BIOTRANSFORMATION OF THE NOVEL INOTROPIC AGENT TOBORINONE (OPC-18790) IN RATS AND DOGS

Evidence for the Formation of Novel Glutathione and Two Cysteine Conjugates

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(Received June 3, 1996; accepted February 7, 1997)

ABSTRACT:

The metabolism of toborinone, (+)-6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-2(1H)-quinolinone, a novel inotropic agent, was studied in rats and dogs after intravenous administration. Chemical structures of the 13 metabolites were characterized by direct-probe FAB/MS and field desorption/MS, LC/FAB/MS, and various NMR measurements. After intravenous dosing of 10 mg/kg [14C]toborinone, fecal and urinary recoveries of the 14C dose were 70% and 25–30%, respectively, in both rats and dogs. The predominant component of radioactivity was the unchanged toborinone in every biological specimen in rats and dogs. Although unchanged toborinone was predominantly observed, toborinone underwent extensive conjugations with glucuronic acid, sulfate, and glutathione, either directly or following phase I reaction. Metabolites resulting from oxidative N-C cleavage were minor both in number and in quantity in every biological specimen in rats and dogs. In rats, toborinone underwent O-demethylation to form M-7 and successive phase II reaction to yield the glucuronide M-1 and the sulfoconjugate M-2, and deconjugation to yield M-7, which was a primary metabolite accounted for 35.67% of the radioactivity excreted in the feces by 48 hr. Conjugates M-1 and M-2 were the major metabolites in rat plasma. In dogs, toborinone was metabolized via mercapturic acid pathway to yield the primary metabolite, cysteine conjugates M-10 and M-11 that accounted for 19.10% and 6.70% of the radioactivity excreted in the feces by 48 hr and that were detected species specifically in dogs. The glutathione conjugate M-13, which was isolated from in vitro incubations using dog liver, led us to consider a possible mercapturic acid pathway from the parent compound to M-10. Metabolites in dog plasma and those in urine in both rats and dogs were minor in quantity. The metabolic pathways of toborinone in rats and dogs are proposed herein.

Toborinone (OPC-18790; fig. 1), is a novel inotropic agent currently under clinical development for the treatment of congestive heart failure. In several mammalian species, toborinone produced a positive inotropic effect with a moderate vasodilating effect and with no direct chronotropic effect in isolated heart preparations or with a little chronotropic effect in the in situ preparations (1–3). The inotropic effect of toborinone is not associated with chronotropic effect in isolated heart preparations or with a little chronotropic effect in the in situ preparations (1–3). The inotropic effect of toborinone is not associated with beta-adrenergic receptor stimulation, alpha-blockade, or Na+K+-ATPase inhibition. Toborinone inhibits PDE1 III, as do other PDE inhibitors; but, it prolongs action potential duration in isolated ventricular muscles (1). These effects may contribute to the positive inotropic action of the compound. When toborinone was administered to healthy human volunteers at doses ranging from 0.5 to 15.0 μg/kg/min for 1-hr intravenous infusion and from 0.05 to 0.4 mg/kg for rapid intravenous administration, the pharmacokinetic profile of toborinone was linear in both instances (4). Although those studies showed few effects on heart rate and blood pressure, the shortened systolic left ventricular dimension measured by an M-mode echocardiogram suggested that toborinone increased cardiac contractile force. The cardiovascular effects of toborinone might demonstrate a beneficial effect in the treatment of congestive heart failure. The present study was conducted to characterize the metabolites of toborinone and to quantitate the metabolites after an intravenous dose of [14C]toborinone to elucidate the biological fate of toborinone in rats and dogs.

Materials and Methods

Chemicals. [14C]Toborinone (racemate) was purchased from Amersham International plc (Buckinghamshire, UK). Specific activity was 4.5 MBq/mg, and radiochemical purity was 97% as determined by TLC (Kieselgel 60F254, 0.25 mm thick; Merck, Darmstadt, Germany) with a developing solvent system of chloroform:methanol:ammonia water (25% as NH3) [70:10:1 (v/v)]. Standard toborinone (racemate), S(-)- and R(-)-toborinone enantiomers, and authentic metabolite standards for M-3, M-4 aglycone, M-5, M-7, M-8, and M-9 were synthesized at our company. HPLC grade acetonitrile and methanol were used as solvents throughout the study. Glucose-6-phosphate was purchased from Boehringer Mannheim (Mannheim, Germany); β-NADP, glucose-6-phosphate dehydrogenase, glutathione (reduced), arylsulfatase, and β-a-glucuronidase were from Sigma Chemical Co. (St. Louis, MO); Sep-Pak Vac C18 cartridges (5 or 10 g) were from Waters Associates (Milford, MA); Amberlite XAD-2 resin was from Organo Corp. (Tokyo, Japan); Sephadex LH-20 resin was from Pharmacia Biotech (Uppsala, Sweden); and ACS-II scintillation solution was supplied by Amersham. All other reagents used were of the highest grade commercially available.

Animals. Six-week-old Sprague-Dawley male rats (Charles River Japan, Inc., Tokyo, Japan) weighing 170–180 g and 6-month-old male beagle dogs (Laboratory Research Enterprises, Kalamazoo, MI) weighing 9–10 kg re-
received \(^{14}C\) toborinone. For the isolation of the metabolites, male rats weighing 150–250 g and male beagle dogs weighing 8–14 kg received toborinone. \(S(-)-\) or \(R(+)-\) toborinone; the strains and suppliers were the same as in the \(^{14}C\) experiment. Rats were allowed free access to a standard pellet diet (MF; Oriental Yeast Co., Tokyo, Japan), and the dogs were fed a measured quantity (300 g/day) of food (CD-5; Clea Japan, Inc., Tokyo, Japan). All animals were allowed free access to water at all times. Animals for the \(^{14}C\) experiment were fasted overnight before drug administration and were fed at 4 hr after dosing.

