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Identification and Determination of Glutathione and Glucuronide Conjugates Formed from Butylated Hydroxytoluene in Rats

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Rats which had received butylated hydroxytoluene (BHT) excreted *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)glutathione (BHT-glutathione) and 3,5-di-*tert*-butylhydroquinone (BHQ) glucuronide in the bile. After being separated by high-performance liquid chromatography, the glutathione conjugate was identified by ^{13}C-nuclear magnetic resonance and field desorption mass spectrometry; the glucuronide was identified by gas chromatography-mass spectrometry. The structures of BHT-glutathione and BHQ glucuronide were further confirmed by comparison with synthetic samples. The excretion rates of BHT-glutathione and BHQ glucuronide in rat bile were 3.5 and 1.6%, respectively, of the dose in 24 h after intraperitoneal administration of BHT at a dose of 400 mg/kg. The occurrence of BHT-glutathione suggests that 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, a toxic metabolite of BHT, is detoxified by glutathione conjugation.

Keywords——butylated hydroxytoluene (BHT); BHT glutathione conjugate; BHT-hydroquinone glucuronide; HPLC; ^{13}C-NMR; field desorption mass spectrometry; GC-MS; rat bile

Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene, BHT) is widely used as an antioxidant in processed foods and in rubber and petroleum products. The toxicity and metabolism of BHT have been reviewed by Branen.[1] The major metabolites of BHT in rats are 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-acid), both free and as a glucuronide (BHT-acid glucuronide), and *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine (BHT-mercapturic acid) with minor amounts of 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde. We previously identified 3,5-di-*tert*-butylhydroquinone (BHQ), 3,5-di-*tert*-butyl-*p*-benzoquinone (BBQ), and 2,6-di-*tert*-butyl-4-[(methylthio)-methyl]phenol as minor metabolites in rats.[2] Daniel *et al.* demonstrated the presence of unidentified glucuronides in the bile and urine of rats given BHT.[3] Moreover, the occurrence of BHT-mercapturic acid suggests the formation of a glutathione conjugate of BHT *in vivo*.

This study was undertaken to examine these unidentified conjugates of BHT in the bile of rats. We found *S*-(3,5-di-*tert*-butyl-4-hydroxybenzyl)glutathione (BHT-glutathione) and BHQ glucuronide, together with BHT-acid glucuronide. This paper deals with the identification and determination of these new metabolites in rat bile.

Materials and Methods

Materials——Chemicals were purchased as follows: BHT from Nakarai Chemicals, Ltd., Kyoto; glutathione from P-L Biochemicals, Inc., Milwaukee, Wi., USA; trimethylsilyl (TMS) ether kit from Shimadzu Seisakusho Co., Ltd., Kyoto. TLC plates (Silica gel HF_{254}, 0.25 mm thick) were obtained from E. Merck A. G., Darmstadt, Germany.

Synthesis of BHT-Glutathione——2,6-Di-*tert*-butyl-4-(2,6-di-*tert*-butyl-4-methylphenoxy)-4-methyl-2,5-cyclohexadienone[4] (1.7 g) dissolved in 32 ml of dioxane was added to a solution of glutathione (1.2 g) in 48 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was stirred at 37 °C for 5 h, added to 100 ml of H_2O, and filtered to remove

undissolved compounds. The filtrate was washed with $CHCl_3$ and percolated through a column packed with 100 ml of Amberlite XAD-2 resin (Rohm and Haas Co., Philadelphia, Penn., USA). The conjugate was eluted with MeOH after the column had been washed with H_2O, and the eluate was evaporated to dryness *in vacuo*. The residue was dissolved in H_2O and further purified by rechromatography on a column containing 70 ml of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) eluting with H_2O. The product (600 mg) was the Na salt of BHT-glutathione, mp 180—185 °C. UV λ_{max}^{EtOH} nm (log ε): 275 (3.08). *Anal.* Calcd for $C_{25}H_{37}N_3Na_2O_7S \cdot H_2O$: C, 51.50; H, 6.69; N, 7.15. Found: C, 51.31; H, 6.88; N, 7.24. A solution of the Na salt in H_2O was acidified and the resulting precipitate was filtered off. The product was recrystallized from MeOH to give colorless crystals, mp 178—180 °C. *Anal.* Calcd for $C_{25}H_{39}N_3O_7S \cdot 1/2\ H_2O$: C, 56.16; H, 7.54; N, 7.86. Found: C, 56.38; H, 7.43; N, 7.75.

