Metabolism of *tert*-Butylhydroquinone to *S*-Substituted Conjugates in the Male Fischer 344 Rat

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tert-Butyl-4-hydroxyanisole (BHA) and its demethylated analog, *tert*-butyl-hydroquinone (TBHQ), are antioxidants used in food. Both BHA and TBHQ have been shown to promote kidney and bladder carcinogenesis in the rat. We have previously demonstrated that glutathione (GSH) conjugates of a variety of hydroquinones are nephrotoxic and proposed that GSH conjugation serves to target these compounds to the kidney. In the present study, we examined the metabolism of TBHQ, focusing on the formation of potentially nephrotoxic sulfur-containing metabolites. 2-*tert*-Butyl-5-glutathion-*S*-ylhydroquinone, 2-*tert*-butyl-6-glutathion-*S*-ylhydroquinone, and 2-*tert*-butyl-3,6-bisglutathion-*S*-ylhydroquinone were identified as biliary metabolites of TBHQ (1.0 mmol/kg, ip) in male F344 rats, accounting for 2.2% of the dose. Liquid chromatography/mass spectroscopic analysis of urine also revealed the presence of additional sulfur-containing metabolites, tentatively identified as 2,5-dihydroxy-3-*tert*-butylthiophenol, 2,5-dihydroxy-4-*tert*-butylthiophenol, and their *S*-methyl derivatives. No mercapturic acids of TBHQ were found in the urine. The major biliary and urinary metabolites were TBHQ−glucuronide and TBHQ−sulfate, with a trace of TBHQ excreted unchanged. The results indicate that TBHQ undergoes oxidation and GSH conjugation *in vivo* in the male F344 rat. These conjugates are excreted into bile and undergo further metabolism prior to excretion in urine. Formation of the *S*-containing metabolites of TBHQ may occur in amounts sufficient to play a role in the toxicity of TBHQ to kidney and bladder.

Introduction

tert-Butyl-4-hydroxyanisole (BHA)[1] and its demethylated analog, *tert*-butylhydroquinone (TBHQ), are two of the few antioxidants permitted for use in foods (*1*). Both compounds appear to lack genotoxicity, but dietary administration of BHA results in papilloma and carcinoma formation in the forestomach of rats, mice, and hamsters (reviewed in ref 2). BHA also enhances the development of preneoplastic and neoplastic lesions in rat kidney and urinary bladder (*3*), while it inhibits the development of hepatocellular carcinomas.

Both BHA and its metabolites have been implicated in the toxicity of BHA in various organs (*4*). TBHQ is known to induce cell proliferation in renal and urinary bladder epithelia in the rat (*5*). In various mammalian species, including humans, BHA is excreted in urine mainly as its glucuronide and sulfate conjugates (*6*, *7*). A portion of BHA undergoes *O*-demethylation to TBHQ. Verhagen and Kleinjans (*8*) identified free TBHQ in the bile of rats following oral administration of BHA. In humans (0.5 mg/kg body weight) and in rats (200 mg/kg body weight), 9% of a single dose of BHA is excreted in urine as glucuronide and sulfate conjugates of TBHQ (*9*). The metabolic fate of TBHQ has also been studied in a number of species. In rats, dogs, and humans, most of an orally administered dose of TBHQ is rapidly absorbed and essentially quantitatively excreted in urine within 48 h, the major metabolite (accounting for between 50 and 80% of the recovered dose) being the 4-*O*-sulfate. Smaller amounts of the 4-*O*-glucuronide conjugate were also found in these species (*10*).

Rat and human cytochrome P450's (*11*) and peroxidases (*12*) catalyze both the *O*-demethylation of BHA to TBHQ and the subsequent oxidation to the corresponding quinone (TBQ). The reactive nature of TBQ may be important in the carcinogenic activity of BHA (reviewed in ref 2). Metabolites of BHA, including TBHQ, TBQ, 3-*tert*-butyl-4,5-dihydroxyanisole, and 3-*tert*-butyl-4,5-quinone, have been shown to induce DNA damage in the forestomach epithelium of rats after oral administration (*13*). Interestingly, depletion of reduced glutathione (GSH) inhibited the proliferative response to BHA, suggesting that GSH may be involved in the carcinogenic process (*14*, *15*).

GSH conjugates have been found as *in vitro* metabolites of TBHQ, and *S*-methyl metabolites were detected in the urine of rats after the administration of BHA or TBHQ (*16*). However, GSH conjugates have yet to be identified as *in vivo* metabolites of BHA or TBHQ. The

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[1] Abbreviations: GSH, glutathione; BHA, *tert*-butyl-4-hydroxyanisole; TBHQ, *tert*-butylhydroquinone; 5-(GSyl)TBHQ, 2-*tert*-butyl-5-glutathion-*S*-ylhydroquinone; 6-(GSyl)TBHQ, 2-*tert*-butyl-6-glutathion-*S*-ylhydroquinone; 3,6-bis-(GSyl)TBHQ, 2-*tert*-butyl-3,6-bisglutathion-*S*-ylhydroquinone; TBQ, *tert*-butyl-1,4-benzoquinone; HQ, hydroquinone; BrHQ, bromohydroquinone, EC, electrochemical; LC/MS, liquid chromatography/mass spectroscopy; CID, collision-induced dissociation; γ-GT, γ-glutamyl transpeptidase.