**Dosages and Sample Collection in Rats and Dogs.** In rats, for the quantitative \(^{14}C\)-HPLC analysis and mass balance experiment, a total of 11 rats were used. Each animal received a single intravenous (bolus) dose of \(^{14}C\) toborinone (10 mg/37 MBq/kg/2 ml). In one group of three rats, urine and feces were collected at 24-hr intervals for 48 hr. In two other groups of four rats each, blood samples were drawn from the inferior vena cava, and the liver, kidney, and heart were collected from each rat killed at 10 min or 1 hr after dosing. For metabolite isolation in rats, during twice-daily intravenous dosing of toborinone or \(R(+)-\) toborinone at 30 mg/kg, urine was collected and pooled daily for 3–5 days, and bile was collected for up to 24 or 48 hr after the first dose. In addition, urine and bile were collected from rats for 24 hr after single intravenous dosing of toborinone or \(R(+)-\) toborinone at 30 mg/kg. In dogs, two animals were used for the quantitative \(^{14}C\)-HPLC analysis and mass balance experiment. Each animal received a single intravenous (bolus) dose of \(^{14}C\) toborinone (10 mg/7.4 MBq/kg/ml). Urine and feces were collected at 24-hr intervals for 72 hr. Samples between 48–72 hr were not used for \(^{14}C\) metabolite assay because of the low amounts of \(^{14}C\), accounting for only 2% of the dosed radioactivity. Blood samples were drawn from the cephalic vein at the specified times (10 and 30 min and 1 and 2 hr postdosing). For metabolite isolation in dogs, fecal samples were obtained after multiple intravenous doses of 10–30 mg/kg of toborinone or \(S(-)-\) toborinone. All samples from rats and dogs were stored at −20°C until analysis. Dosing solutions for rats and dogs were prepared by adding 10–20% \((v/v)\) of 1 M DL-lactic acid to toborinone [including \(^{14}C\), \(R(+)-\), and \(S(-)-\)] as a percentage of the final volume, then diluting with 5% xylitol to yield the final concentration.

**Determination of Total Radioactivity.** Total radioactivity in urine and plasma was determined by direct liquid scintillation counting of a 0.05–0.1 (ml or g) aliquot of samples in 1 ml of water and 8 ml of ACS-II scintillator using an LSC-1050 or LSC-3500 liquid scintillation counter (Aloka, Tokyo, Japan). Quenching was corrected by the external standard method. Feces were mixed with a mixture of methanol:water \((1:1, \text{ v/v})\), then homogenized with a Polytron. The radioactivity in fecal homogenates was then determined by liquid scintillation counting of 0.025–0.1 g of each sample in duplicate or triplicate in 1 ml of methanol and 8 ml of ACS-II scintillator as described for the urine and plasma. The liver and heart were homogenized to prepare 25% homogenates in 5% xylitol. The kidney was prepared as a 20% homogenate. These homogenates were prepared for counting using a model 306 Tri-Carb Sample Oxidizer (Packard, Downers Grove, IL).

**Preparation of Samples for Quantitative HPLC Analysis of \(^{14}C\) Metabolites.** To a weighed aliquot of plasma or homogenized tissue samples, a mixture of methanol:acetoneitrile \((1:1, \text{ v/v})\) was added. The mixture was well mixed and sonicated, followed by centrifugation at 1800g for 5 min. The supernatant was removed, and the solid-pellet residue was re-extracted. This operation was repeated 4 times, then all of the supernatants were combined and lyophilized. The residue was reconstituted in a mobile phase \((\text{component A—acetoneitrile:methanol:water:acetic acid (5:5:90:0.5, v/v)}\); component B—25:25:50:0.5, A:B = 1:1, v/v). To a weighed aliquot of fecal homogenates, a mixture of solvent of methanol:water was added. The extraction procedure was repeated 6 times, then all of the supernatants were combined and lyophilized. The residue was reconstituted in a mobile phase \((\text{components A:B = 1:1, v/v})\), and urine was either applied directly to HPLC analysis without treatment, or lyophilized and reconstituted in a mobile phase \((\text{components A:B = 1:1, v/v})\), then applied to HPLC analysis.

**In Vitro Incubation Experiment.** The incubation experiment was conducted using a 0.5 ml aliquot of 9000g supernatant of 20% homogenate of dog liver, cofactor solution containing 2.5 mM MgCl\(_2\), 2.5 mM \(\beta\)-NADP, 2.5 mM glucose-6-phosphate in 200 mM potassium phosphate buffer (\(pH 7.4\)), and 100 \(\mu\)M toborinone or \(S(-)-\) toborinone. After 2 min of preincubation at 37°C, 5 units of glucose-6-phosphate dehydrogenase was added. After 1 hr of incubation, a 1.5-ml aliquot of acetoneitrile:methanol \((1:1, \text{ v/v})\) was added and mixed, followed by centrifugation. The supernatant was diluted one-third with water, then directly analyzed by HPLC. A glutathione-added system, blank incubation without drug, and control incubations with boiled 9000g supernatant were also analyzed.

**Preparative Metabolite Isolation Procedure.** Pooled rat urine was either added to a mixture of potassium/sodium phosphate powder followed by extraction with chloroform or added to Sep-Pak Vac C\(_{18}\) cartridges preconditioned with washing with water. The carbohydrates were washed with water, then eluted with methanol:water \((1:1, \text{ v/v})\). Urinary extracts were reduced in volume by rotary evaporation. The residue was added to TLC plates (Kieselgel 60F\(_{254}\); 0.25 mm thick, Merck) with a developing solvent system of chloroform:methanol:ammonia water \((50:10:0.2\) for M-3 and M-8; 70:10:1 for M-7 and M-8, v/v). The area containing metabolites [highlighted with UV light radiation (254 and 365 nm), compared with the control extract] was removed from the plate by scraping. The obtained sample was extracted with a chloroform:methanol mixture, the solvent was evaporated to dryness, and applied to the preparative HPLC procedure. Pooled bile from toborinone- or \(R(+)-\) toborinone-treated rats was applied to Amberlite XAD-2 resin packed in an open glass column \((30 \text{ mm i.d.} \times 300 \text{ mm})\) preconditioned with water. The column was washed with water, then elution was performed sequentially with methanol:water \((3:7, \text{ v/v})\), methanol:water \((5:5, \text{ v/v})\), and an appropriate volume of 1% acetic acid in methanol. Each fraction was analyzed by HPLC, and the resulting chromatograms were compared with those of a control specimen run in parallel. The methanol:water \((3:7)\) and \((5:5)\) fractions were combined, reduced in volume by rotary evaporation to remove the methanol, then applied to Sephadex LH-20 resin. This procedure yielded the rat biliary metabolites M-1, M-2, M-4, and M-6.

Pooled fecal homogenate from dogs treated with toborinone or \(S(-)-\) toborinone was extracted several times with methanol and a methanol:water mixture. The extracted solvent was combined and reduced in volume by rotary evaporation. The residues were then applied to Sep-Pak Vac C\(_{18}\) cartridges or Sephadex LH-20 resin preconditioned by washing with water. The Sep-Pak Vac C\(_{18}\) cartridges were washed with water, then elution was performed sequentially with methanol:water \((2:8\) to \(5:5, \text{ v/v})\), methanol, and 1% acetic acid in methanol \((\text{v/v})\). Each fraction was analyzed by HPLC to compare the chromatograms with those of the control. Fractions containing metabolites were combined and reduced in volume by rotary evaporation to remove the methanol, then applied to Sephadex LH-20 resin. This procedure yielded the biliary metabolites M-9, M-10, M-11, and M-14s.

