DMD Fast Forward. Published on October 21, 2005 as doi:10.1124/dmd.105.007229
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Slow Elimination of Nonylphenol from Rat Intestine

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Running title: Slow Elimination of Nonylphenol from Intestine

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Manuscript:

Text, 26 pages

Tables, 0

Figures, 6

References, 26

Abstract, 233 words

Introduction, 402 words

Discussion, 1584 words

Abbreviations: UGT, UDP-glucuronosyltransferase; HPLC, high performance liquid chromatography

Abstract

Nonylphenol, a possible endocrine disrupter, tends to persist in rat liver tissue after detoxification as a glucuronide conjugate by UDP-glucuronosyltransferase 2B1 expressed in the liver. In the intestine, however, the metabolism and dynamics of nonylphenol remain to be elucidated. The objectives of this study were to clarify the metabolism and excretion of nonylphenol having a long alkyl chain in the first barrier intestine, and to estimate whether the nonylphenol alkyl chain governs the speed of excretion from intestinal tissue. Organ tissue glucuronidation activity toward alkylphenols (C2, C9) was investigated using microsomes prepared from intestinal tissue. To elucidate the elimination pathway of alkylphenols (C2, C4, C6, C9), a perfusion study was conducted on everted intestine. After oral administration (5 mg) of alkylphenols (C2, C9) to rats, gastrointestinal contents and related organ-tissues (gastrointestinal tissue, liver, kidney), blood, and urine were analyzed for alkylphenols (C2, C9) and glucuronides. The intestine showed strong glucuronidation activity toward alkylphenols (C2, C9). In everted intestinal assay, nonylphenol was glucuronidated within the intestinal wall, as is the case for other alkylphenols (C2, C4, C6), but nonylphenol-glucuronide was not excreted from intestinal tissue. Orally administered nonylphenol remained for long periods in gastrointestinal tissue as both the parent compound and glucuronide. The present study confirmed that intestinal tissue possesses an alkylphenol elimination system employing UDP-glucuronosyltransferase; however, this system is impaired by the marginal transport of alkylphenol-glucuronide possessing long alkyl chain, such as nonylphenol.

Introduction

Nonylphenol, possessing long alkyl chain, is used in a wide variety of detergents and plastics and has been reported to be environmentally persistent (Petrovic *et al*., 2002; Ying *et al*., 2002). Nonylphenol is also a possible endocrine disrupter, based on data regarding its estrogenic effects in MCF7 cell proliferation assays (Soto *et al*., 1991), binding to the estrogen receptor (White *et al*., 1994) and uterotropic assays in mice (Shelby *et al*., 1996). Exposure of male rainbow trout (*Oncorhynchus mykiss*) to four different alkylphenolic chemicals, including nonylphenol, resulted in synthesis of vitellogenin, a process normally dependent on endogenous estrogens, and a concominant inhibition of testicular growth (Jobling *et al*., 1996). The number of 4-5 day estrous cycles in rats was reduced during 25-day exposure to nonylphenol (100 mg/kg) by oral gavage (Laws *et al*, 2000). Early neonatal exposure to nonylphenol has been reported to cause dysfunction of postpubertal reproductive function in female rats and to disrupt development of gonads in male and female rats (Nagao *et al*., 2000).

In vertebrates, endogenous and exogenous compounds are metabolized by phase I and phase II enzyme reactions such as oxidation, sulfation and glucuronidation, with glucuronidation being the major pathway for metabolism of both endogenous and exogenous compounds in vertebrates (Dutton, 1980). It has been reported that the nonylphenol metabolite nonylphenol-glucuronide is only able to bind weakly to estrogen receptor (Moffat *et al*., 2001). Therefore, in order to elucidate the mechanism responsible for the adverse effects of nonylphenol on reproductive organs, it is essential to clarify both the actual metabolism and distribution of the compound in the body before its arrival at the target organs, such as the testis and the uterus.

Nonylphenol introduced orally into the body must pass through the intestine and liver before arriving at the reproductive organs, where irreversible damage may be inflicted. Previously, we found that nonylphenol excretion from liver was difficult, despite the fact that the compound was effectively metabolized to glucuronide conjugate by UDP-glucuronosyltransferase (UGT) 2B1 (Daidoji *et al*., 2003). In the intestine, however, which provides the first barrier against ingested drugs, the metabolism and dynamics of nonylphenol have not been elucidated.

We focused on the characteristics of metabolism and excretion of nonylphenol in the intestine in order to evaluate the effects of the compound on target organs. The present study was conducted to estimate whether the alkyl chain belonging to nonylphenol governs the speed of excretion from intestinal tissue.