Synthesis of Methyl [2,3,4-Tri-*O*-acetyl-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-β-D-glucopyranosid]uronate——BHQ[5] (1.7 g) was reacted with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate[6] (6.0 g) by a modified Koenigs–Knorr reaction procedure.[7] The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The oily residue was subjected twice to silica gel column chromatography eluting with hexane–AcOEt (6 : 1). The product was recrystallized from EtOH to yield 840 mg of BHQ glucuronide acetate-methyl ester, mp 96—97 °C. UV λ_{max}^{EtOH} nm (log ε): 281 (3.40). $[\alpha]_D^{28}$ −33.2° (c=0.50, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.43 (18H, s, $C(CH_3)_3$), 2.03, 2.05, 2.09 (each 3H, s, 3×$COCH_3$), 3.76 (3H, s, $COOCH_3$), 4.08—4.18 (1H, m, pyranose-C_5–H), 4.98 (1H, s, OH), 5.03 (1H, d, J=7 Hz, pyranose-C_1–H), 5.10—5.42 (3H, m, pyranose-C_2–, C_3– and C_4–H), 6.89 (2H, s, aromatic protons). *Anal.* Calcd for $C_{27}H_{38}O_{11}$: C, 60.21; H, 7.11. Found: C, 60.20; H, 7.01.

Synthesis of Na Salt of BHQ Glucuronide——A solution of 470 mg of methyl [2,3,4-tri-*O*-acetyl-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-β-D-glucopyranosid]uronate in 30 ml of MeOH was treated with 0.5 ml of 2 N NaOH. The mixture was stirred at room temperature for 5 h and concentrated to a small volume. The resulting precipitate was filtered off and recrystallized from MeOH to give 136 mg of the Na salt of BHQ glucuronide, mp 220—230 °C (dec.). $[\alpha]_D^{25}$ −55.6° (c=0.50, H_2O). *Anal.* Calcd for $C_{20}H_{29}NaO_8 \cdot 1/4\ H_2O$: C, 56.53, H, 7.00. Found: C, 56.37; H, 7.00.

High-Performance Liquid Chromatography (HPLC)——HPLC was performed on a Hitachi model 638-30 liquid chromatograph equipped with a UV spectrophotometer (280 nm). Two columns, a 25 cm × 8 mm i.d. column for preparative separation and a 30 cm × 4 mm i.d. column for quantitative analysis, were packed with NUCLEOSIL 10 C_{18} (particle size 10 μm, Macherey-Nagel, Düren, Germany).

Nuclear Magnetic Resonance (NMR) Spectrometry——^{13}C-NMR spectra were measured on a JEOL model JMN-FX 100 spectrometer at 25 MHz employing the deuterium field-frequency lock system. The sample was dissolved in 0.3 M sodium phosphate in D_2O (pH 6.9). Dioxane (δ=67.4 ppm) was used as an internal standard (IS).

^{1}H-NMR spectra were measured on a JEOL JMN-MH-100 spectrometer at 100 MHz. Chemical shifts are given relative to internal tetramethylsilane.

Mass Spectrometry——Gas chromatography-mass spectrometry (GC-MS) was carried out on a JEOL JMS-D 100 mass spectrometer coupled with a JGC-20 K gas chromatograph operating at an ionizing voltage of 22 eV and a total ionizing current of 300 μA. GC conditions were as follows: column, 1.5% SE-30 on Chromosorb W, 1 m × 2 mm i.d.; column temp., 250 °C; carrier gas, He 0.25 kg/cm^2.

Field desorption mass spectrometry (FDMS) was performed on a Shimadzu model GC-MS 9020-DF mass spectrometer operating at an accelerating voltage of 3 kV and an emitter current of 15—17 mA.