Pooled dog liver homogenate incubated with toborinone (as described in In Vitro Incubation Experiment) was extracted several times with a solvent of acetoneitrile:methanol \((1:1, \text{ v/v})\). The extracted solvent was combined and reduced in volume by rotary evaporation, then applied to Sephadex LH-20 resin. This procedure yielded the dog in vitro metabolite M-13.

The preparative purification procedure with Sephadex LH-20 resin was used for all specimens previously mentioned. The residue from each specimen was then applied to LH-20 resin packed in an open glass column \((20 \text{ mm i.d.} \times 200 \text{ mm})\) preconditioned with water. Elution was performed sequentially with 25–30 ml methanol:water \((2:8, \text{ v/v})\), 25–30 ml methanol:water \((5:5)\), 50–100 ml methanol, and an appropriate volume of 1% acetic acid in methanol \((\text{v/v})\). Immediately after the colored eluate was seen, 5 ml each of elution fractions \(1 \text{ to } 13\) and 10 ml each of elution fractions nos. 14 and higher were collected. Each fraction was analyzed by HPLC, and the chromatograms were compared with those of the corresponding fractions of the control specimen.

![Fig. 1. Chemical structure of toborinone.](https://example.com/fig1.png)
similarly analyzed. LH-20 column effluent was collected until no peaks were observed on the chromatograms by simultaneous HPLC analysis. Crude metabolites were purified by preparative HPLC and then structurally characterized by several spectrometric measurements.

**HPLC.** The HPLC system consisted of two model 510 pumps, a model 680 gradient controller, a WISP 712 or WISP 710B autosampler, and a U6K universal injector (Waters Associates, Milford, MA), together with a RF-535 fluorescence HPLC monitor and a Chromatopac C-R1B, C-R3A, C-R5A, or C-R6A integrator (Shimadzu Corp., Kyoto, Japan). Fluorescence detection was performed at an excitation wavelength of 355 nm and an emission wavelength of 405 nm as the main monitor at all times. A model 490E multi-wavelength detector (280, 254, and 340 nm) or a model 440 absorbance detector (280 and 254 nm) was also used for metabolite identification as well as isolation.

**Profiles of 14C Metabolites.** The metabolites of toborinone were separated at a flow rate of 1.0 ml/min by a gradient elution of component A [acetonitrile: methanol:water:acetic acid (5:5:90:0.5, v/v)] and component B [acetonitrile: methanol:water:acetic acid (25:25:50:0.5, v/v)] using a Develosil ODS-5 column (4.6 mm i.d. × 250 mm; Nomura Chemical Co., Ltd., Seto, Japan). Gradient conditions were 100% component A (0% B) at first, changing to 10% B over 20 min, followed by a 10% B isocratic condition for 10 min (20–30 min from the start), then changing to 50% B over 10 min (30–40 min), to 50% B over 5 min (40–45 min), and back to the initial condition of 100% A for 0.1 min, with a flow rate of 1.0 ml/min throughout. The column temperature was maintained at 30°C. A total of 100 HPLC-eluted fractions were collected into scintillation vials at 0.5-min intervals. To each eluate, 8 ml of scintillation solution (ACS-II) was added, and then the radioactivity in each vial was measured by several spectrometric measurements.

**Characterization of the Metabolites.** The metabolites of toborinone were purified by preparative HPLC and then structurally characterized by several spectrometric measurements.

**NMR Spectrometry.** NMR spectra (1H-NMR, 13C-NMR, distortionless enhancement by polarization procedure, HMBC, and/or HMQC) were recorded on a Bruker WH-400 FT NMR spectrometer (frequency: 1H = 400.14 MHz, 13C = 100.61 MHz), a JNM-A500 FT NMR spectrometer (frequency: 1H = 500.00 MHz, 13C = 125.65 MHz; JEOL, Tokyo, Japan), or a JNM-GSX500 FT NMR spectrometer (frequency: 1H = 500.0 MHz, 13C = 125.8 MHz; JEOL) in dimethylsulfoxide-d6 or deuterium oxide as a solvent.

**MS.** Direct-probe MS was performed as follows. FD/MS spectra of M-1, M-2, M-4, M-6, M-7, M-8, and M-14s were obtained on a 80-MU-detecting spectrometer (Hitachi, Tokyo, Japan). FAB/MS spectra of M-3, M-5, and M-9 were generated on a 2AB-HF mass spectrometer (VG Analytical, Manchester, UK), and those of M-10, M-11, and M-12 on a JMS-SX102A mass spectrometer (JEOL) using glycerol or meta-nitrobenzylalcohol as matrices, with an accelerating voltage of 7–8 kV using xenon as the primary beam gas. LC/FAB/MS spectra of M-11, M-12, and M-13 were obtained on a JMS-SX102A mass spectrometer equipped with a Frit-FAB MS interface, with LC-6A HPLC pumps (Shimadzu), and an Inertisol ODS-2 column (1.5 mm i.d. × 150 mm; GL-Science, Osaka, Japan), using xenon as the primary beam gas. The conditions were as follows. The mobile phase used was comprised of components A and B defined under HPLC in Materials and Methods. A 30-min linear gradient was applied from 0% B to 15% B at a flow rate of 80 μl/min [for M-13, the mobile phase consisted of water:acetic acid (100:1, v/v) (A) and acetonitrile (B), and a 30-min linear gradient was applied from 2% B to 30% B at a flow rate of 40 μl/min]. The column effluent was mixed with 5% glycerol in methanol at a flow rate of 20 or 10 μl/min as a matrix. The mixture was split 1:20 or 1:10 and directed to a JMS-SX102A mass spectrometer through a Frit-FAB/MS interface at a flow rate of 5 μl/min, with an accelerating voltage of 6 or 5 kV.

**UV Spectrometry.** Data of UV spectra were obtained using a UV-260 UV Visible Recording Spectrophotometer (Shimadzu).

**Results**

HPLC 14C radiochromatograms of rat plasma (1 hr) and dog feces (0–24 hr) after an intravenous dose of [14C]toborinone at 10 mg/kg are shown in fig. 2. In rat plasma, toborinone and metabolites M-1, M-2, M-6, and M-3 were observed, and in dog feces, toborinone, and metabolites M-10, M-11, M-9, M-7, M-14, M-2, and M-6 were observed. These two biological specimens contained more metabolites both in number and in quantity than other specimens, including dog plasma, rat feces, and rat and dog urine.

**Characterization of the Metabolites.** The metabolites in the biological specimens from rats and dogs were isolated and characterized as described in Materials and Methods. M-1, M-2, M-4, and M-6 were isolated from rat biliary excreta, and M-3, M-5, M-7, and M-8 were isolated from rat urinary excreta. M-7 for [1H-NMR was obtained by hydrolysis of M-1, whereas M-9, M-10, M-11, and M-14s were isolated from dog fecal excreta and M-13 from the in vitro incubation mixture using dog liver, as described in Materials and Methods.