Materials and Methods

Chemicals. Cholic acid was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), then purified further and converted to its sodium salt (Imai, 1979). UDP-glucuronic acid was obtained from Nakarai Pharmaceutical Co., Ltd. (Kyoto, Japan); 4-ethylphenol (C2), 4-*n*-butylphenol (C4), 4-*n*-hexylphenol (C6), and 4-*n*-nonylphenol (C9) from Kanto Chemical Co., Ltd. (Tokyo, Japan); high-performance liquid chromatography (HPLC)-grade acetonitrile from Labscan Co., Ltd. (Dublin, Ireland); and β-glucuronidase from Sigma-Aldrich Co., Ltd. (St. Louis, MO). Nonylphenol-glucuronide purified from resultant supernatant after microsomal reaction was identified by liquid chromatography coupled to mass spectrometry (LC-MS), and quantified by HPLC by using the difference between β-glucuronidase-treated sample and untreated sample. Nonylphenol-glucuronide was used as a standard compound. All other reagents were of the highest grade available.

Animals. Male Sprague-Dawley rats, 9–13 weeks old (300–350 g), were used in all experiments. The rats were housed under standard conditions, given food and water *ad libitum*, and handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which are based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Preparations of Microsomes. The rats were anesthetized by intraperitoneal injection of 60% urethane and euthanized by exsanguination. The small and large intestines each were excised and individually minced and homogenized with 4 volumes of 1.15% KCl solution (mass/vol.) containing 10 mM EDTA (pH 7.4). The homogenate was centrifuged at 9000 g for 15 min at 4 °C, and

the supernatant fraction was centrifuged at 105,000 g for 60 min at 4 °C to isolate the microsomes. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

UGT Enzyme Analysis. The microsomes of each organ tissue were activated with a final concentration of 0.05% sodium cholate. UDP-glucuronosyltransferase activitiy was determined by incubation (1 h) of the microsomes of each organ tissue at 37 °C in 0.1 M Tris/HCl buffer (200 μl, pH 7.4) containing lysophosphatidylcholine (50 μg), $MgCl_2$ (5 mM), ethylphenol or nonylphenol (0.5 mM each), and UDP-glucuronic acid (3 mM). Linearity of the enzyme reactions was confirmed during incubation (data not shown). In preparation for HPLC assay for the substrate metabolites, acetonitrile (200 µl) was added to the reaction solution (200 µl), and the mixture was centrifuged at 9000 xg for 10min at room temperature. The supernatant was eluted by HPLC as described below. Ethylphenol-glucuronide and nonylphenol-glucuronide produced by UGT enzyme reaction were verified by β-glucuronidase reaction as explained below.

Metabolite Verification. After UGT enzyme analysis, the resultant medium (100 μl) was mixed with β-glucuronidase (2.5 mg/ml) in 0.5 M acetate buffer solution (20 μl, pH 4.5) and incubated for 30 min at 37 °C (Shibata et al., 2002). Acetonitrile (40 μl) was added to the reaction solutions, and the mixture was boiled for 5 min. This mixture was centrifuged for 10 min at 9000 g in room temperature. The supernatant was eluted by HPLC according to the analytical condition described below.

Preparation of Everted Intestine. Krebs Ringer's bicarbonate buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) was used in all experiments. The buffer solution was aerated by 95% O_2 +

5% CO_2 and the pH was adjusted to 7.4. The rats were anesthetized by intraperitoneal injection of 60% urethane, euthanized by exanguination, and the jejunum, ileum, cecum, and colon were flushed with cold Krebs Ringer's buffer. The bowels of the animals were excised and prepared according to a modification of the segmentation and eversion method described previously (Inoue et al., 1999). Briefly, with the exception of the duodenum, the excised small intestine was lavaged and divided into four sections of equal length as quickly as possible. The distal portion of each section was excised and trimmed to 15 cm and designated I, II, III, and IV in distal order, with segment I being from jejunum and segment IV from the distal ileum. In the same manner, the cecum was excised, washed; the colon was excised, washed, and trimmed to 10 cm taken from the distal end. The six trimmed segments were turned inside out and fixed on a polyethylene tube in the mucosal buffer solution (40 ml). Serosal buffer solution (40 ml) was pumped through the everted bowels (tube pump MP-32N; EYELA, Tokyo, Japan) at 5 ml per min via polyethylene tubes. Alkylphenols (C2, C4, C6, C9) were added individually to the mucosal buffer solution in concentrations of 50 μM, and reaction products were collected independently from the serosal and mucosal sides at 100 min after each addition of the compound. The mucosal and serosal samples collected were supplemented with 25% acetonitrile for ethylphenol or with 50% acetonitrile for butylphenol, hexylphenol, and nonylphenol (final conc.), and the mixture was centrifuged at 9000 xg for 10min at room temperature. The supernatants were eluted by HPLC as described below.

The substrate recovery after 100-min incubation with 2 μmol was 53-66%: ethylphenol; 60-81%: butylphenol. After 100-min incubation, we could not obtain stable parameter of hexylphenol and nonylphenol concentration in the mucosal

buffer solution, suggesting that both compounds formed an aggregate during long incubation. Therefore, the recoveries of hexylphenol and nonylphenol could not be calculated.