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relevance of this metabolic pathway is exemplified by the finding that the nephrotoxicity of hydroquinone (HQ) and bromohydroquinone (BrHQ) is mediated by conjugation of the corresponding quinone with GSH (*17*). Administration of 2-Br-bis-(GSyl)HQ and 2,3,5-tris-(GSyl)HQ to rats by tail vein injection indicated that these metabolites were much more potent nephrotoxicants than the parent compounds (*18*−*20*). Similarly, *p*-aminophenol, a nephrotoxic metabolite of acetaminophen, is metabolized in the rat to 4-amino-2,5-bis-(GSyl)phenol, 4-amino-2,3,5-tris-(GSyl)phenol, and 4-amino-2,3,6-tris-(GSyl)phenol (*21*). Administration of 4-amino-2-(GSyl)phenol produced nephrotoxicity at doses that were 3−4 times lower than those for *p*-aminophenol (*22*). Because the selective nephrotoxicity and nephrocarcinogenicity of quinones/quinonimines may be a consequence of their conjugation with GSH, we investigated the metabolism of TBHQ, a metabolite of BHA in humans, and a known urinary tract tumor promoter, to potentially nephrotoxic thioethers.

Materials and Methods

Caution: *TBHQ is toxic in kidney and bladder in rats and must be handled with care. In addition, the synthetic thioethers are potent nephrotoxicants and bladder toxicants in the rat. All of these compounds therefore should be handled with protective clothing in a well-ventilated fume hood.*

Chemicals. BHA (Fluka Chemical AG, Buchs, Switzerland), TBHQ (Aldrich Chemical Co., Milwaukee, WI), GSH, γ-glutamyl transpeptidase (γ-GT), aryl sulfatase (Sigma Chemical Co., St. Louis, MO), and β-glucuronidase/aryl sulfatase (Boehringer-Mannheim, Indianapolis, IN) were purchased commercially. All other reagents were of the highest grade available.

Synthesis of GSH Conjugates. 2-*tert*-Butyl-5-glutathion-*S*-ylhydroquinone [5-(GSyl)TBHQ], 2-*tert*-butyl-6-glutathion-*S*-ylhydroquinone [6-(GSyl)TBHQ], and 2-*tert*-butyl-3,6-bis-glutathion-*S*-ylhydroquinone [3,6-bis-(GSyl)TBHQ] were synthesized as previously described (*23*). In short, TBQ was obtained by the oxidation of TBHQ with potassium bromate in 1 N sulfuric acid. An aqueous solution of 0.8 mmol of GSH was added to a solution of 0.8 mmol of TBQ in methanol. The reaction mixture was stirred for 22 h under N_2. After reduction of the reaction mixture with ascorbic acid, the conjugates formed were purified by preparative HPLC using a Zorbax 22 × 250 mm RP18 column and elution with 55% water containing 0.5% formic acid and 45% methanol at a flow rate of 5 mL/min. Structural characterization of the products was performed by fast atom bombardment mass spectrometry, with positive ion detection (Finigan-Matt HSQ-30, BEQQ geometry), and proton NMR (Varian Unity-400).

Identification of *tert*-Butylhydroquinone Thioethers by NMR. The ^{1}H NMR spectra for 5- and 6-(GSyl)TBHQ were described previously (*23*). The ^{13}C spectrum of 60 mg of 3,6-bis-(GSyl)TBHQ in 0.7 mL of D_2O was recorded over a period of 36 h on a Varian Unity 400. The positions of the two GSH moieties were assigned based on the basis of the observed two- and three-band couplings. Resonances were recorded at 152.1 (C−O, with $^2J_{[C-CH]}$ coupling of 3.5 Hz), 151.7 (C−O, with $^3J_{[C-C-H]}$ coupling of 10.1 Hz), 141.1 (C-butyl), 124.2 (C−S, with ca. 4 Hz triplet from $^3J_{[CH_2-S-C]}$ coupling of 6.9 Hz), 120.4 (C−S, with ca. 4 Hz triplet from $^3J_{[CH_2-S-C]}$ coupling of 6.9 Hz and $^3J_{[C-C-C-H]}$ coupling of 6.9 Hz), 117.6 (C−H), and 35.8 ppm (C_{2butyl}).

Animals and Dosing. Male Fischer 344 (160−200 g) rats were obtained from Harlan Sprague−Dawley (Houston, TX) and allowed food and water *ad libitum* prior to and during the experiments. Rats received a single ip dose of 1.0 mmol/kg TBHQ in 0.15 mL of ethanol, which was the maximum dose that was not lethal in these studies. Control rats for each group received dosing vehicle only. For the collection of urine, rats were housed individually in metabolism cages, and urine was collected over dry ice and in the dark for 19 h. Urinary metabolites of TBHQ were determined by HPLC, as described below.

Surgical Technique. Rats were anesthetized with equithesin (a mixture of 35 mg/kg pentobarbital and 140 mg/kg chloral hydrate, 3.5 mL/kg in ethanol, ip) and maintained under anesthesia with equithesin (20 mg/kg sodium pentobarbital and 80 mg/kg chlorate hydrate, im) administered every hour after the surgery. The abdomen was sterilized, an incision was made, and the bile duct was cannulated with PE-10 tubing. After the surgery was complete, 1.0 mmol/kg TBHQ was administered by intraperitoneal injection. Bile samples were collected at hourly intervals, on ice, for a period of 4 h and analyzed by HPLC with both UV and EC detection, as described below.