Structural assignments of metabolites for which no reference standards were available were based on interpretation of the NMR (including 1H-NMR (table 1), 13C-NMR, and/or two-dimensional heteronuclear measurements), MS (FAB, LC/FAB, and/or FD), and UV spectra (6, 7). For metabolites having corresponding reference standards, various spectral data and retention times by HPLC were compared with those of the reference standards.

**M-1, M-7, and M-2.** The FD/MS spectrum of M-1 showed a protonated quasi-molecular ion [M + H]+ at m/z 547 along with a diagnostic fragment ion [M − 176 + H]+ at m/z 371 consistent with a loss of a gluconic acid portion. The 13C-NMR spectrum of M-1 showed a distinguishing feature of six additive carbon signals assigned to a gluconic acid molecule (chemical shifts: G1, 99.6 ppm; G2, 73.0 ppm; G3, 76.6 ppm; G4, 71.9 ppm; G5, 74.3 ppm; and G6, 50.0 ppm).
additive ions at m/z confirmed to be protonated quasi-molecular ion \([M-7]\) hr postdosing. Consistent with the loss of a sulfate portion. 1 H-NMR spectrum of 1 H-NMR and HPLC data with that of the corresponding authentic treatment with a loss of a carbon signal consistent with a methyl carbon. After 172.0 ppm; positions were labeled as shown in fig. 3) accompanied by a loss of a carbon signal consistent with a methyl carbon. After treatment with \(\beta\)-d-glucuronidase, the hydrolysate release (M-7) was confirmed to be O-demethylated at the 4'-position by comparing 1 H-NMR and HPLC data with that of the corresponding authentic standard. The FD/MS spectrum of M-7 from rat urine showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 371, along with additive ions at m/z 393 [M + Na]\(^+\) and at m/z 409 [M + K]\(^+\). The FD/MS spectrum of M-2 showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 451, additive ions at m/z 473 [M + Na]\(^+\) and 489 [M + K]\(^+\), and a diagnostic fragment ion at m/z 371 [M – 80 + H]^+ consistent with the loss of a sulfate portion. 1 H-NMR spectrum of M-2 showed that the 2(1H)-quinolinone nucleus and the side chain were unaltered except for the disappearance of a singlet integrating three protons, indicating that one of the two methoxy groups in the 3,4-dimethoxybenzylamino portion was O-demethylated. The nature of the 4'-substituent was determined based on the downfield shift of 0.54 ppm corresponding to the adjacent 5'-proton (M-2: 7.39 ppm; parent: 6.85 ppm). When M-2 was treated with arylsulfatase, the retention time of the hydrolysate was identical to that of the corresponding authentic standard of M-7 by HPLC cochromatography. Based on these results, metabolites M-1, M-2, and M-7 were assigned the structures shown in fig. 3 {M-1: 6-[2-hydroxy-3-(4-hydroxy-3-methoxybenzylamino)propoxy]-2(1H)-quinolinone-4'-sulfate, MW = 450; M-7: 6-[2-hydroxy-3-(4-hydroxy-3-methoxybenzylamino)propoxy]-2(1H)-quinolinone, MW = 370}.

M-3. The FAB/MS spectrum of M-3 showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 236. The parent compound has an even-numbered molecular weight (MW = 384); therefore, the proposed odd-numbered molecular weight of M-3 indicated that this metabolite had lost one nitrogen atom. This observation was also supported by the 1 H-NMR spectrum, providing evidence that a 3,4-dimethoxybenzyl portion was lost (table 1). The amine part in the side chain was considered to have been lost, in accordance with the mass spectral data. Based on this information, as well as on comparison of spectra and cochromatography with those of corresponding authentic standard, M-3 was identified as 6-(2,3-dihydroxypropoxy)-2(1H)-quinolinone (MW = 235), as shown in fig. 3.

M-4. The FD/MS spectrum of M-4 showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 577 and a diagnostic fragment ion at m/z 401 [M – 176 + H]^+, which was assumed to be consistent with the loss of a glucuronic acid portion. These findings suggested that M-4 was a glucuronide of a hydroxylated form of toborinone. 1 H-NMR data showed that both the 3,4-dimethoxybenzylamino and the side chain portions were unaltered. The site of conjugation was determined based on the loss of a resonance corresponding to the 8-proton in the 2(1H)-quinolinone nucleus and the slight upfield shift of 0.26 ppm of the adjacent 7-proton from 7.13 ppm (parent) to 6.87 ppm (M-4). After treatment with \(\beta\)-d-glucuronidase, the hydrolysate released had the same retention time as the authentic standard of 8-hydroxytoborinone by HPLC cochromatography. Based on this evidence, M-4 was assigned the structure shown in fig. 3 {6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-2(1H)-quinolinone-8-glucuronide, MW = 576}.

M-5. The FAB/MS spectrum of M-5 showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 577 and an additive ion \([M + Na]^+\) at m/z 299. The even-numbered molecular weight indicated that the nitrogen atom in the side chain remained in place. This observation was also supported by the 1 H-NMR spectrum, providing that the carbon atom adjacent to this methyl group had no protons. Based on this evidence, as well as on the comparison of spectra and HPLC cochromatography with the corresponding authentic standard, M-5 was identified as 6-(3-acetylamino-2-hydroxypropoxy)-2(1H)-quinolinone (MW = 276), as shown in fig. 3.

M-6 and M-8. The FD/MS spectrum of M-6 showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 451 and a diagnostic fragment ion \([M – 80 + H]^+\) at m/z 371 consistent with the loss of a sulfate portion, similar to M-2. A distinguishing feature in the 1 H-NMR spectrum was the disappearance of a singlet signal integrating three protons, indicating that one of the two methoxy groups was demethylated. The nature of the electron-withdrawing 3° substituent was determined based on the downfield shift of 0.64 ppm for the adjacent 2° proton from 6.93 ppm in the parent to 7.57 ppm in M-6. M-6 was treated with arylsulfatase, and the retention time of the hydrolysate released was identical to that of the corresponding authentic standard having an O-demethylated structure at the 3°-position in the 3,4-dimethoxybenzyl portion, which was named M-8. In comparison, the FD/MS spectrum of M-8 purified from rat urine showed a protonated quasi-molecular ion \([M + H]^+\) at m/z 371 and additive ions at m/z 393 [M + Na]\(^+\) and m/z 409 [M + K]\(^+\). Based on these results, metabolites M-6 and M-8 were assigned the structures shown in fig. 3.

**Fig. 2.** HPLC 14C radiochromatograms of biological samples after intravenous administration of [14C]toborinone at 10 mg/kg to rats and dogs.