The Analysis of Gastrointestinal Contents for Alkylphenols (C2, C9) and the Metabolites. Ethylphenol and nonylphenol were independently dissolved in olive oil (25 mg/ml olive oil), and each solution (0.2 ml) was administrated orally to each rat. At 1, 3, and 6 h after alkylphenol (C2, C9 each) administration, the animal were killed under anesthesia with 60% urethane (0.3 ml/100 g of body weight) by exsanguination via the abdominal aorta. After ventrotomy, the small intestine below the duodenum, cecum, and colon were take out, and the small intestine was divided into two sections of equal length (upper and lower part of the small intestine). Each part of the small intestine was slit open with scissors, and the feces and intestinal contents (0.1 g each) were suspended in 0.1 M potassium phosphate buffer (250 μl, pH 7.0). Suspension (50 μl) of the feces and intestinal contents were added to 100% acetonitrile (200 μl), and the mixture was shaken vigorously for 10 min at room temperature and centrifuged at 9000 xg for 10 min at room temperature. A separate aliquot of the supernatant (200 μl) was diluted with redistilled water (1300 μl) and analyzed by HPLC for free alkylphenols (C2, C9) and their metabolites as described below.

The Analysis of Organ-Tissue for Alkylphenols (C2, C9) and the Metabolites. Free alkylphenols (C2, C9) and their metabolites were extracted from the resultant gastrointestinal tissue after removal of digestive contents, liver, kidney, and blood with 67% acetonitrile in 0.06 M acetate buffer solution (pH 4.5). The extracts were centrifuged at 25,000 xg for 10 min at room temperature, and the supernatants were injected into the HPLC system and eluted as described below.

The Analysis of Urine for Alkylphenols (C2, C9) and the Metabolites. Each urine was collected at 3, 6, 8, and 10 h after administration of ethylphenol or nonylphenol (5 mg each). Free alkylphenols (C2, C9) and their metabolites were extracted from the collected urine with 50% acetonitrile in 0.05 M acetate buffer solution (pH 4.5). The extracts were centrifuged at 9000 xg for 10 min at room temperature, and the supernatants were injected into the HPLC system and eluted as described below.

High Performance Liquid Chromatography. The supernatants were filtered by a disposable disc filter (HLC-DISK3; Kanto Co., Ltd. Tokyo, Japan) and the filtered samples were injected into the HPLC system (UV8020, DP8020, SD8022, CO8020, Tosoh, Tokyo, Japan) equipped with Tosoh TSK-gel 80TS C18 reverse-phase column (4.6 mm x 30 cm). The column temperature was maintained at 40 °C, and the samples were eluted on a constant flow rate at 1.0 ml/min. Reaction products were eluted by HPLC with acetonitrile solutions. The ethylphenol products were eluted with the solution of 30% acetonitrile : 70% water : 0.1% acetic acid; the butylphenol products with the solution of 50% acetonitrile : 50% water : 0.1% acetic acid; the hexylphenol products with the solution of 60% acetonitrile : 40% water : 0.1% acetic acid; and nonylphenol with the solution of 70% acetonitrile : 30% water : 0.1% acetic acid. Detection of the eluted metabolites was made by measuring absorption at 222 nm. Nonylphenol and nonylphenol-glucuronide were identified according to the retention time of standard compound; other alkylphenols (C2, C4, C6) were identified according to the same method, and other alkylphenol (C2, C4, C6)-glucuronides were identified according to the retention time of these glucuronides included in the resultant buffer after microsomal reaction (data not shown). Nonylphenol and nonylphenol-glucuronide

were quantified by using standard compound; other alkylphenols (C2, C4, C6) were quantified according to the same method, and other alkylphenol (C2, C4, C6)-glucuronides were quantified by reference to a standard curve based on the amount of the deconjugated each alkylphenol (C2, C4, C6) remaining after β-glucuronidase treatment of the resultant buffer after microsomal reaction.

Results

Alkylphenol (C2, C9) Glucuronidation in Intestinal Microsomes. We focused on glucuronidation in small and large intestines in order to assess the role of the intestine as a first barrier. We noted intense UGT activity toward ethylphenol and nonylphenol in both the small and large intestines using microsomes prepared from rat intestine, with the large intestine showing slightly higher activity than the small intestines (Fig. 1).

Alkylphenol (C2, C4, C6, C9) Glucuronidation and Excretion from Everted Intestine. In order to elucidate the elimination pathway of alkylphenols (C2, C4, C6, C9) from intestinal tissues, a perfusion study was conducted on everted intestine. Upon addition of 50 μM alkylphenol to the mucosal side, concentration in the mucosal fluid decreased over time (data not shown). Alkylphenol absorbed into the intestine was primarily metabolized to alkylphenol-glucuronide with glucuronide excreted into both the mucosal and serosal sides at different levels based on alkyl chain length (Fig. 2). The large amounts of short alkyl chain alkylphenol-glucuronides (C2 and C4) were readily excreted from the intestine, while the small amounts of long alkyl chain alkylphenol-glucuronide (C6) were scarcely excreted from the intestine. Nonylphenol was not detected on either side.