Isolation and Determination of BHT Conjugates in Rat Bile——Bile was collected from rats cannulated as described previously[8] for 24 h after intraperitoneal administration of BHT (400 mg/kg) and percolated through SEP-PAK C_{18} cartridges (Waters Associates, Milford, Mass., USA). The cartridges were washed with H_2O and the conjugates were eluted with MeOH. The eluate was concentrated to dryness *in vacuo* and the residue was dissolved in MeOH. The sample was injected onto the preparative HPLC column and eluted with MeOH–1% AcOH (55 : 45, v/v) at a flow rate of 3.0 ml/min. Under these conditions, the eluate was divided into three fractions: fraction I (12—14 min), fraction II (16—19 min), and fraction III (24—28 min). Each fraction was lyophilized. Fraction I was trimethylsilylated directly, but fraction II was trimethylsilylated after being esterified with CH_2N_2 for GC-MS examination. Fraction III was subjected to ^{13}C-NMR and FDMS.

For quantitative studies of BHT-glutathione and BHQ glucuronide in the bile, a sample purified on a SEP-PAK C_{18} cartridge was injected onto the analytical HPLC column and eluted under the conditions described in the caption of Fig. 4. The amounts of the two conjugates were calculated from the peak heights.

Results

Fractions I, II, and III obtained by preparative HPLC of the biliary extract contained metabolites M-I, M-II, and M-III, respectively. Metabolites M-I and M-II gave positive reactions for glucuronides, and M-III gave a positive reaction for amino acids in thin-layer chromatography (TLC) (Table I).

TABLE I. TLC Comparison of Synthetic BHQ Glucuronide and BHT-Glutathione and Metabolites M-I, M-II, and M-III

	TLC (Rf)[a]		
	A	B	C
BHQ glucuronide[b]	0.05	0.63	0.54
BHT-glutathione[c]	0.02	0.49	0.32
Metabolite M-I[b]	0.06	0.62	0.53
Metabolite M-II[b]	0.06	0.59	0.47
Metabolite M-III[c]	0.02	0.49	0.32

a) A: *n*-PrOH–27% NH_3 (8:1, v/v), B: *n*-BuOH–EtOH–H_2O (2:1:1, v/v), C: *n*-BuOH–AcOH–H_2O (4:1:1, v/v).
b) Detected by spraying with naphthoresorcinol.
c) Detected by spraying with ninhydrin.