HPLC conditions are described in Materials and Methods (Analytical A2). (Top) Rat plasma 1 hr postdosing. (Bottom) Dog feces collected during 0–24 hr postdosing.
TABLE 1
Proton NMR spectra of toborinone and its metabolites

Samples were dissolved in deuterated dimethyl sulfoxide (DMSO-\textsubscript{d\textsubscript{6}}) or deuterium oxide (D\textsubscript{2}O) and analyzed on a 400 MHz or 500 MHz spectrometer.

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<td>G2'' 3.00</td>
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<td>G3'' 3.24–3.27</td>
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<td>G4'' 3.05</td>
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<td>Gly-\beta 2.01</td>
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<td>G5'' 3.34</td>
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<td>G5'' 3.19</td>
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<td>Gly-\gamma 2.37</td>
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</table>

Cys, cysteine or cysteinyl unit of glutathione; Gly, glyciny lunit of glutathione; \(\gamma\)-Glu, \(\gamma\)-glutamyl unit of glutathione.

\(a\) M-7 was prepared by enzymatic hydrolysis of M-1.
\(b\) M-14-1 and M-14-2 were assigned as the respective glucuronides of \(\text{R}(-)\) - and \(\text{S}(-)\) -isomers of toborinone.
\(c\) G1-5 correspond to the positions of protons for the glucuronic acid portion as shown in fig. 3.
\(d\) Values denote interference with H\textsubscript{2}O protons.
(M-6: 6-[2-hydroxy-3-[(3-hydroxy-4-methoxybenzylamino)propoxy]-2(1H)-quinolinone-3'-sulfate, MW = 450; M-8: 6-[2-hydroxy-3-[(3-hydroxy-4-methoxybenzylamino)propoxy]-2(1H)-quinolinone, MW = 370).

M-9. The negative-ion FAB/MS mass spectrum of M-9 showed a quasimolecular ion [M – H]⁻ at m/z 248. Attempts to obtain the positive-ion FAB/MS and FD/MS spectra were unsuccessful. The ¹H-NMR spectrum showed that the 2(1H)-quinolinone nucleus was unaltered, and that there were no soluble protons except for signals at 4.28 and 4.33 ppm attributed to 1° methylene protons and at 4.43 ppm attributed to a 2° methine proton. The ¹³C-NMR spectrum showed evidence that the 2(1H)-quinolinone nucleus was unaltered and revealed two signals, one methylene carbon and one methine carbon (both at 71.9 ppm), corresponding to the 1° and 2° carbons in the side chain, which was consistent with the results of ¹H-NMR measurement. In addition, a signal corresponding to a carbonyl group (178.2 ppm) was observed in the ¹³C-NMR spectrum. Based on these results, as well as on the comparison of the spectral data and cochromatography with the corresponding authentic standard, M-9 was identified as 2-hydroxy-3-[2(1H)-quinolinon-6-yloxy]propanoic acid (MW = 249), as shown in fig. 3.

M-10. The FAB/MS spectrum of M-10 showed a protonated quasimolecular ion [M + H]⁺ at m/z 504 and diagnostic fragment ions at m/z 417, 385, and 151. The ion at m/z 417 [M – 86]⁺ was considered to correspond to a loss of the adduct portion. The ¹H-NMR spectrum of M-10 showed no changes other than the lack of signals for the 3-proton in the 2(1H)-quinolinone nucleus, the conversion of a doublet to a singlet (7.67 ppm) for the 4-proton, and newly observed signals attributed to the additional one methylene group protons and one methine group proton. The ¹³C-NMR spectrum of M-10 also indicated signals assigned to the additional one methylene group carbon and one methine group carbon (33.7 and 54.2 ppm, respectively), as well as a carbonyl group carbon (174.2 ppm). The HMBC spectrum showed a long-range coupling between additional methylene protons in the substituent and the 3-carbon signal, suggesting that M-10 might have obtained some adduct at the 3-position. The high-resolution FAB/MS spectrum of M-10 showed a quasimolecular ion at m/z 504.1826 [M + H]⁺, with an empirical formula of C₂₂H₂₄N₂O₇S (error, +2.2 mmu), indicating that the substituent at the 3-position was a cysteine. Based on this evidence, metabolite M-10 was assigned the structure shown in fig. 3 (3-(cystein-S)-yl)-6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-2(1H)-quinolinone, MW = 503).

M-11. The positive-ion mode LC/FAB mass spectrum of M-11 showed a protonated quasimolecular ion [M + H]⁺ at m/z 502 and a diagnostic fragment ion [M – 86]⁺ at m/z 435 corresponding to the loss of a conjugated portion. The negative-ion mode FAB/MS spectrum of M-11 showed a quasimolecular ion [M – H]⁻ at m/z 520. In the early stage, a problem arose in which M-11 was decomposed during NMR measurement. As a result, measurements of several spectra of the decomposed product (MD-12) also assisted in the characterization of M-11. The mass spectra of MD-12 by positive-ion mode LC/FAB/MS and negative-ion mode FAB/MS, respectively, showed ions at m/z 401 and m/z 399 that were attributed to [M + H]⁺ and [M – H]⁻. The high-resolution FAB/MS spectrum of MD-12 showed a quasimolecular ion [M + H]⁺ at m/z 401.1719 with an empirical formula of C₂₂H₂₄N₂O₇S (error, +0.6 mmu). Chemical shift for 3-carbon appeared in the low field (116.4 ppm) compared with those of 3-substituent M-10 (M-10: 3-C, 128.2 ppm; 4-C, 137.9 ppm). The MS and NMR spectra of MD-12 indicated that substitution by a hydroxyl group occurred at the 3-position. NMR spectra of intact M-11 could be obtained by lowering the temperature (5°C). ¹H-NMR data for M-11 showed evidence that the 3,4-dimethoxybenzylamino...
portion and side chain were unaltered, that a new singlet at 4.32 ppm integrating two protons had appeared, accompanied by the loss of the aromatic signals corresponding to protons for the 3- and 4-positions, indicating that these positions were substituted, and that there were additive signals at 3.73 ppm that corresponded to protons of one methine group and signals at 3.05 and 3.09 ppm that corresponded to protons of one methylene group. The 13C-NMR spectrum of M-11 showed two methine carbon signals at 70.4 and 47.1 ppm attributed to 3- and 4-position carbons, indicating that these positions were saturated. Further assignment of 1H and 13C resonances was achieved by the HMBC experiment for multiple-bond connectivity. The proton signal at 4.32 ppm showed connectivities to the 2-, 4a-, and 5-carbon signals at 170.8, 123.2, and 116.6 ppm, respectively. The methylene proton signal at 3.05 ppm in the substituent showed connectivity to the signal at 47.1 ppm assigned to the 4-carbon. In the HMBC spectrum of MD-12, the 4-carbon signal did not show connectivity to methylene proton signals in the adduct. The high-resolution FAB/MS spectrum of M-11 showed a quasimolecular ion at m/z 522.1926 with an empirical formula of C24H32N3O8S (error, +1.6 mmu), indicating that the substituents were a cysteine and a hydroxyl group. Based on these results, M-11 was assigned the structure shown in fig. 3 {4-(cysteinyl-S-yl)-6-[3-(3,4-dimethoxybenzylamino)-2-hydroxypropoxy]-3-hydroxy-3,4-dihydro-2H-quinolinone, MW = 521}. The stereochemistry of the 3- and 4-positions was not established.