In the small intestine, large amounts of alkylphenol-glucuronide were secreted into the mucosal side and small amounts of glucuronide were secreted into the serosal side. In the large intestine, mucosal secretion of glucuronide was diminished, while serosal secretion increased (Fig. 2).

Furthermore, when we analyzed the intestinal tissue for alkylphenol and glucuronide in order to clarify whether these compounds remain in the tissue,

alkylphenols having short alkyl chain were present at low levels in the tissue, but alkylphenols having long alkyl chain were remained at high levels in the tissue as both free compounds and glucuronide conjugates (Fig. 3).

Alkylphenol (C2, C9) Metabolism in Gastrointestinal Tract. Because of the lack of the information regarding metabolism governed by the gastrointestinal tract *in vivo*, an oral administration study using ethylphenol and nonylphenol was conducted in rats. On oral administration (5 mg) of ethylphenol and nonylphenol to rats, we observed that both ethylphenol and nonylphenol were metabolized to glucuronide conjugates in the gastrointestinal tract. Other metabolites of ethylphenol and nonylphenol than glucuronide were not detected in the gastrointestinal tract (data not shown).

In the stomach, the parent compound ethylphenol was found extensively among gastric contents at 1 h after administration, and had decreased gradually for 6 h, whereas ethylphenol-glucuronide was observed at 1 h after administration, and decreased gradually for 6 h (Fig. 4A-C); high levels of free nonylphenol were also seen in the gastric contents at 1 h after administration, and decreased gradually for 6 h, while nonylphenol-glucuronide was not observed among the stomach contents (Fig. 4D-F). In the small intestine, free ethylphenol and ethylphenol-glucuronide were detected among the intestinal contents at 1 h after administration and decreased with time (Fig. 4A-C); nonylphenol was detected at low levels among intestinal contents at 1 h and 3 h after administration, while nonylphenol-glucuronide was observed among intestinal contents at 1 h and 3 h after administration and decreased over time (Fig. 4D-F). In the cecum, which is known to be a reservoir of enterobacteria having de-conjugating enzyme (β-glucuronidase) activity, little free ethylphenol was detected among the contents,

and no ethylphenol-glucuronide was shown in the cecal contents at any time (Fig. 4A-C); free nonylohenol was detected at later times (3 h and 6 h after administration), but no glucuronide conjugate of nonylphenol was detected among the cecal contents (Fig. 4D-F). In the colon, only amounts of free ethylphenol and ethylphenol-glucuronide were detected among the contents (Fig. 4A-C); free nonylohenol was detected at later times (3 h and 6 h after administration), but no glucuronide conjugate of nonylphenol was detected (Fig. 4D- F). In feces, none of the compounds or metabolites were found (Fig. 4A-F).

Remaining Alkylphenols (C2, C9) and their Glucuronides in Organ-Tissue. We analyzed gastrointestinal tissues for ethylphenol, nonylphenol and their glucuronide conjugates in order to clarify whether the compounds and glucuronides remained in the tissues. We also analyzed the liver for ethylphenol, nonylphenol and their glucuronide conjugates. To follow the fate of glucuronides transported into the inner body, we analyzed the blood and kidney, which are known as excretion organs, for ethylphenol-glucuronide and nonylphenol-glucuronide. On oral administration (5 mg) of ethylphenol and nonylphenol to rats, free alkylphenols (C2, C9) and alkylphenol (C2, C9) glucuronides were detected in gastrointestinal tissue, liver, kidney and blood, although other metabolites of nonylphenol, which were detected as some faint peaks, were only observed in intestinal tissue (data not shown).

In the stomach, free ethylphenol and ethylphenol-glucuronide were detected at all time points (Fig. 5A-C), and although nonylphenol was detected at all time points, nonylphenol-glucuronide was not detected (Fig. 5D-F). In the small intestine, ethylphenol and ethylphenol-glucuronide were detected at 1 h after administration and decreased with time, as was observed for intestine contents (Fig.

5A-C). Nonylphenol was detected in intestinal tissue at 1 h after administration and subsequently decreased, while large amounts of nonylphenol-glucuronide remained in the intestinal tissue at 1 h after administration, but decreased gradually as was seen for the intestine contents. However, nonylphenol-glucuronide remained in the intestinal tissue, even at 6 h after administration (Fig. 5D-F). In the cecum and colon, neither ethylphenol nor ethylphenol-glucuronide were detected at any time (Fig. 5A-C). As was seen for the digestive contents of the cecum, small amounts of free nonylphenol were detected at later times (9 h after administration), but the glucuronide was not detected (Fig. 5D-F). In the liver, no parent compound was detected, but small amounts of ethylphenol-glucuronide were detected at 1 h after administration (Fig. 5A-C). In addition, no free nonylphenol was detected but nonylphenol-glucuronide was abundant in the liver at 1 and 3 h after administration (Fig. 5D-F). In blood and kidney, no parent compounds (C2, C9) were detected, but ethylphenol-glucuronide was observed at all time points (Fig. 5A-C), while nonylphenol-glucuronide was only detected at 1 h after administration (Fig. 5D-F).