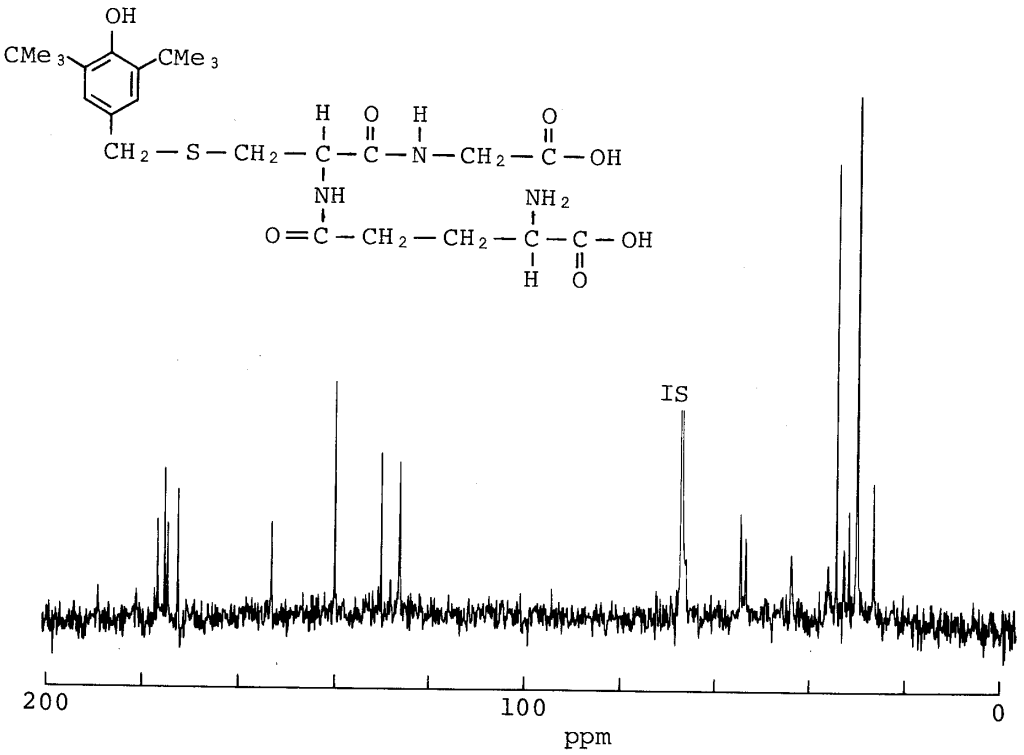

Fig. 1. ^{13}C-NMR Spectrum of Metabolite M-III Isolated by Preparative HPLC

Chemical shifts in ppm from tetramethylsilane (0.0 ppm): Glu (β), 27.1; $C(\underline{C}H_3)_3$, 30.6; Glu (γ), 32.3; Cys (β), 33.4; $\underline{C}(CH_3)_3$, 34.9; CH_2, 36.7; Gly (α), 44.3; Cys (α), 53.9; Glu (α), 54.9; C_2 and C_6, 126.6; C_1, 130.4; C_3 and C_5, 140.1; C_4, 153.2; Cys CO, 172.8; Glu COOH, 175.0; Glu CO, 175.5; Gly COOH, 177.0. Conditions: spectral width, 6024 Hz; pulse width, 8 μs (pulse flipping angle, *ca.* 55°); temp., 23 °C; pulse repetition, 3.0 s; number of pulses, 52000.

Identification of Metabolite M-III by ^{13}C-NMR and FDMS

The ^{13}C-NMR spectrum (Fig. 1) of metabolite M-III revealed signals corresponding to Glu (β), Glu (γ), Gly (α), Glu (α), and the four downfield carbonyl carbons of glutathione.[9,10] The signals of the Cys (β) and Cys (α) carbons of M-III were shifted 7.2 ppm downfield and 2.5 ppm upfield, respectively, compared with those of glutathione. These shifts are consistent with substitution at the cysteinyl sulfur atom.[10] The more intense signal at 30.6 ppm, together with the signal at 34.9 ppm, indicated the presence of two *tert*-butyl groups on the basis of the