M-13. M-13 had twin peaks on the HPLC chromatograms (HPLC condition Analytical A2, data not shown). Incubation of S-(-)-toborinone formed a metabolite showing only a single peak on the chromatogram that corresponded to the earlier of the two peaks seen with racemic toborinone (data not shown). The positive-ion mode LC/FAB mass spectrum of M-13 (fig. 4, middle) showed a quasimolecular ion [M + H]+ at m/z 708 and diagnostic fragment ions at m/z 558, 402, 308, 261, and 151. The negative-ion mode LC/FAB/MS spectrum of
M-13 (fig. 4, bottom) showed a quasimolecular ion \([M+H]^+\) at \(m/z\) 706 and diagnostic fragment ions at \(m/z\) 556, 306, and 259. The ions at \(m/z\) 308 (positive-ion mode) and \(m/z\) 306 (negative-ion mode) were, respectively, thought to be positive and negative quasimolecular ions of the additive group. The positive-ion mode high-resolution FAB/MS spectrum of M-13 showed a quasimolecular ion \([M+H]^+\) at \(m/z\) 708.2555 with an empirical formula of \(C_{15}H_{22}N_3O_7S\) (error, +0.4 mnu). The \(^1H\)-NMR spectrum (fig. 4, top) of M-13 showed the presence of new characteristic signals corresponding to nonexchangeable protons of the cysteiny1, glycinyl, and \(\gamma\)-glutamyl units of gluthathione, accompanied by the loss of the aromatic signals for the 3- and 4-positions, thus indicating that both of these positions were substituted. Allylic decoupling (-1 Hz) between the signal for the 5-position and the signal at 4.20 ppm indicated that the signals at 4.20 and 4.24 ppm were attributed to the 4- and 3-protons, respectively. In the HSQC measurement, the signal at 4.24 ppm (3-position) showed connectivity to the carbon signal at 49 ppm, and the signal at 4.20 ppm (4-position) showed connectivity to the signal at 68 or 73 ppm (this ambiguity was due to the overlapping of signals near 4.2 ppm in the \(^1H\)-NMR spectrum). Based on these results, M-13 was assigned the structure shown in fig. 3. \(3\{\text{glutathion-S-yl}\}-6\{3,4\)-dimethoxybenzylamino\}-2-hydroxypropoxy\}-4-hydroxy\}-3,4-dihydro\}-2(1H)\}-quinolinin\}, \(MW = 707\), suggesting that the glutathione and hydroxyl groups were, respectively, attached to the 3- and 4-positions, thus resulting in the opposite regiochemistry from that of M-11. Attempts to isolate the presumed 4-glutathione conjugate was unsuccessful.

M-14. The FD/MS spectrum of M-14-1 showed a quasimolecular ion \([M+H]^+\) at \(m/z\) 561 and a diagnostic fragment ion \([M-176+H]^+\) at \(m/z\) 385 consistent with the loss of a glucuronic acid portion. The FD mass spectrum of M-14-2 showed also the same ions, except for an additional additive ion \([M+Na]^+\) at \(m/z\) 583. A distinguishing feature in the \(^1H\)-NMR spectra of M-14-1 and M-14-2 was the presence of several additive protons corresponding to a glucuronic acid in each case. The chemical shift of the anomeric proton was 6.99 ± 0.14 (55.8) ppm, 1.70 ± 0.24 (56.2), 2.09 ± 0.23 (7.01), 0.10 ± 0.01 (0.34), 0.13 ± 0.02 (0.45), 0.46 ± 0.03 (1.53), 2.10 ± 0.33 (6.95), 0.11 ± 0.02 (0.36), 1.01 ± 0.09 (3.42), 18.76 ± 0.87 (62.73), 24.56 ± 1.70 (36.57). Values represent the mean ± SE from data from three animals. Values in parentheses indicate the percentage of radioactivity of each component to the total radioactivity in each specimen. ND, not detected.

Discussion

The present study has demonstrated the structural characterization of metabolites and elucidated the metabolic pathways of toborinone...
The predominant route of elimination of radioactivity was via M-7 and (feces) in both rats and dogs.

(M-7)

Activity in each specimen. ND, not detected.

Indicate the percentage of radioactivity of each component to the total radioactivity of ([14C]toborinone) after intravenous administration in rats and dogs.

M-10 cleavage yielding cysteine conjugates ([14C]glucuronide (M-4)); sulfates: M-5); deamination and subsequent redox reactions; M-2; and M-6) and subsequent conjugation (glucuronide: M-4); (fig. 3). M-3 was observed only in rats, whereas M-10 and M-11 were observed only in dogs, suggesting species-selective metabolite formation of toborinone. The predominant component of radioactivity after intravenous administration of ([14C]toborinone was the parent drug in every biological specimen both in rats and dogs. Toborinone and most of its metabolites could be detected by HPLC and fluorescence detection. Simultaneous UV detection at 254, 280, and 340 nm was also helpful in distinguishing the metabolites both from endogenous materials and from other metabolites having similar retention times or being coeluted. These observations were especially applicable to the compounds possessing substituents in the 2(1H)-quinolinone nucleus, namely M-4, M-10, M-11, M-13, and a decomposed product of M-11 (named MD-12). Saturation of the 3- and 4-positions caused the disappearance of UV absorption near 340 nm

### TABLE 3

Concentration of total radioactivity and percentage of radioactivity of each component to total radioactivity in each specimen after a single intravenous dose of 10 mg/kg [14C]toborinone to male rats (10 mg/37 MBq)

<table>
<thead>
<tr>
<th>Component</th>
<th>Plasma 10 Min</th>
<th>Plasma 1 Hr</th>
<th>Liver 10 Min</th>
<th>Liver 1 Hr</th>
<th>Kidney 10 Min</th>
<th>Kidney 1 Hr</th>
<th>Heart 10 Min</th>
<th>Heart 1 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μg eq/g tissue)*</td>
<td>±0.907</td>
<td>±0.206</td>
<td>±3.628</td>
<td>±0.229</td>
<td>±11.048</td>
<td>±1.028</td>
<td>±1.491</td>
<td>±0.090</td>
</tr>
</tbody>
</table>