Alkylphenol (C2, C9) Excretion into Urine. In this study, we focused on urine as an excretion pathway in addition to the gastrointestinal tract. Ethylphenol orally administered to rats was excreted into urine as unaltered compound or as ethylphenol-glucuronide. No other metabolites of ethylphenol were detected in urine (data not shown). Ethylphenol was expelled into urine at 3 h as free compound, and glucuronide secretion peaked at 8 h after administration (Fig. 6). On the other hand, nonylphenol orally administered to rats was not detected in urine, either as the parent compound or as nonylphenol-glucuronide, at any time (Fig. 6).

DMD Fast Forward. Published on October 21, 2005 as DOI: 10.1124/dmd.105.007229
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Discussion

The present study had three main findings based on experiments on the metabolism and excretion of nonylphenol in Sprague-Dawley rats *in vitro* and *in vivo*. First, the intestine exhibited strong glucuronidation activity toward nonylphenol as well as ethylphenol. Second, in intestinal tissue of everted intestine, although nonylphenol was glucuronidated within the intestinal wall as is the case with other alkylphenols (C2, C4, C6), the resultant glucuronide was not excreted into the mucosal or serosal side. Finally, orally administered nonylphenol was absorbed by the gastrointestinal tract, and persisted for long periods in the gastrointestinal tissue as both the parent compound and the glucuronide.

Alkylphenol Glucuronidation in Rat Intestine. As suggested by the present results, the intestine plays an important role in the metabolism of ethylphenol and nonylphenol. Thus, the intestine not only absorbs nutrients from food, but also plays an important role in the detoxification of drugs and xenobiotics. Rat intestine is reported to express UDP-glucuronosyltransferase (UGT) isoforms (UGT1A1, UGT1A2, UGT1A6, UGT1A7), which catalyze glucuronidation (Shelby *et al*., 2003). It is of particular interest to clarify which isoform is responsible for the glucuronidation of ethylphenol and nonylphenol in rat intestine. We previously showed that nonylphenol glucuronidation in rat liver is mediated by UGT2B1 (EC 2. 4. 1. 17), an isoform of UDP-glucuronosyltransferase (Daidoji *et al*., 2003). However, UGT2B1 is not expressed in gastrointestinal tissue (Shelby *et al*., 2003). Generally, the UGT2B family glucuronidates steroid hormones and bulky phenol compounds (Bock *et al*., 1979; King *et al*., 2000; Turgeon *et al*., 2001), while the UGT1A family glucuronidates bilirubin and smaller phenol compounds, such as 4-nitrophenol (Bock *et al*., 1979; King *et al*., 2000). This substrate specificity leads

us to conjecture that (i) that an unknown UGT belonging to the UGT2B subfamily is responsible for the glucuronidation of nonylphenol, or (ii) that UGT1A1, UGT1A2, UGT1A6 or UGT1A7 is able to glucuronidate nonylphenol. Further studies are necessary to identify the isoform(s) responsible for glucuronidation of nonylphenol in the intestine.

Alkylphenol Glucuronidation and Excretion from Intestine. Because alkylphenols (C2, C4, C6, C9) absorbed into mucosa were readily glucuronidated and excreted into the mucosal and serosal sides at different levels, rat intestine appears to have a characteristic transport system for alkylphenol-glucuronide in enterocytes. This suggests that the transport of alkylphenol-glucuronide, which is too hydrophilic to penetrate the cell membrane, is accomplished by transporters having substrate specificity for alkylphenol-glucuronide.

Recently, ATP-dependent transporters have been described as mediating the transport of glucuronide across the cell membrane (Oude Elferink *et al*., 1995). In rat liver, a member of the ATP-binding cassette family, namely, multidrug resistance associated protein (MRP), is reported to be capable of mediating transmembrane excretion of wide range of amphiphathic compounds, including the glucuronide of bilirubin, estrogen and xenobiotics (Yamazaki *et al*., 1996). In rat intestine, MRP2, localized in the apical domain of enterocytes, is distributed in the proximal intestine (Mottino *et al*., 2001) and MRP3, localized in the basolateral domain, is distributed mainly in the ileum and colon (Rost *et al*., 2002). These reports support the hypothesis that, in the proximal intestine, alkylphenol-glucuronide is transported from inner enterocytes to the mucosal side by a transporter such as MRP2 located in the apical membrane, and that in the distal intestine, alkylphenol-glucuronide is transported from inner enterocytes to the

serosal side by a transporter such as MRP3 located in the basolateral domain. The low elimination efficiency toward alkylphenol-glucuronides possessing long alkyl chain, such as hexylphenol and nonylphenol, indicate that the limiting factor in the elimination of these two derivatives is not UGT activity, but rather is the function of transporters such as MRP2 or MRP3, as large amounts of glucuronides remained in intestinal tissue. The difficulty in transporting alkylphenol-glucuronides possessing long alkyl chain, such as hexylphenol and nonylphenol, indicate that MRP2 and MRP3 are unable to readily transport alkylphenols having long alkyl chain.