HPLC−Electrochemical Analysis of TBHQ−GSH Conjugates. The metabolism of TBHQ was assessed by HPLC (Shimadzu LC-10AS) coupled to UV (280 nm) (Shimadzu SPD-10A) and electrochemical (EC) detectors (ESA Coulochem, Model 5100A). The EC detector was equipped with two porous graphite test electrodes connected in series (detector 1 at −0.20 V and detector 2 at +0.30 V) with a glassy carbon reference electrode. Aliquots (10 μL of a 1:40 dilution of urine and bile samples with mobile phase) were injected onto a Beckman Ultrasphere 5 μm ODS reverse-phase analytical column (4.6 × 150 mm), and each sample was eluted with a mixture of 80% citric acid (4 mM)/ammonium acetate (8 mM) and 20% methanol (pH 4.0) containing 20 mg/L EDTA, at a flow rate of 1 mL/min. Under these conditions, authentic samples of 5-(GSyl)THBQ, 6-(GSyl)TBHQ, 3,6-bis-(GSyl)TBHQ, and TBHQ were eluted with retention times of 36.9, 60.9, 12.3, and 56.0 min, respectively. Thioethers of TBHQ were identified by (i) comparison of HPLC retention times with those of authentic standards, (ii) their electrochemical properties upon HPLC-EC analysis, and (iii) mass spectroscopic analysis (described below). TBHQ thioethers were quantified by HPLC−EC using Shimadzu EZChrom software. Standard curves were prepared from each authentic standard, and quantitation was achieved by linear regression analysis (*24*).

Characterization of *tert*-Butylhydroquinone Conjugates in Bile and Urine. TBHQ−glucuronide, TBHQ−sulfate, and TBHQ−GSH conjugates in urine and bile were identified by analyzing samples after β-glucuronidase/aryl sulfatase, aryl sulfatase, or γ-GT hydrolysis. The commercial β-glucuronidase/aryl sulfatase preparation was purified prior to use by gel filtration (Sephadex G25M, Pharmacia) and elution with 0.1 M sodium acetate buffer (pH 5.0). Aliquots (50 μL) of bile or urine were diluted to 1 mL with either (a) 150 μL of β-glucuronidase (4.5 IU/mL, containing 14 IU/mL aryl sulfatase) in 0.1 M sodium acetate buffer pH 5.0 and incubated at 37 °C for 16 h, (b) 50 μL of aryl sulfatase (14.5 IU/mL) in 0.01 M Tris buffer (pH 7.0) and incubated at 37 °C for 16 h, or (c) γ-GT (4 IU/mL) in 20 mM HEPES containing 1.15% KCl (pH 7.4) at 37 °C for 1 h. Control incubations were performed by omitting either urine, bile, or enzyme from the incubation mixtures. Samples were centrifuged (11000*g*, 15 min, Beckman, Microfuge G), and 10 μL aliquots of the supernatant in a 1:20 dilution with the mobile phase were analyzed by HPLC−UV−EC.

Identification of *tert*-Butylhydroquinone Thioethers by Liquid Chromatography/Mass Spectroscopy. Confirmation of the HPLC−EC data was achieved using liquid chromatography (LC)/ion spray mass spectroscopy (IS-MS) and LC/MS/MS analysis. Mono- and bis-substituted GSH conjugates of TBHQ were analyzed in both positive and negative ion modes by loop injection and following liquid chromatographic separation using a Sciex API III mass spectrometer with an ion spray interface coupled to a Hitachi (L6200, 6000) liquid chromatograph. The loop injection eluent contained 0.1 M ammonium acetate/methanol adjusted to pH 5.0 with acetic acid (50:50) at a flow rate of 60 μL/min. The HPLC eluent contained 0.01 M ammonium acetate/methanol (pH 4.0 adjusted with acetic acid) (75:25) at a flow rate of 1 mL/min, with a split of 50 μL/min to the mass spectrometer through a Beckman Ultrasphere ODS HPLC column (4.6 × 150 mm). For collision-induced dissocia-

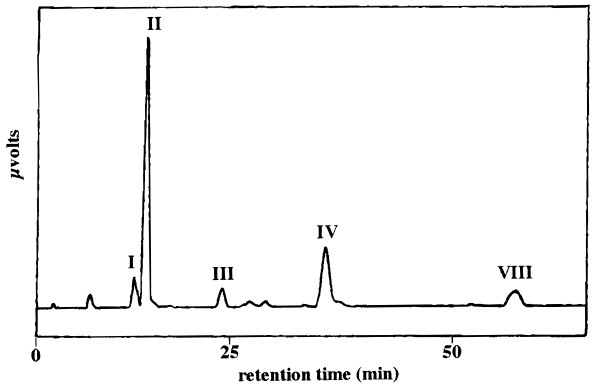


Figure 1. Biliary excretion of *tert*-butylhydroquinone and *tert*-butylhydroquinone metabolites in a male Fischer rat, 30−60 min after a single *ip* dose of 1.0 mmol/kg *tert*-butylhydroquinone. HPLC−UV and HPLC−EC analysis used an RP18, 25 × 4.6 mm id column and elution with 20% methanol/80% citric acid (4 mM)/ammonium acetate (8 mM) containing 20 mg/L EDTA (pH 4.0) at 1 mL/min. UV detection was monitored at 280 nm (not shown); EC detection was monitored at −0.20 (detector 1) and +0.30 V (detector 2). Individual peaks represent the following: (I) 3,6-bis(glutathion-*S*-yl)-*tert*-butylhydroquinone, 12.3 min; (II) *tert*-butylhydroquinone−glucuronide, 13.6 min; (III) *tert*-butylhydroquinone−sulfate, 23.5 min; (IV) 5-(glutathion-*S*-yl)-*tert*-butylhydroquinone, 36.9 min; (VIII) 6-(glutathion-*S*-yl)-*tert*-butylhydroquinone, 60.9 min.