M-1 7.61 7.05 18.08 12.39 1.55 4.69 1.26 1.65
M-2 3.10 11.94 12.11 16.33 1.10 4.94 0.40 2.53
M-3 1.26 2.33 2.04 1.72 0.58 5.46 1.27 2.97
M-4 0.09 0.05 0.47 ND ND 0.18 ND ND
M-5 0.04 0.08 0.59 0.13 ND ND ND ND
M-6 1.48 6.46 1.74 1.49 0.45 1.46 0.21 1.20
M-7 0.51 0.48 6.88 6.36 3.01 4.57 0.08 0.15
M-8 0.09 0.39 0.14 0.21 ND ND ND ND
M-9 0.72 0.45 0.60 0.42 1.08 2.46 0.07 ND
Toborinone 80.87 67.05 49.00 52.89 86.00 70.08 84.16 78.29

Total 14C 95.77% 96.28% 91.65% 91.94% 93.77% 94.16% 87.45% 86.79%

Values represent the mean of data from four animals. ND, not detected.

a Values represent the mean ± SE of data from four animals.

### TABLE 4

Concentrations of total radioactivity and percentage of radioactivity of each component to total radioactivity in each specimen after a single intravenous dose of 10 mg/kg [14C]toborinone to male dogs (10 mg/7.4 MBq)

<table>
<thead>
<tr>
<th>Component</th>
<th>Plasma 10 Min</th>
<th>Plasma 1 Hr</th>
<th>Liver 10 Min</th>
<th>Liver 1 Hr</th>
<th>Kidney 10 Min</th>
<th>Kidney 1 Hr</th>
<th>Heart 10 Min</th>
<th>Heart 1 Hr</th>
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<td>1 Hr</td>
</tr>
<tr>
<td>(μg eq/g tissue)*</td>
<td>67.05</td>
<td>67.05</td>
<td>66.24</td>
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<td>66.24</td>
<td>65.55</td>
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</table>

M-1 7.61 7.05 18.08 12.39 1.55 4.69 1.26 1.65
M-2 3.10 11.94 12.11 16.33 1.10 4.94 0.40 2.53
M-3 1.26 2.33 2.04 1.72 0.58 5.46 1.27 2.97
M-4 0.09 0.05 0.47 ND ND 0.18 ND ND
M-5 0.04 0.08 0.59 0.13 ND ND ND ND
M-6 1.48 6.46 1.74 1.49 0.45 1.46 0.21 1.20
M-7 0.51 0.48 6.88 6.36 3.01 4.57 0.08 0.15
M-8 0.09 0.39 0.14 0.21 ND ND ND ND
M-9 0.72 0.45 0.60 0.42 1.08 2.46 0.07 ND
Toborinone 80.87 67.05 49.00 52.89 86.00 70.08 84.16 78.29

Total 14C 95.77% 96.28% 91.65% 91.94% 93.77% 94.16% 87.45% 86.79%

Values represent the mean of data from two animals. ND, not detected.

### TABLE 5

Quantitation of toborinone and its metabolites in dog excreta after a single intravenous dose of 10 mg/kg [14C]toborinone (10 mg/7.4 MBq)

<table>
<thead>
<tr>
<th>Component</th>
<th>Urine (0–48 Hr)</th>
<th>Feces (0–48 Hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>0.63 (2.46)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>M-2</td>
<td>0.63 (2.45)</td>
<td>0.61 (0.90)</td>
</tr>
<tr>
<td>M-3</td>
<td>0.73 (2.88)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>M-5</td>
<td>0.09 (0.35)</td>
<td>0.15 (0.22)</td>
</tr>
<tr>
<td>M-6</td>
<td>0.04 (0.16)</td>
<td>0.23 (0.33)</td>
</tr>
<tr>
<td>M-7</td>
<td>0.20 (0.80)</td>
<td>2.43 (3.55)</td>
</tr>
<tr>
<td>M-8</td>
<td>0.02 (0.06)</td>
<td>0.04 (0.06)</td>
</tr>
<tr>
<td>M-9</td>
<td>1.96 (7.69)</td>
<td>2.90 (4.25)</td>
</tr>
<tr>
<td>M-10</td>
<td>0.24 (0.94)</td>
<td>13.04 (19.10)</td>
</tr>
<tr>
<td>M-11</td>
<td>0.28 (1.12)</td>
<td>4.58 (6.70)</td>
</tr>
<tr>
<td>M-14</td>
<td>0.21 (0.82)</td>
<td>1.65 (2.41)</td>
</tr>
<tr>
<td>Toborinone</td>
<td>18.43 (72.57)</td>
<td>33.79 (49.50)</td>
</tr>
</tbody>
</table>

Total 23.46% (92.30%) 59.42% (87.02%)

Values represent the mean of data from two animals. ND, not detected.

6 Values represent the mean ± SE of data from four animals.
and of fluorescence for M-11 and M-13. The structural change in the aromatic region of the 2(1H)-quinolinone nucleus explains the change in the UV spectrum because the chromophore has altered. The NMR resonances for metabolites, including MD-12, were compared with those of the parent compound, and distinguishing features were demonstrated: the elimination of the signal corresponding to the H-3 proton for M-10 and MD-12; the presence of methine groups at both the 3- and 4-positions for M-11 and M-13; and large upfield shifts of proton and carbon signals for the 3- and 4-positions for M-11 and M-13. These NMR spectral alterations were in accordance with both the structural and UV spectral alterations. A similar phenomenon concerning the alteration of UV spectra and NMR resonances has been seen with the quinoline derivative verlukast, with substitution occurring at the 5- and/or 6-positions in the quinoline nucleus (16). Various polar metabolites of toborinone were hardly extracted into low-polar organic solvent. However, wide distribution in the molecular size of the toborinone metabolites suggested the possibility of using the characteristics of the metabolites themselves for isolation. The use of Sephadex LH-20 resin, which has a molecular sieve effect, helped to confirm indirectly the approximate structures of the metabolites by the order of elution before NMR and/or MS measurements. Toborinone metabolites could be classified into four fraction groups according to the order of elution in the Sephadex LH-20 resin separation procedure: 1) M-1, M-4, and M-14 as glucuronic acid conjugates and M-13 as a glutathione conjugate were eluted (MW range: 546–707); 2) M-2 and M-6 as sulfooconjugates and M-11 and M-10 as cysteine conjugates were eluted (MW range: 450–521); 3) the parent compound M-7, and M-8 were eluted (MW range: 370–384); and 4) M-3 and M-5 were eluted (MW range: 235–276). The order of elution was approximately corresponded to molecular weight, in decreasing order. These characteristics of the metabolites themselves helped to identify metabolites indirectly, especially those metabolites for which no reference standards were available, including the conjugated metabolites M-1, M-2, M-4, M-6, M-10, M-11, M-13, and M-14.