We previously observed that in rat liver, the main metabolite of bisphenol A, which is also a known endocrine disrupter, was a glucuronide conjugate, and this glucuronide was immediately excreted into the bile duct (Inoue *et al*, 2001). This indicates that the enzyme activity of UGT glucuronidating bisphenol A is critical for the excretion and clearance of bisphenol A from the body. However, in this study, we believe that the critical factor in the elimination of drug or xenobiotics is not only the metabolism but also the transport system responsible for the excretion of metabolites, as we observed previously in the rat liver (Daidoji *et al*., 2003).

Glucuronidation and Accumulation of Nonylphenol in Gastrointestinal Tract. When nonylphenol and ethylphenol were independently orally administered to rats, nonylphenol persisted as nonylphenol-glucuronide in the gastrointestinal tissue, and ethylphenol was eliminated as ethylphenol-glucuronide from the gastrointestinal tissue into the luminal side, or into urine. The difference in excretion pathways, suggest that gastrointestinal tissue is able to eliminate readily alkylphenols possessing short alkyl chains, but eliminate slowly alkylphenols possessing long alkyl chains, as shown in the everted intestinal

assay.

Generally, glucuronide conjugate excreted into the luminal side of intestine is reabsorbed after deconjugation in the cecum and colon, and moves to the liver via the portal vein. In the liver, the compound derived from the cecum and colon is reconjugated and excreted into bile. A series of these processes are known as enterohepatic circulation. We previously showed that orally administered bisphenol A enters enterohepatic circulation, based on the fact that bisphenol A gulucuronide, which gradually decreased, increased in the upper part of the small intestine at 12 h after oral administration (Sakamoto *et al*., 2002). The present findings differ from those reported by Sakamoto *et al*. (2002); neither nonylphenol-glucuronide nor ethylphenol-glucuronide increased at later times in the upper part of the small intestine. Different characteristics among these three compounds indicate that the three compounds are excreted through distinct elimination pathway.

Whether compounds enter enterohepatic circulation may be dependent on the distribution of glucuronidated compounds from liver. Inoue *et al*. (2001) reported that bisphenol A entering the liver *via* the portal vein was excreted mainly into bile (42.0%) and veins (16.6%) as bisphenol A-glucuronide. On the other hand, Daidoji *et al*. (2003) showed that, according to the method of Inoue *et al*., ethylphenol was excreted into bile (20.0%) and veins (20.0%) equally as ethylphenol-glucuronide, while nonylphenol remained as nonylphenol-glucuronide in the liver tissue instead being excreted by either pathway, which supports the present result, nonylphenol-glucuronide remaining in the liver at 1 h and 3 h after administration. Based on these reports (Inoue *et al*., 2001; Daidoji *et al*., 2003), it can be assumed that the bisphenol A being excreted into bile can enter

enterohepatic circulation, while nonylphenol remaining in the liver cannot enter enterohepatic circulation and ethylphenol being excreted into bile behind excretion into veins can marginally enter enterohepatic circulation. Among the small intestinal contents at times later than 6 h, which was the time limit in our study, ethylphenol-glucuronide reconjugation and excretion from liver may again increase.

Generally, the effects of enterohepatic circulation on excretion of drug into feces depends on the activity of β-glucuronidase, which catalyzes the release of toxic aglycones detoxified by glucuronidation in the liver and is most highly present in enterobacteria and clostridia (Hawksworth *et al*., 1971). A number of studies using animal models have demonstrated that consumption of probiotic bacteria can reduce the risk of colon cancer (Goldin *et al*., 1980). However, for compounds not entering enterohepatic circulation, such as nonylphenol, exclusion from the body by controlling enterobacteria is difficult.

Although nonylphenol persisted for long periods in the gastrointestinal tissue and liver as the parent compound and glucuronide conjugate, the glucuronide disappeared over time, but the glucuronide was not subsequently detected in gastrointestinal contents or urine. Given that the chromatogram of extract from intestinal tissue after oral ingestion of nonylphenol showed some faint peaks other than nonylphenol-glucuronide (data not shown), we believe that nonylphenol may change slightly over time to further derivatives or metabolites of nonylphenol-glucuronide (sulfate). This supports the report by Zalko *et al*. (2003), in which urine from a male rat at 0-24 h after oral dosing with 1 μg/kg 4-*n*-nonylphenol contained glucuronides and sulfates of nonylphenol derivatives, such as 3-(4-hydroxy-phenyl)-2-propenoic acid, 3-(4-Hydroxy-phenyl)-2-propinoic acid, *para*-hydroxy benzoic acid.

A limitation of the present work was that faint peaks in extract from intestinal tissue were not identified. Further studies directed at clarifying these faint peaks, which are assumed to be associated with nonylphenol, need to be undertaken.