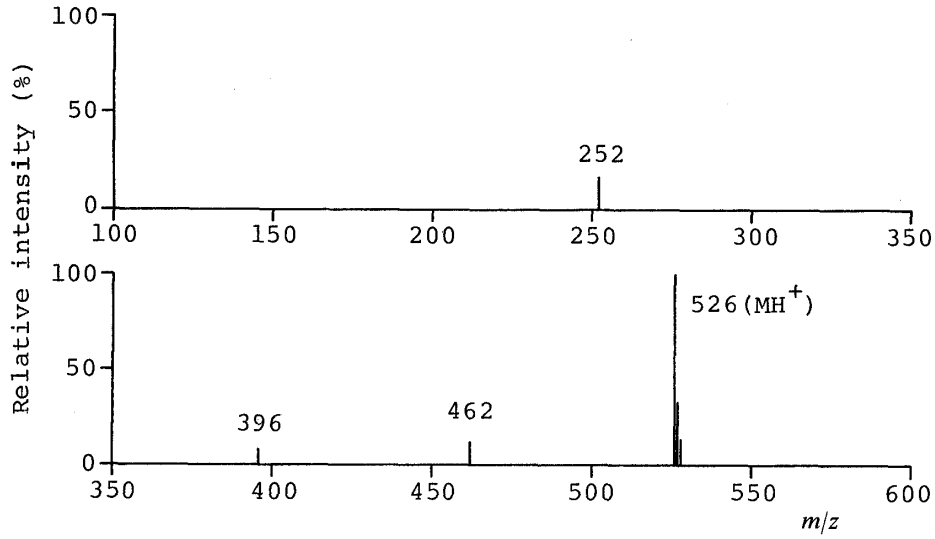

Fig. 2. FD Mass Spectrum of Metabolite M-III Isolated by Preparative HPLC

chemical shifts and relative peak heights. In addition, the triplet at 36.7 ppm obtained from the off-resonance decoupled spectrum was assigned to the methylene carbon bonded to the sulfur atom of the glutathionyl moiety. There were signals of six aromatic carbons of M-III in the region of 126-154 ppm. The signals at 130.4, 140.1, and 153.2 ppm were assigned to carbons bonded to methylene (C_1), *tert*-butyl (C_3, C_5), and hydroxy (C_4) substituents, respectively, based on the substitution effects of substituted benzenes.[11] The doublet at 126.6 ppm obtained from the off-resonance decoupled spectrum was assigned to C_2 and C_6 of the aromatic ring. The structure of M-III was thus assigned as BHT-glutathione.

The FD mass spectrum of M-III (Fig. 2) showed a intense protonated molecular ion at *m/z* 526 as a base peak whose elemental composition was consistent with that of BHT glutathione conjugate. The fragment ion at *m/z* 462 ($[MH-NHCH_2COOH]^+$) represents the loss of a Gly residue, and the ion at *m/z* 396 ($[MH-COCH_2CH_2CH(NH_2)COOH]^+$) corresponds to the loss of a Glu residue from the glutathionyl moiety. The peak ion at *m/z* 252, which represents [3,5-di-*tert*-butyl-4-hydroxybenzyl (BHT-benzyl) mercaptan]$^+$, represents strong evidence that glutathione is conjugated with BHT-benzyl *via* the sulfur atom. An authentic sample of BHT-glutathione was synthesized and proved to be identical with metabolite M-III by comparison of the spectral data and the mobility in TLC (Table I).

Identification of Metabolites M-I and M-II by GC-MS

Metabolite M-I (purified by HPLC) was converted to a trimethysilyl derivative (TMS-M-I) and analyzed by GC-MS. The mass spectrum of TMS-M-I (Fig. 3 (1)) showed a molecular ion at *m/z* 686 with fragment ions at *m/z* 671 ($[M-CH_3]^+$) and 581 ($[M-CH_3-TMSOH]^+$). Moreover, the fragment ions at *m/z* 217, 375, and 464 are typical ions produced from the TMS-glucuronic acid moiety.[12] The base peak at *m/z* 294 corresponds to [TMS-BHQ]$^+$, and the ion at *m/z* 222 represents $[BHQ]^+$. These data suggest TMS-M-I to be TMS-BHQ glucuronide. This suggestion was confirmed through comparison of M-I with an authentic sample of BHQ glucuronide by GC-MS and TLC (Table I).

Metabolite M-II was assigned as BHT-acid glucuronide by GC-MS. The mass spectrum of the methylated-trimethylsilylated derivative (TMS-Me-M-II) of M-II (Fig. 3 (2)) showed a molecular ion at *m/z* 656 with fragment ions at *m/z* 217 and 407, which are attributable to TMS-Me-glucuronides.[12] The base peak at *m/z* 233 corresponds to [3,5-di-*tert*-butyl-4-hydroxybenzoyl]$^+$ resulting from the ester-bonding cleavage of the glucuronide. The assignment of M-II as BHT-acid glucuronide was supported by the formation of BHT-acid and