tion (CID) spectral analysis, the Sciex ion spray voltage was optimized at 4800 V, with a collision energy of 90 (for positive ion CID analysis) or 125 V (for negative ion CID analysis). The collision gas thickness was 400×10^{12} molecules/cm^2. Aliquots (1 μL) of each synthetic standard (approximately 1 mg/mL concentration) were injected directly into the ion source for full-scan LC/MS and CID analyses. Aliquots (95−100 μL) of urine or bile were analyzed under the HPLC conditions described earlier. In selected cases, control bile or urine was spiked with authentic synthetic standard for comparison of LC/MS/MS spectra and HPLC retention time with those components observed in the biological samples.

Statistics. All data are expressed as mean ± SD. Statistical comparisons between two groups was made by a two-tailed Student's *t*-test with significance defined as $p < 0.05$. Multigroup studies were analyzed by ANOVA followed by Student Newman-Kuel's test.

Results

Identification of *tert*-Butylhydroquinone Thioethers as *in Vivo* Metabolites of *tert*-Butylhydroquinone by HPLC−UV−EC. The extent to which TBHQ (1.0 mmol/kg) undergoes oxidation and subsequent conjugation with GSH *in vivo* was examined in bile duct-cannulated rats. Preliminary identification of 5-(GSyl)TBHQ, 6-(GSyl)TBHQ, and 3,6-bis-(GSyl)TBHQ was achieved by HPLC utilizing UV and EC detection (Figure 1). This technique identified the presence of five major electroactive compounds in the bile of TBHQ-treated rats, none of which were present in bile collected prior to TBHQ administration. The compounds eluting at 12.3, 36.9, and 60.9 min corresponded in retention time with authentic 3,6-bis-(GSyl)TBHQ, 5-(GSyl)TBHQ, and 6-(GSyl)TBHQ, respectively. These were further characterized as GSH conjugates by their disappearance upon incubation with γ-GT (data not shown). Confirmation of the TBHQ−GSH conjugates was achieved by LC/MS as described below. A trace amount of TBHQ eluting at 56.0 min was also detected. Several other electroactive metabolites were excreted in the bile at later times (>1 h after 1.0 mmol/kg TBHQ, ip) and their identities are

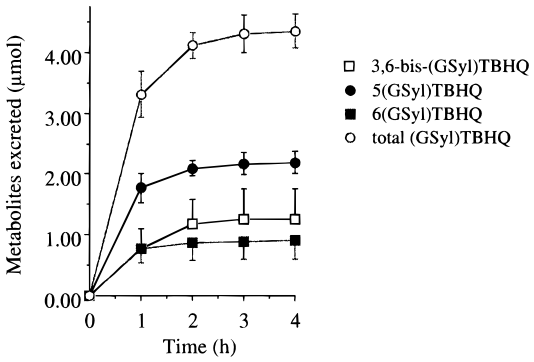


Figure 2. Cumulative biliary excretion of 2-*tert*-butylglutathion-*S*-ylhydroquinones in male F344 rats, 0−4 h after a dose of 1.0 mmol/kg *tert*-butylhydroquinone ip.

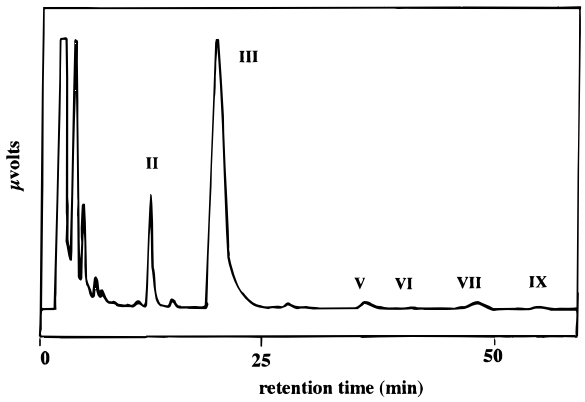


Figure 3. Urinary excretion of *tert*-butylhydroquinone and *tert*-butylhydroquinone metabolites in male F344 rats, 19 h after a single ip dose of 1.0 mmol/kg *tert*-butylhydroquinone. HPLC−UV and HPLC−EC analysis used an RP18, 25 cm × 4.6 mm id column and elution with 20% methanol/80% citric acid (4 mM)/ammonium acetate (8 mM) containing 20 mg/L EDTA (pH 4.0) at 1 mL/min. UV detection was monitored at 280 nm (not shown); EC detection was monitored at −0.20 (detector 1) and +0.30 V (detector 2). Individual peaks represent the following: (II) *tert*-butylhydroquinone−glucuronide, 12.1 min; (III) *tert*-butylhydroquinone−sulfate, 19.5 min. By using LC/MS, other unknowns were tentatively identified as (V) 2,5-dihydroxy-4-*tert*-butylthiophenol, 36.3 min; (VI) 2,5-dihydroxy-*tert*-butylmethylthiophenol, 41.2 min; (VII) *tert*-butylhydroquinone, 48.8 min; and (IX) 2,5-dihydroxy-3-*tert*-butylthiophenol, 55.6 min.