Nonylphenol is used for a wide variety of purposes, and has been reported to be environmentally persistent (Petrovic *et al*., 2002; Ying *et al*., 2002). If nonylphenol is ingested through water or food, the intestine, as a first barrier, absorbs and glucuronidates the compound. However, the transport system for alkylphenols is impaired by long alkyl chain, and thus the elimination of nonylphenol from intestine is slow, which can lead to accumulation of nonylphenol in intestinal tissue. This accumulation of nonylphenol gives rise to persistent release of the harmful compound into blood flow, and nonylphenol subsequently arrives at the liver. The liver is able to metabolize nonylphenol, but also possesses a transport system vulnerable to long alkyl chain (Daidoji *et al*., 2003). These tissue accumulations of nonylphenol results in protracted exposure to target organs such as testis or ovary.

Conclusions

We confirmed that rat intestine possesses an alkylphenol elimination system comprising the detoxification enzyme UGT; however, this elimination system is impaired by the marginal transport of glucuronide conjugates of alkylphenol having long carbon chain, such as nonylphenol. Further studies are warranted to identify the transporter responsible for excretion of alkylphenol-glucuronide from the intestine.

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Footnotes

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Legends for Figures

FIG. 1. UDP-glucuronosyltransferase activity in rat intestinal microsomes toward ethylphenol and nonylphenol (0.5 mM each). Results are shown as means ± S.E. (n=3 animals). SI: Small Intestine; LI: Large Intestine.

FIG. 2. Alkylphenol (C2: ethylphenol; C4: butylphenol; C6: hexylphenol; C9: nonylphenol) glucuronidation and excretion to the mucosal side and transport to the serosal side based on length of alkyl chain during 100-min incubation. Alkylphenols (C2, C4, C6, C9) were added individually to the mucosal buffer solution of each segment at a concentration of 50 μM. I, II, III, IV indicate intestinal site in distal order from the ligament of Trietz. Results are means ± S.E. (n=3 animals).

FIG. 3. Free alkylphenols (C2: ethylphenol; C4: butylphenol; C6: hexylphenol; C9: nonylphenol) (□) and glucuronides (■) remaining in everted intestinal segments after 100-min incubation together with alkylphenols (C2, C4, C6, C9) (50 μM). Samples were derived from the intestinal sites in distal order from the ligament of Trietz (I, II, III, IV), cecum, and colon. Results are means ± S.E. (n=3 animals).

FIG. 4. Distribution of free alkylphenols (C2: ethylphenol; C9: nonylphenol) (□) and glucuronides (■) in gastrointestinal contents of stomach (St), upper part of small intestine (Up), lower part of small intestine (Lo), cecum, colon (Co), and feces (Fe), after oral administration of ethylphenol (5 mg) or nonylphenol (5 mg). Samples were collected at 1 (A, D), 3 (B, E) or 6 h (C, F) after administration. Results are means ± S.E. (n=3 animals).

FIG. 5. Distribution of free alkylphenols (C2: ethylphenol, C9: nonylphenol) (□) and glucuronides (■) in gastrointestinal tissue: stomach (St), upper part of small intestine (Up), lower part of small intestine (Lo), cecum, colon (Co), liver tissue (Li), kidney (Ki), and blood (Bl) after oral administration of ethylphenol (5 mg) or nonylphenol (5 mg). Samples were collected at 1 (A, D), 3 (B, E) or 6 h (C, F) after administration. Results are means ± S.E. (n=3 animals).

FIG. 6. Free alkylphenols (C2: ethylphenol, C9: nonylphenol) (□) and glucuronides (■) in urine after oral-administration of ethylphenol (5 mg) or nonylphenol (5 mg). Samples were collected at 3, 6, 8 or 10 h after administration. Results are means ± S.E. (n=3 animals). N.D.: not detected.

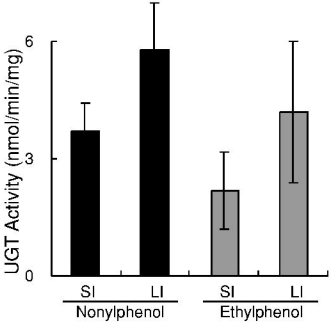
UGT Activity (nmol/min/mg)
6
3
0
SI
LI
SI
LI
Nonylphenol
Ethylphenol

FIG. 1.

Alkylphenol Glucuronide (nmol/bowel/100min)
800
400
0
400
800
Mucosal Side
Serosal Side
C2
C4
C6
C9
I
II
III
IV
Cecum
Colon

FIG. 2.

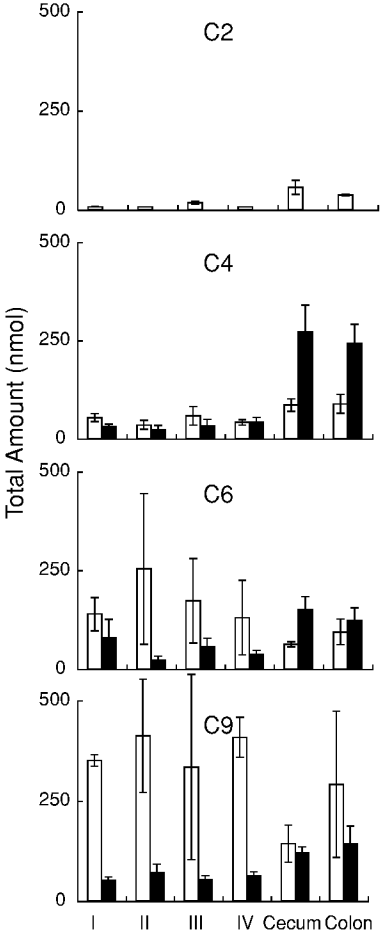

FIG. 3.

