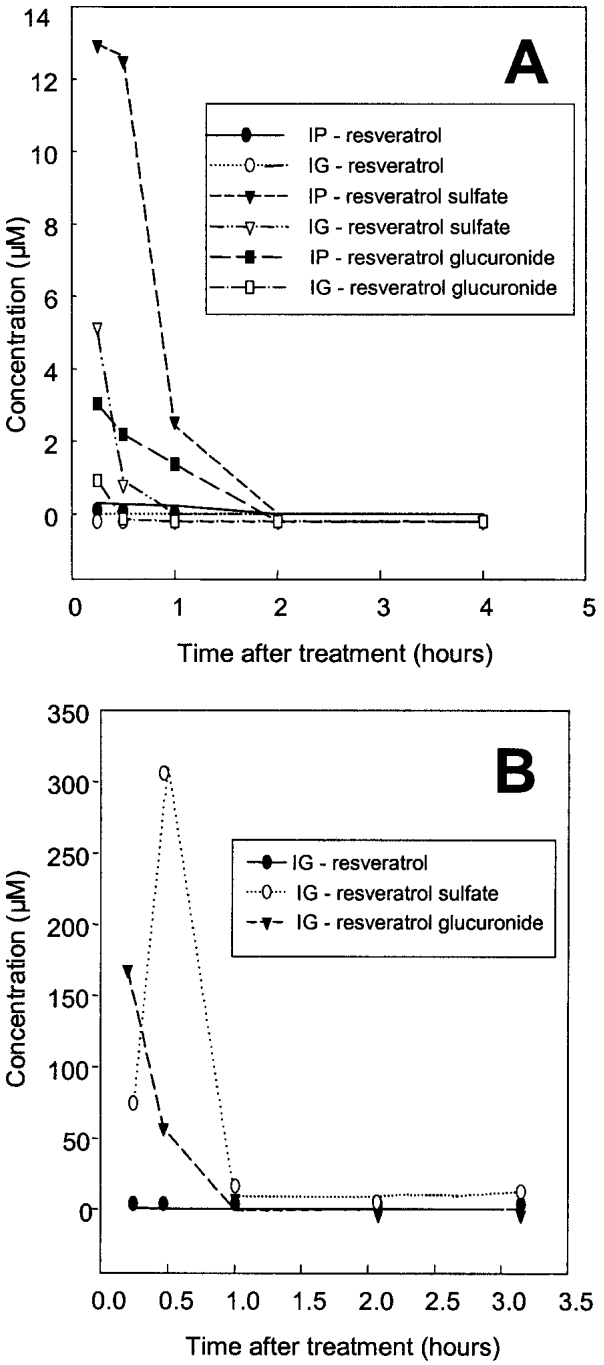


Fig. 5. Resveratrol metabolites in mouse serum following doses of (A) 20 mg/kg via IP injection or gavage (IG), and (B) 60 mg/kg via gavage (IG). Each data point represents the analysis of pooled blood samples from five control or five treated mice. Because no resveratrol or resveratrol conjugates were detected in the control serum, these data are not plotted.

In the second set of experiments, 60 mg/kg of resveratrol was given only via gavage because oral administration would be more relevant to future clinical investigations, and serum was collected serially up to 3 h. LC-DAD-MS and LC-UV-