In rats, the major metabolites were M-7 in the feces, M-1, M-2, and M-6 in both the bile and plasma, and M-1, M-2, and M-7 in the liver. These observations suggested that the formation of M-7 from toborinone and the successive phase II reactions yielding M-1 and M-2 were all conducted in the liver. Most of the conjugated metabolites of toborinone observed in both rats and dogs were excreted in the bile (MW range: 400–800). These observations were consistent with the established observations of the molecular weight threshold for biliary excretion (325 ± 50 in rats and 350 ± 50 in dogs) (17–20). M-7 might be present in the liver as a precursor of both M-1 and M-2, and it may have remained in the liver without being transferred to the bile or to systemic circulation due to its low molecular weight (MW = 370) and relatively low polarity. However, M-7 in the intestines, present as a deconjugated product of M-1 and M-2, lacked a polar group by presumed bacterial participation in the intestines, and was excreted in large quantity in the feces without reabsorption. Enterohepatic circulation of toborinone has been reported in rats, although the ratio is low (~15% by 24 hr postdosing) (21). In the kidney, the high radioactivity (10 min) and the metabolite profiles suggested that rapid excretion of radioactivity into the urine occurred and that the metabolic activity in the kidney was likely to be low. Consequently, it was suggested that the major metabolic pathways in the rat were O-demethylation at the 4'-position on the 3,4-dimethoxybenzyl portion (M-7) and the successive phase II reactions yielding M-1 and M-2, and deconjugation of M-1 and M-2 to form M-7. There was a predominance of 4'-O-demethylation over 3'-O-demethylation (which yielded M-6 and M-8), suggesting regiochemistry.

In dogs, the major metabolites were the cysteine conjugates M-10 and M-11 observed in fecal excreta. The major metabolic pathway in dogs was apparently different from that in rats. The presence of metabolite M-13, having a structure corresponding to an intermediate between the parent compound and metabolite M-10, provided evidence of a possible mercapturic acid pathway. The formation of M-10 and M-11 may occur through an attack of glutathione on the 3- or 4-position carbon of the presumed 3,4-epoxide intermediate to form the corresponding glutathione adducts, followed by several subsequent deaminations, resulting in the formation of cysteine conjugates (16, 22–27). The relative stability of the two cysteine conjugates (3-cysteine adduct: M-10, 4-cysteine adduct: M-11) and the presumable steric hindrance at the 4-position may explain why the 3-glutathione and 3-cysteine adducts were formed with greater predominance than the 4-glutathione and 4-cysteine adducts. Toborinone formed glutathione adducts at both the 3- and 4-positions, suggesting that the difference in electrophilic nature between the 3- and 4-carbons was not large. It has been reported that, although the quinoline derivative verlukast yielded a 5° glutathione adduct, no 6° glutathione adduct was observed (16), and the opposite regiochemistry might be expected with naphthalene and anthracene (28), thus suggesting a difference in electron density between the two positions in each case. Thus, the regiochemistry, stereochemistry, and mechanism of subsequent nucleophilic substitution or deamination reactions may determine which metabolite is formed. The amount of M-13 formed was low, and other peaks formed were lower than that of M-13. Even the addition of glutathione as a reagent to the incubation mixture did not affect the amount of M-13 formed, suggesting that the amount of glutathione originally contained in the S9 fraction was likely to be enough for the formation of M-13, unlike the case when using purified microsomal fraction. The in vitro experiment showed that both the formation of the presumed 3,4-epoxide of the 2(1H)-quinolinone nucleus and the glutathione attack might occur in the liver; however, no other metabolite was obtained except for M-13, which was involved in the mercapturic acid pathway and further successive pathways, suggesting that it was not clear whether these deamination reactions yielding cysteine conjugates might occur in the liver or in the intestines. The glutathione conjugate M-13, with a higher molecular weight of 707, would be excreted via the bile. M-10 and M-11, with respective molecular weights of 403 and 421, were observed in dog feces, but not in the urine, which seems to satisfy the requirement for the molecular weight threshold for biliary excretion (17–20), as described in the profile for rats. However, these thresholds might be limited as far as biliary excretion is concerned; therefore, the overall metabolic profile should be constructed by investigation of the metabolite profiles comprising metabolism in the intestines, including bacterial participation, as well as enterohepatic circulation.

In vitro experiments with dog liver S9 fractions, the formation ratio of S(−)−R(−)-M-13 (glutathione adducts) from racemic toborinone was ~1:1, suggesting no substrate stereoselectivity in vitro. The absence of interconversion was observed in M-13, M-4, M-10, M-11, and other metabolites. These findings were used to avoid overlapping of signals in the 1H-NMR measurements by separate administration of the enantiomers. A previous study revealed no interconversion for the parent drug in rats, dogs, and monkeys (29). However, in vivo, on dosing of the racemate to dogs, the amount of S(−)-M-10 was larger than that of R(+)-M-10. After intravenous dose of racemic toborinone, the relationship of the concentrations of S(−) and R(+) toborinone in the plasma was R(+) > S(−) in rats, S(−) > R(+) in dogs, and R(+) ≥ S(−) in humans (5, 29). Incidentally, in an in
vitro study, R- (+) -toborinone was showed to be ~ 10 times more potent than S- (−) -toborinone (30). Thus, differences in regioselectivity, enantioselectivity, and species specificity may all play roles in the in vivo biotransformation of toborinone.

In conclusion, in the metabolism of toborinone in rats and dogs, various phase II metabolites were observed, including glucuronides, sulfates, cysteine conjugates, a glutathione conjugate, and an acetyl conjugate. The metabolites M-1, M-2, M-4, M-6, and M-14 rendered hydrolysates by β- d-glucuronidase or arylsulfatase; however, M-10, M-11, and M-13 apparently do not produce hydrolysis products. Thus, it was suggested that research on polar metabolites should not be conducted merely by enzymatic hydrolysis experiments. In the present study, each enzymatic hydrolysis for a glucuronide or sulfate metabolite was performed after the characterization had been completed in each intact metabolite by mass and NMR spectroscopy to avoid the possibility of misinterpreting the results.

Acknowledgments. We gratefully acknowledge Ms. Y. Seo (Otsuka Chemical Co., Ltd., Tokushuka Research Institute, Tokushuka, Japan) for FD/MS analysis; Dr. A. Abe and Dr. T. Tajima (Toray Research Center, Kamakura, Japan) for NMR analysis and FAB/MS analysis, respectively; and Dr. E. Ozaki (Toray Research Center, Ohtsu, Japan) for LC/FAB/MS analysis. We would also like to thank Mr. N. Kusumoto and Mr. Y. Abe for their skillful technical assistance.

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