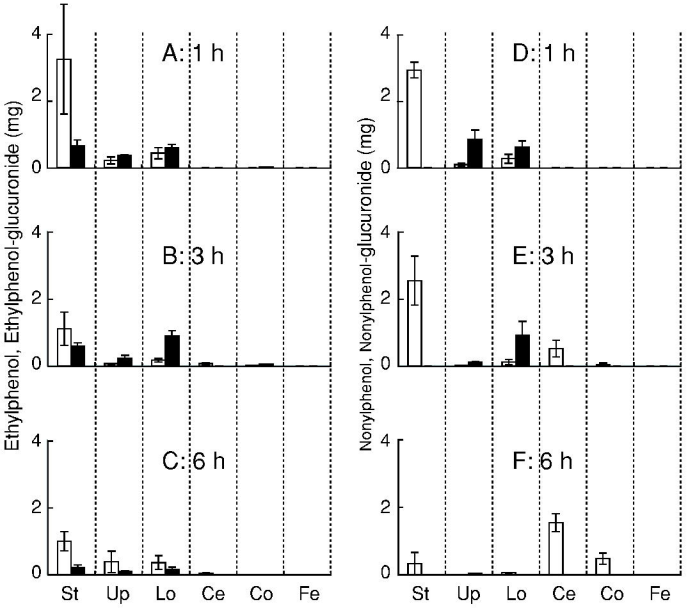
Ethylphenol, Ethylphenol-glucuronide (mg)
Nonylphenol, Nonylphenol-glucuronide (mg)
A: 1 h
B: 3 h
C: 6 h
D: 1 h
E: 3 h
F: 6 h
0
2
4
St
Up
Lo
Ce
Co
Fe

FIG. 4.

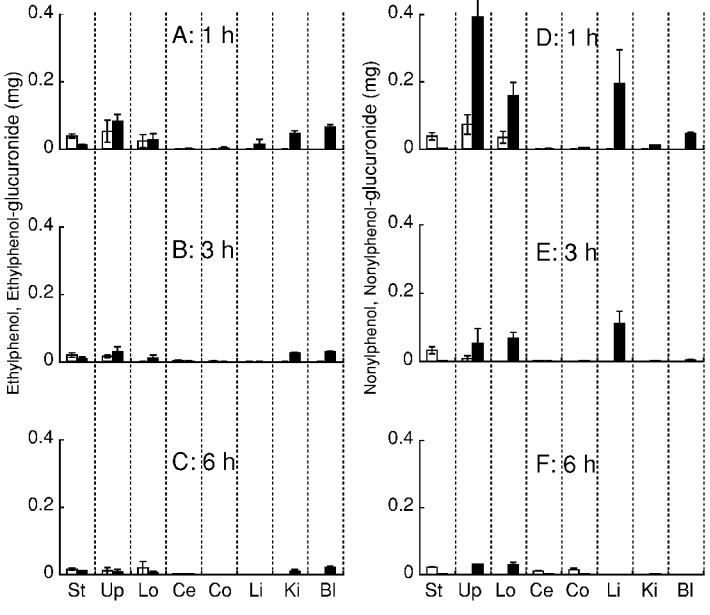
A: 1 h
B: 3 h
C: 6 h
D: 1 h
E: 3 h
F: 6 h
Ethylphenol, Ethylphenol-glucuronide (mg)
Nonylphenol, Nonylphenol-glucuronide (mg)
0.4
0.2
0
St
Up
Lo
Ce
Co
Li
Ki
Bl

FIG. 5.

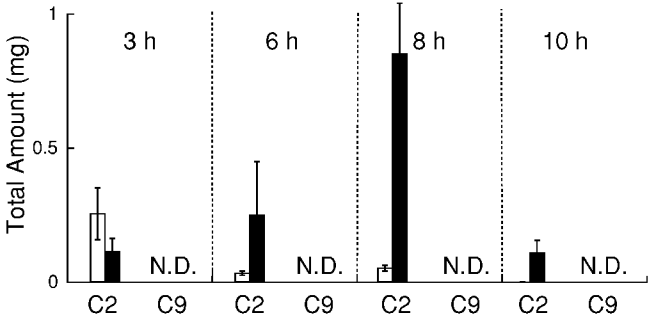
Total Amount (mg)
1
0.5
0
3 h
6 h
8 h
10 h
N.D.
N.D.
N.D.
N.D.
C2
C9
C2
C9
C2
C9
C2
C9

FIG. 6.