unknown. In addition, TBHQ−glucuronide (13.6 min) and TBHQ−sulfate (23.5 min) were identified by HPLC−UV−EC and enzymatic methodology. These metabolites selectively disappeared upon incubation with either β-glucuronidase and/or aryl sulfatase, with a concomitant increase in the recovery of TBHQ (data not shown). TBHQ−glucuronide was the major metabolite in bile, while a smaller amount of TBHQ−sulfate was present. Quantitation of metabolites excreted in bile showed that the major GSH conjugate formed was 5-(GSyl)TBHQ (1.11% of dose 0−4 h), with lesser amounts of 3,6-bis-(GSyl)TBHQ (0.65% of dose 0−4 h) and 6-(GSyl)TBHQ (0.46% of dose 0−4 h) formed. In total, GSH conjugates accounted for 2.22% of the dose. The biliary excretion of TBHQ−GSH conjugates was essentially complete by 4 h (Figure 2).

Analysis of rat urine by HPLC−UV and HPLC−EC (Figure 3) 19 h after TBHQ (1.0 mmol/kg, ip) injection showed the presence of at least six electroactive metabolites that were absent from urine collected prior to TBHQ administration. Metabolites identified in urine by enzymatic methodology were TBHQ−glucuronide (12.1 min) and TBHQ−sulfate (19.5 min), the latter being the major metabolite. A trace of TBHQ (48.8 min) was

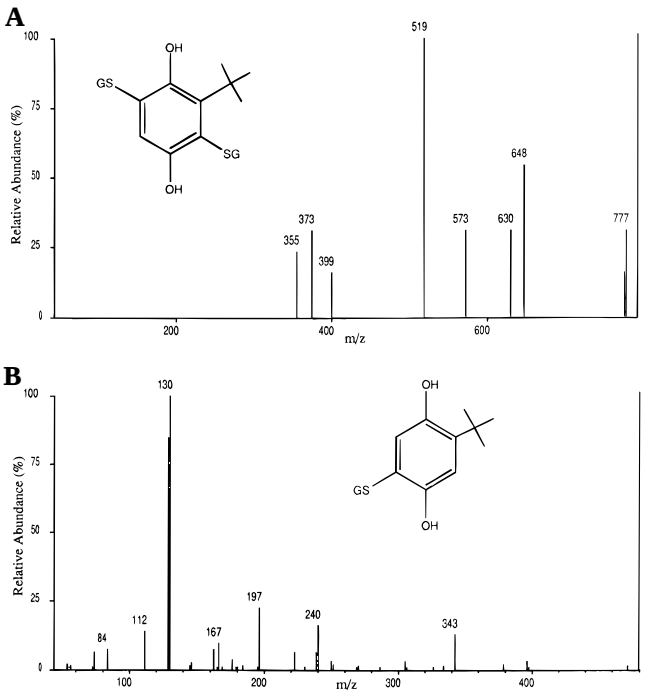


Figure 4. Collisionally induced daughter ion spectrum obtained by on-line LC/MS/MS analysis of authentic standards of (A) 3,6-bis(glutathion-*S*-yl)-*tert*-butylhydroquinone (*m*/*z* 777; positive ion) and (B) 5-(glutathion-*S*-yl)-*tert*-butylhydroquinone (*m*/*z* 472; positive ion).

excreted in the urine unchanged. Additional urinary metabolites, some of which were also seen in the bile, eluted at 27.7, 29.8, 36.3, 41.2, and 55.9 min. These metabolites did not coelute with any of the available standards and therefore were tentatively identified by LC/MS as described below.

Identification of *tert*-Butylhydroquinone Thioethers as *in Vivo* Metabolites of *tert*-Butylhydroquinone by LC/MS. Structural elucidation of the TBHQ metabolites observed in bile and urine were confirmed by ion spray mass spectrometry under CID conditions. The CID fragmentation product spectrum obtained from authentic 3,6-bis-(GSyl)TBHQ [$(M + H)^+$ *m*/*z* 777] spiked into control rat bile is shown in Figure 4A. Product ions at *m*/*z* 648 and 519 were consistent with fragmentation via loss of glutamic acid (one or two molecules, respectively). The ion spray CID mass spectra of authentic 5-(GSyl)TBHQ (Figure 4B) and 6-(GSyl)-TBHQ (not shown) produced identical molecular ions at *m*/*z* 472, with fragmentation characteristic of the loss of glycine (M − 75) and glutamic acid (M − 129). The glutamic acid fragment at *m*/*z* 130 was the base peak in the spectra of both regioisomers; however, the compounds were distinguished by the relative intensity of the *m*/*z* 223 fragment ion (stronger for the 6-isomer than for the 5-isomer).