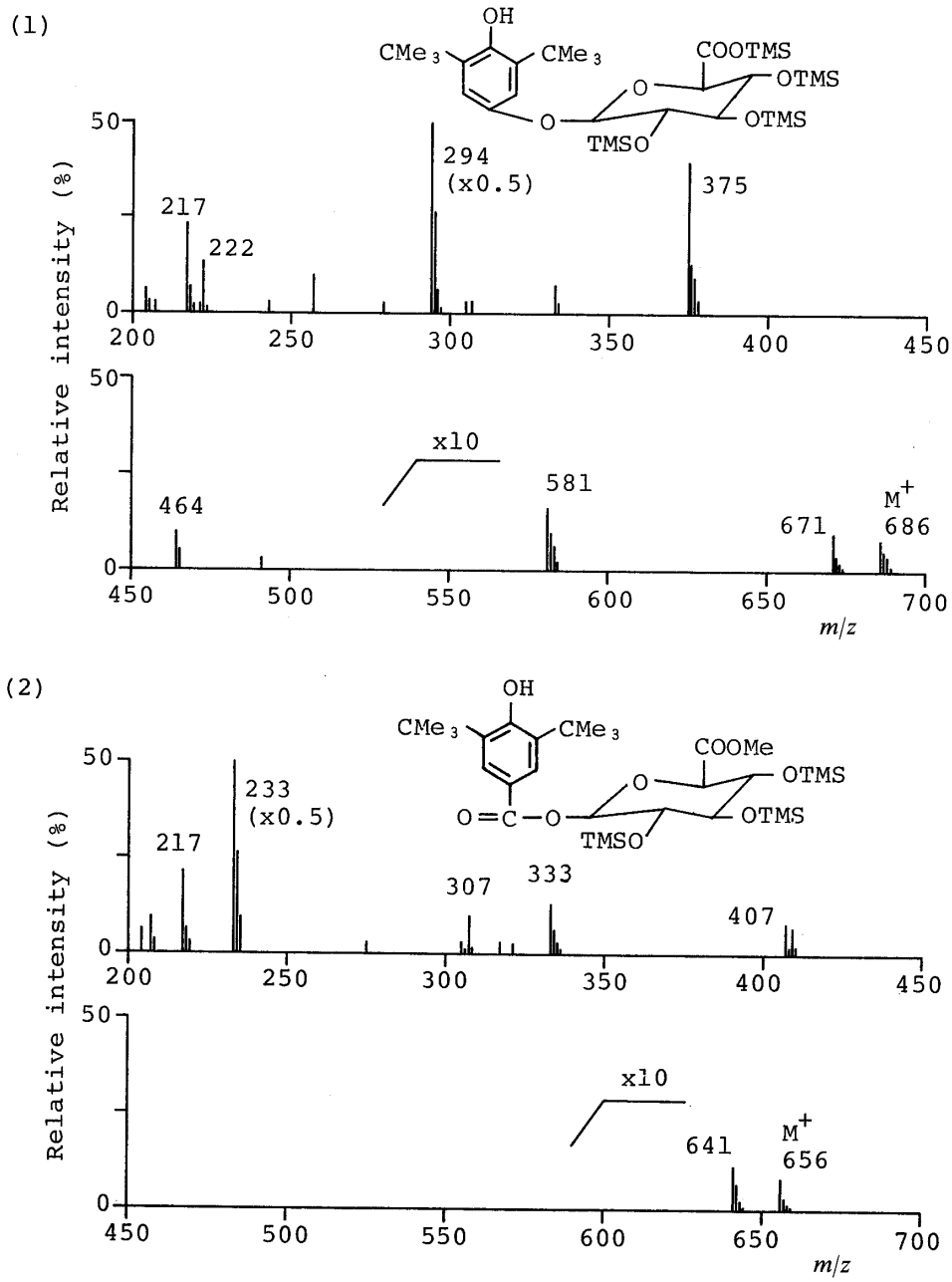

Fig. 3. Mass Spectra of (1) Trimethylsilylated Derivative of Metabolite M-I and (2) Methylated-Trimethylsilylated Derivative of Metabolite M-II

glucuronic acid from M-II on incubation with β-glucuronidase.

Determination of BHT-Glutathione and BHQ Glucuronide in Rat Bile by HPLC

As shown in Fig. 4, BHT-glutathione and BHQ glucuronide were separated satisfactorily on a NUCLEOSIL 10 C_{18} column when MeOH–0.1 M $NH_4H_2PO_4$ (52:48, v/v) was employed as a mobile phase at a flow rate of 1.0 ml/min. The UV absorbance (280 nm) was linearly related to amount in the ranges of 5—20 μg for BHT-glutathione and 2.5—10 μg for BHQ glucuronide. The recoveries of BHT-glutathione and BHQ glucuronide added to normal bile were 69 and 91%, respectively. The excretion rates of BHT-glutathione and BHQ glucuronide in 24 h after intraperitoneal administration of BHT were 3.5 and 1.6%, respectively (Table II).