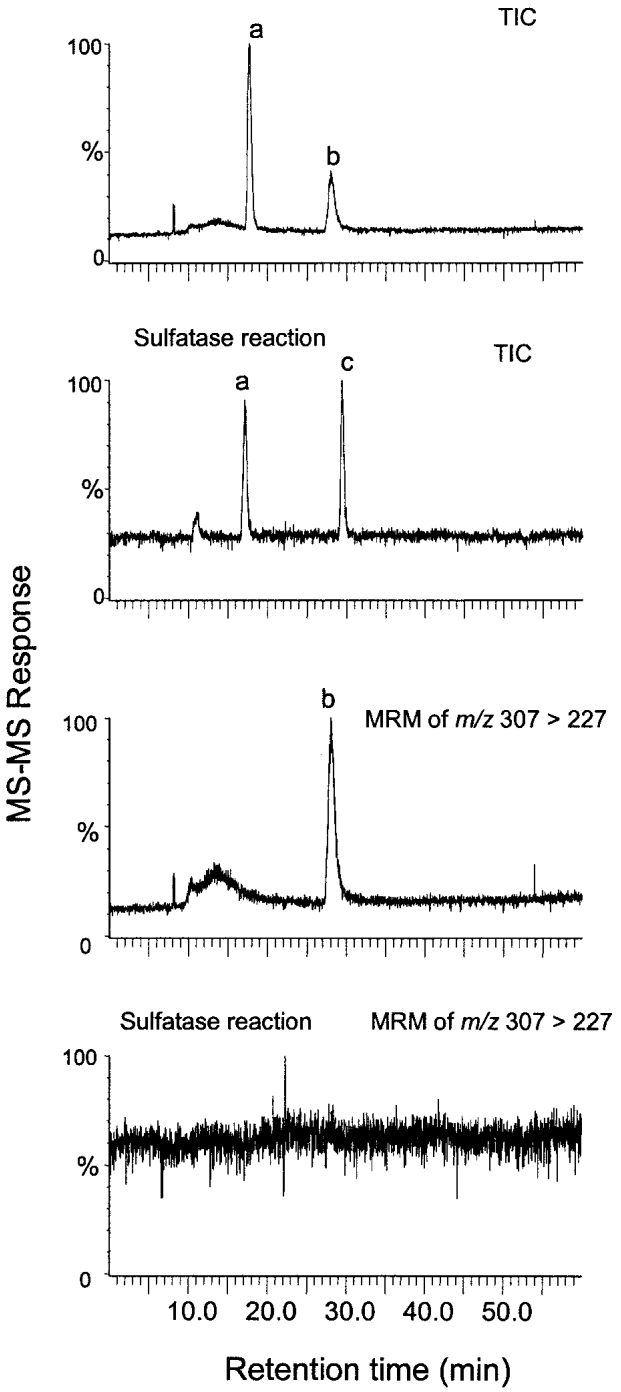


Fig. 6. LC-UV-MS-MS analysis of mouse serum collected after 15 min of administrating resveratrol via gavage and the same mouse serum sample incubated with sulfatase for 18.5 h. Peaks were assigned to the following compounds. (a) *trans*-resveratrol-3-*O*-glucuronide; (b) *trans*-resveratrol-3-sulfate; (c) *trans*-resveratrol.

MS-MS analysis were performed using the same conditions as described above. The same resveratrol sulfate and resveratrol glucuronide metabolites were detected in these analyses as were observed in the analyses of serum samples following administration of the lower dose of resveratrol. The resveratrol sulfate concentration reached a maximum value in mouse serum after 30 min instead of 15 min as observed for the lower dosage, probably because more time was required to absorb the large volume that was administered. Note that serum resveratrol disappeared after 30 min but that resveratrol sulfate and resveratrol glucuronide were still detectable 3 h after the highest dosage (Fig. 5B). The prolonged detection of low levels of resveratrol sulfate and resveratrol glucuronide in serum following the highest dose suggests that resveratrol was distributed to various tissues and was being cleared slowly. Perhaps only the highest dosage produced tissue levels that were high enough for subsequent detection of the cleared metabolites.

CONCLUSION

Although many studies have implicated a role for resveratrol in disease prevention, information on *in vivo* bioavailability and metabolism is incomplete and the structures of the resveratrol metabolites were uncertain. Our LC-DAD-MS and LC-UV-MS-MS data for the human, rat, and mouse experiments indicate that *trans*-resveratrol-3-*O*-glucuronide is the primary metabolite of resveratrol in human liver, and that *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate are both significant metabolites in rat urine, mouse serum, and formed by rat hepatocytes. It is important to note that no phase I metabolites of resveratrol such as oxidations, reductions or hydrolyzes were detected in any of these systems. Furthermore, conjugated resveratrol and not its free form were found to predominate in the circulation. These data suggest that the potential biologic activity of resveratrol conjugates should be considered in future investigations. Furthermore, the form of resveratrol in cells and tissues after oral or IP administration should be investigated to determine the levels of conjugated and unconjugated resveratrol at these potential points of action.

ACKNOWLEDGMENT

These studies were funded by grants R24 CA83124 and P01 CA48112 awarded by the National Cancer Institute.

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