The confirmatory CID analysis ion trace for the presence of 5-(GSyl)TBHQ, 6-(GSyl)TBHQ, and 3,6-bis-(GSyl)-TBHQ in rat bile following the administration of TBHQ (1.0 mmol/kg, ip) was achieved by comparison with the spectra of rat bile spiked with authentic synthetic standards. By using multiple reaction monitoring, signals corresponding to the molecular ions at *m*/*z* 472, which produced product ions at *m*/*z* 130 characteristic of 5-(GSyl)TBHQ and 6-(GSyl)TBHQ, were observed in rat bile samples (Figure 5). Relative retention times for 5-(GSyl)TBHQ and 6-(GSyl)TBHQ were 29.2 and 47.4

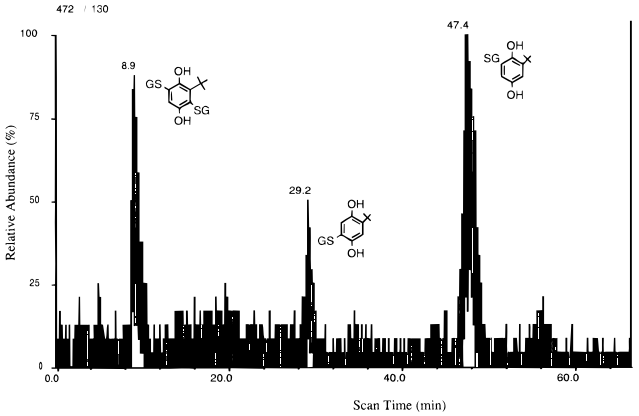


Figure 5. Reconstructed ion chromatogram depicting the reaction of *m*/*z* 472, identified in rat bile, to *m*/*z* 130. The ion signals observed produced characteristic fragmentation in the CID mode corresponding to 3,6-bis(glutathion-*S*-yl)-*tert*-butylhydroquinone (8.9 min), 5-(glutathion-*S*-yl)-*tert*-butylhydroquinone (29.2 min), and 6-(glutathion-*S*-yl)-*tert*-butylhydroquinone (47.4 min).

min, respectively. The ion signal observed at 8.9 min corresponded to 3,6-bis-(GSyl)TBHQ, which produced characteristic fragmentation in the CID mode (not shown).

Additional metabolites were observed in rat urine following TBHQ administration (1.0 mmol/kg) by using ion spray LC/MS and CID analyses (Figure 6). The metabolite at HPLC retention time 22.6 min was tentatively identified as 2,5-dihydroxy-4-*tert*-butylthiophenol by full-scan LC/MS analysis. A molecular ion was observed at *m*/*z* 197 in the negative ion mode. The full-scan negative ion LC/MS spectrum of the component eluting at retention time 53.4 min also presented a molecular ion at *m*/*z* 197. It is likely that this component corresponds to 2,5-dihydroxy-3-*tert*-butylthiophenol on the basis of the relative HPLC retention times of the related regioisomeric GSH conjugates [5-(GSyl)TBHQ eluted earlier than the 6-(GSyl)TBHQ isomer]. The negative full-scan ion spray mass spectrum indicated a putative methylthioconjugate of TBHQ (HPLC retention time = 26.7 min), with a molecular ion observed at *m*/*z* 211. The corresponding positive ion ion spray mass spectrum showed a molecular ion at *m*/*z* 213. Figure 6A displays the single negative ion reconstructed ion chromatograms for the urinary metabolites 2,5-dihydroxy-4-*tert*-butylthiophenol and 2,5-dihydroxy-3-*tert*-butylthiophenol and the corresponding *S*-methyl conjugate. The ion chromatogram signal was very weak for the molecular ion corresponding to the *S*-methyl conjugate at *m*/*z* 213; the positive ion chromatogram is shown for comparison in Figure 6B. LC/MS did not identify any mercapturic acids of TBHQ.

Both TBHQ−glucuronide and TBHQ−sulfate conjugates were also detected in rat urine after 1.0 mmol/kg TBHQ injection by negative ion ion spray LC/MS analysis. The negative ion ion spray spectrum corresponding to TBHQ−glucuronide (not shown), with an HPLC retention time of 13.6 min, exhibits a molecular ion species at *m*/*z* 341 ($[M - H]^-$) and a doubly charged ion species at *m*/*z* 683 ($[2M - H]^{2-}$). Characteristic product ions were observed at *m*/*z* 165 and 175, indicating the presence of TBHQ and glucuronic acid, respectively. The negative ion spray mass spectrum of TBHQ−sulfate (not shown), with a retention time of 23.5 min, showed a molecular ion species at *m*/*z* 245, with facile loss of SO_3 to produce a fragmentation ion at *m*/*z* 165. These fragmentation