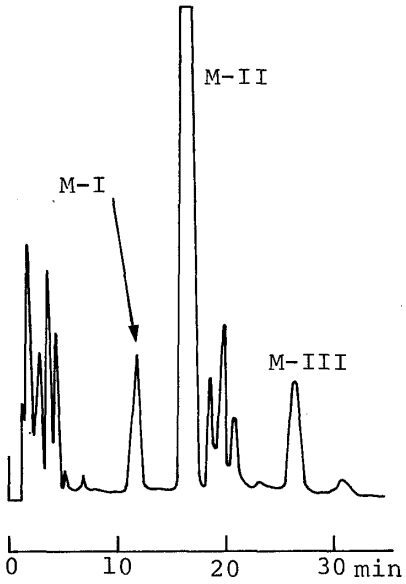

Fig. 4. High-Performance Liquid Chromatogram of the Biliary Extract Obtained during 8 h after Intraperitoneal Administration of BHT

M-I, BHQ glucuronide; M-II, BHT-acid glucuronide; M-III, BHT-glutathione. Conditions: column, NUCLEOSIL 10 C_{18}, 30 cm × 4.0 mm i.d.; mobile phase, MeOH–0.1 M $NH_4H_2PO_4$ (52:48, v/v); flow rate, 1.0 ml/min; detection, UV 280 nm; 0.08 AUFS.

TABLE II. Biliary Excretion of BHT-Glutathione and BHQ Glucuronide after Intraperitoneal Administration of BHT[a]

Period (h)	Excretion rate (% of dose)[b]	
	BHT-glutathione	BHQ glucuronide
0— 8	1.00±0.44	0.51±0.28
8—24	2.51±0.62	1.08±0.35

a) BHT was administered as a single dose of 400 mg/kg dissolved in an emulsion of DMSO and olive oil.
b) Each value is the mean±S.D. of 4 rats.

Discussion

In this study, we isolated two new conjugates, BHT-glutathione and BHQ glucuronide, from the bile of rats given BHT and identified them on the basis of chromatographic and spectral comparisons with authentic samples. Moreover, we separated these metabolites on a reversed-phase HPLC column (Fig. 4) and determined them in rat bile (Table II). Recently, ^{13}C-NMR, FDMS, and GC-MS have been shown to be useful for the structural analysis of xenobiotic conjugates. Glutathione conjugates of acetaminophen,[10] *N,N*-dimethyl-4-aminoazobenzene,[13] and bromobenzene[14] were identified by ^{13}C-NMR, and those of acetaminophen[15] and *N*-hydroxy-2-acetylaminofluorene[16] were identified by FDMS. Fenselau and Johnson have reviewed the analysis of intact glucuronides by GC-MS.[12]

The excretion rate of BHT-glutathione (Table II) is comparable to that (3% of dose) of BHT-mercapturic acid in rat bile after administration of BHT.[3] Daniel *et al.* tentatively proposed that BHT-mercapturic acid originates from nonenzymic conjugation of BHT-benzyl radical with cysteine, followed by acetylation.[3] The occurrence of BHT-glutathione, however, suggests another pathway whereby BHT-mercapturic acid is formed from BHT-glutathione by catabolic reactions.

We have recently suggested that 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (BHT-quinone methide) or closely related metabolites are responsible for BHT-induced lung damage in mice.[17,18] Concerning the hemorrhagic toxicity of BHT in rats, Takahashi and

Hiraga suggested that BHT-quinone methide inhibits the activity of vitamin K epoxide reductase by binding with the SH groups and, as a result, prolongs the plasma prothrombin times.[19] The metabolic reaction forming BHT-glutathione most likely involves the reaction of BHT-quinone methide, an electrophile,[20] with glutathione and may play a significant role in preventing the toxicity of BHT. In this context, we recently found a protective effect of cysteine, a glutathione precursor, against BHT-induced lung damage in mice (unpublished data).

We previously reported that the excretion rate of BBQ in rat feces was 0.34% of the dose in 24 h and was maximal (0.63%) during 24—48 h after administration of BHT, while the excretion of BHQ was far less than that of BBQ.[2] The delayed excretion of BBQ suggests that BHQ glucuronide undergoes an enterohepatic circulation and, in part, is oxidized to BBQ after being hydrolyzed.

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