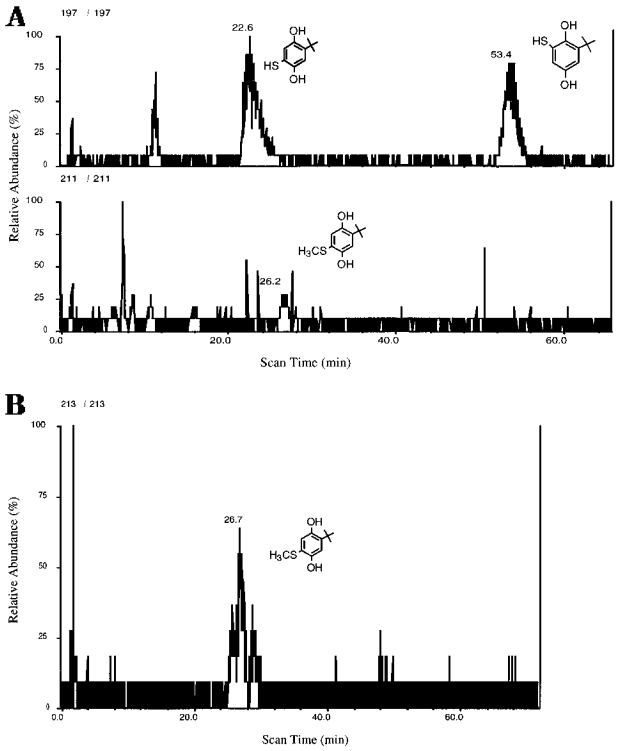


Figure 6. Reconstructed ion chromatograms of (A) *m*/*z* 197 and 211 obtained from rat urine by negative ion ion spray LC/MS analysis. The negative ion ion spray (IS) mass spectra were consistent with the presence of 2,5-dihydroxy-4-*tert*-butylthiophenol (22.6 min), 2,5-dihydroxy-3-*tert*-butylthiophenol (53.4 min), and 2,5-dihydroxy-*tert*-butylmethylthiophenol (26.2 min). (B) Reconstructed ion chromatograms of *m*/*z* 213, obtained from rat urine, by positive ion IS LC/MS analysis. The positive ion IS mass spectrum was consistent with the presence of 2,5-dihydroxy-*tert*-butylmethylthiophenol (26.7 min).

pathways were also observed in rat bile, indicating the presence of these conjugates in bile.

Discussion

In the present article, we report the identification of 5-(GSyl)TBHQ, 6-(GSyl)TBHQ, and 3,6-bis-(GSyl)TBHQ as *in vivo* metabolites of TBHQ. Tajima *et al.* (*17*) identified 5-(GSyl)TBHQ and 6-(GSyl)TBHQ as metabolites of TBHQ in microsomal incubations, but the formation of 3,6-bis-(GSyl)TBHQ as a metabolite of TBHQ has not previously been reported. GSH conjugates are formed from TBHQ after its oxidation to the corresponding quinone, followed by direct addition of GSH. The reduced TBHQ−GSH conjugate is still redox-active and undergoes a second oxidation and a second conjugation with GSH to yield 3,6-bis-(GSyl)TBHQ. Addition of the first GSH molecule occurs preferentially at the less sterically hindered 5- and 6-positions, while addition of the second molecule apparently only occurs *para* to the first GSH group to give 3,6-bis-(GSyl)TBHQ. The proposed pathway by which TBHQ−GSH conjugates are formed is presented in Figure 7. We have previously demonstrated the formation of 2,3-bis-(GSyl)HQ, 2,5-bis-(GSyl)HQ, 2,6-bis-(GSyl)HQ, and 2,3,5-tris-(GSyl)HQ as biliary metabolites of HQ in rats (*25*). Moreover, the relative nephrotoxicity of the conjugates increased with an increase in the degree of GSH substitution of the benzene ring. If such a relationship extended to the metabolism of TBHQ, the 3,6-bis-(GSyl)TBHQ conjugate could represent a potentially nephrotoxic metabolite of TBHQ, which may be of relevance to the ability of BHA and TBHQ to promote urinary tract tumors.

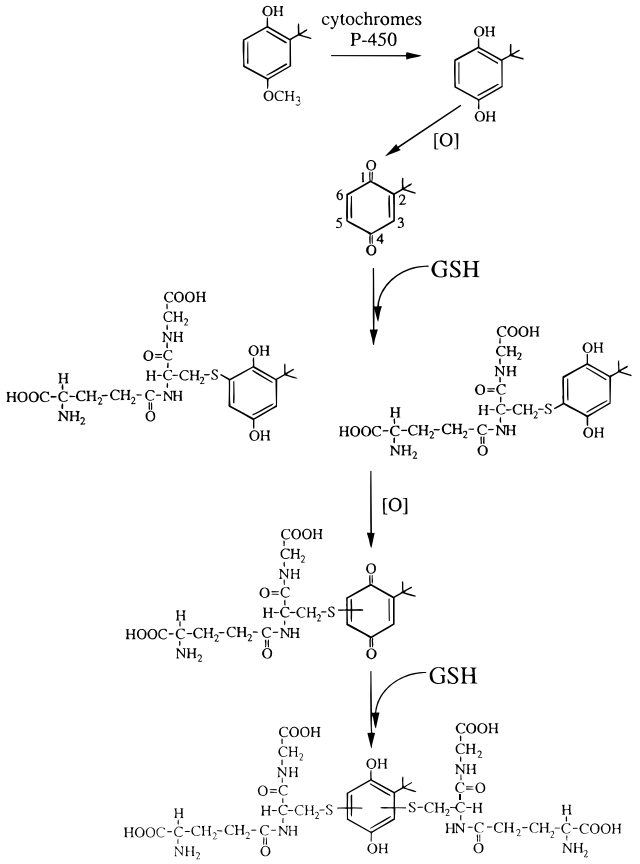
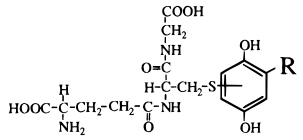


Figure 7. Metabolism of *tert*-butylhydroxyanisole to potentially nephrotoxic quinone thioethers.

Although 5-(GSyl)TBHQ, 6-(GSyl)TBHQ, and 3,6-bis-(GSyl)TBHQ are quantitatively minor metabolites of TBHQ (2.2% of dose), similar metabolites of 2-BrHQ (*18*, *19*) and HQ (*20*) are known to be very potent nephrotoxicants. For example, 2,3,5-tris-(GSyl)HQ causes proximal tubular necrosis at a dose of 7.5 μmol/kg (*26*). Following the administration of HQ to rats (1.8 mmol/kg, ip), 4% of the dose was recovered in bile, within 4 h, as GSH conjugates (*25*, *27*). Thus, HQ thioethers are formed in amounts sufficient to contribute to the acute and chronic effects of HQ on the kidney. Consistent with our finding of both 5-(GSyl)TBHQ and 6-(GSyl)TBHQ isomers in rat bile, Tajima *et al.* (*17*) reported the presence of 2-*tert*-butyl-5-(methylthio)hydroquinone and 2-*tert*-butyl-6-(methylthio)hydroquinone in the urine of Wistar rats after ip administration of BHA or TBHQ. However, LC/MS analysis of Fischer 344 rat urine only demonstrated the presence of one thiomethyl metabolite, perhaps because the two isomers coelute under the LC conditions used. LC/MS analysis also indicated the presence of two additional sulfur-containing urinary metabolites, 3- and 4-*tert*-butyl-2,5-dihydroxythiophenol, which were not previously reported. The combined activity of γ-GT and dipeptidase converts GSH conjugates to the corresponding cysteine conjugates, which can be converted either to (i) mercapturic acids by *N*-acetyltransferases and excreted in urine (*28*) or (ii) to thiols by cysteine conjugate β-lyases. The free thiols may be excreted or undergo further metabolism by thiol *S*-methyltransferases to form thiomethyl metabolites (*29*−*32*). About 6% of a single dose of HQ (1.8 mmol/kg, po), the unsubstituted analog of TBHQ, is excreted as the corresponding mercapturic acid (*N*-acetylcystein-*S*-yl)HQ, and this was the only sulfur-containing metabolite

Table 1. Substituent-Directed Partitioning of Polyphenolic−Glutathione Conjugates through either the Mercapturic Acid Biosynthetic Pathway and/or the *β*-Lyase (Thiomethyl Shunt) Pathway



	metabolites in urine	
	mercapturates	thiomethyl (*β*-lyase)
R = *tert*-butyl	no	yes
R = bromine	yes	?
R = hydrogen	yes	no

identified in the urine of rats (*25*, *27*). In contrast, we did not identify mercapturic acids of TBHQ in the urine of rats, suggesting that the *tert*-butyl substituent either inhibits *N*-acetylation of the cysteine moiety or facilitates its metabolism by *β*-lyase(s). Thus, the nature of the substituents on the aromatic ring of polyphenolic−GSH conjugates appears to play an important role in partitioning their metabolism through either the mercapturic acid biosynthetic pathway or through the alternate thiomethyl shunt (*β*-lyase) pathway (Table 1).

Free thiol and thiomethyl metabolites of bromobenzene have been reported (*33*), and thiophenols and thioanisoles have been described as urinary metabolites of 1,2,4-trichlorobenzene (*34*) and dichlorobenzenes (*35*). Interestingly, the only major sulfur metabolite found in urine after the administration of hexachlorobenzene to rats was a mercapturic acid, whereas no mercapturic acid was found after pentachlorobenzene administration (*36*), indicating that small differences in structure may direct a compound via a different pathway of sulfur metabolism, with possible implications for toxicity. In contrast to the *β*-lyase-derived metabolites of halogenated alkanes and alkenes, which mediate their nephrotoxicity (*37*), the metabolism of aromatic cysteine *S*-conjugates by *β*-lyases usually results in the urinary excretion of stable thiols, *S*-glucuronides, *S*-oxides, or *S*-methyl compounds. Thus, pentachlorothiophenol and benzenethiol, metabolites of *S*-(pentachlorophenyl)-L-cysteine and *S*-benzyl-L-cysteine, respectively, do not exhibit the mutagenic and nephrotoxic properties of the halogenated alkane and alkene metabolites (*37*). Renal damage was not observed when *N*-acetyl-*S*-(pentachlorophenyl)-L-cysteine was administered to rats (*38*, *39*). However, little information is available on the potential toxicity of thiols derived from the *β*-lyase-mediated metabolism of aromatic cysteine conjugates.

In summary, we have shown that GSH conjugates of TBHQ, 5-(GSyl)TBHQ, 6-(GSyl)TBHQ, and 3,6-bis-(GSyl)-TBHQ are formed *in vivo*. Recent studies from our laboratory indicate that these GSH conjugates are nephrotoxic and bladder toxic,[2] supporting the contention that these metabolites may be responsible for the tumor-promoting effects of TBHQ and BHA in rat kidney upon long-term administration. Differences in the metabolism of TBHQ−GSH conjugates, a fraction of which appears to be *β*-lyase-mediated, may have relevance for the finding that the bladder forms an additional target for TBHQ toxicity.

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[2] Peters, M. M. C. G., Meussen, E., Rivera, M. I., Jones, T. W., Monks, T. J., and Lau, S. S., Unpublished data.

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