Identification of Metabolites of 4-Nonylphenol Isomer 4-(3’,6’-Dimethyl-3’-Heptyl) Phenol by Rat and Human Liver Microsomes

Xiaoyun Ye, Amber M. Bishop, Larry L. Needham, and Antonia M. Calafat

Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia

Received March 2, 2007; accepted April 23, 2007

ABSTRACT:

Nonylphenol (NP) has been widely used for more than 50 years in the synthesis of NP ethoxylates, which are important nonionic surfactants. NP is considered an endocrine disruptor based on in vitro and in vivo animal studies. However, the toxic effects of NP in humans are unknown. Information regarding the metabolic fate of 4-t-nonylphenol (4-tNP), a mixture of commercial NP branched isomers, in mammalian species is limited. This information is critical for the identification of adequate biomarkers of exposure to NP that could be used for exposure and risk assessment. We identified metabolites of one 4-tNP isomer, namely, 4-(3’,6’-dimethyl-3’-heptyl) phenol (P363-NP), using rat and human liver microsomes. The P363-NP metabolites were extracted by on-line solid-phase extraction and then separated and detected using high-performance liquid chromatography/tandem mass spectrometry. Using the genuine standard, we unambiguously identified 4-(3’,6’-dimethyl-3’-heptyl) catechol (P363-NC) as the main P363-NP metabolite when using human liver microsomes. Based on their chromatographic behavior and mass spectral fragmentation patterns, several other metabolites were tentatively identified, including a hydroxylated P363-NP with the alcohol functional group on the branched alkyl chain and its oxidative metabolite, a catechol with a hydroxylated alkyl side chain. Furthermore, the metabolite profile of P363-NP using rat and human enzymes was compared. Our findings suggest that P363-NC could be used as a biomarker to assess exposure to 4-tNP, although additional research to evaluate its suitability as a biomarker is warranted.

Nonylphenols (NP) are widely used for the synthesis of nonylphenol ethoxylates (NPE), which are one of the most important types of nonionic surfactants, with an annual worldwide production of approximately 650,000 tons (Guenther et al., 2002). Small quantities of NP are also used as stabilizers or antioxidants in the manufacture of plastics, such as polystyrene and polyvinyl chloride. The major source of NP contamination in the environment is the microbial degradation of NP released from polystyrene were found to stimulate the growth of human MCF-7 breast cancer cells, and they increased uterine mitotic activity in rats treated with NP (Soto et al., 1991). The estrogenic potency of NP was also confirmed from a series of in vitro (White et al., 1994; Flouriot et al., 1995; Jobling et al., 1995; Routledge and Sumpter, 1996) and in vivo studies (Colerangle, 1996; Lee and Lee, 1996; Harries et al., 1997). NP could stimulate the synthesis of vitellogenin in rainbow trout hepatocytes (Jobling and Sumpter, 1993; White et al., 1994). NP displaced estradiol from fish and human cell line estrogen receptors at 1500- to 3000-fold concentrations or nominally at about 10^-5 M (White et al., 1994). In vivo data suggested that NP could induce uterine growth in immature female rats, albeit estrogenic potency of NP is many orders of magnitude lower than that of estradiol (Lee and Lee, 1996).

The potential adverse health effects of NP in humans are unknown. However, the estrogenic activity of NP has raised concerns about human exposure to these compounds; this exposure has been estimated to be approximately 2 μg/kg b.wt./day (Bolt et al., 2001). Exposure to NP can occur through dermal absorption, ingestion, or inhalation. In particular, exposure to NP through drinking water

ABBREVIATIONS: NP, nonylphenol(s); NP, nonylphenol ethoxylates(s); 4-tNP, 4-t-tert nonylphenol; 4-nNP, 4-normal nonylphenol; P363-NP, 4-(3’,6’-dimethyl-3’-heptyl) phenol; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; P363-NC, 4-(3’,6’-dimethyl-3’-heptyl) catechol; au, arbitrary unit(s); RT, retention time; EPI, enhanced product ion; MRM, multiple-reaction monitoring; 4-tOP, 4-tert-octylphenol.
(Clark et al., 1992) or contaminated seafood (Guenther et al., 2002) could be of potential relevance. For exposure and risk assessment, information on the bioavailability, fate, and metabolism of NP is necessary.

As a result of the method used to produce NP industrially, NP consist of predominantly branched alkyl chain isomers of 4-NP, 4-tert nonylphenol (4-tNP). At least 22 isomers of 4-tNP have been reported (Wheeler et al., 1997; Thiele et al., 2004). So far, data on the metabolic fate of either linear or branched NP in mammalian species are very limited. In Sprague-Dawley rats, after p.o. or i.v. administration of 4-tNP, NP glucuronide was the main urinary metabolite in females; the metabolites in males were not identified (Green et al., 2003). Lee et al. (1998) reported the in vitro metabolism of NP by rat and human microsomes, but the studies were focused on interactions of NP and hepatic cytochrome P450, and NP metabolites were not identified. Zalko et al. (2003) studied the metabolic fate of 4-normal nonylphenol (4-nNP) in Wistar rats and reported the identification of about 10 different metabolites, most of them formed by the ω- or β-oxidation of the 9-carbon linear alkyl chain. The metabolic profile for the branched 4-NP may be different because the β-oxidation pathway will only proceed on the linear alkyl chain (Zalko et al., 2003), as illustrated by the fact that NP and NP-catechol glucuronides were tentatively identified as the main metabolites of 4-tNP in rats (Doerge et al., 2002).

Previously, we measured the urinary concentrations of 4-nNP from a reference population of 394 adults in the United States (Calafat et al., 2005). The relative low concentration and low frequency of detection of 4-nNP suggested that either exposure to NP is low or that 4-nNP might not be an adequate biomarker of exposure because 4-nNP represents only a very small percentage of the NP in the commercial mixtures (Calafat et al., 2005). Because branched alkyl chain isomers of 4-NP are predominant, these isomers or their metabolites could be good biomarkers of exposure to NP. In the present study, we chose one of these branched 4-tNP isomers, namely, 4-(3′,6′-dimethyl-3′-heptyl)-phenol (P363-NP), one of the three major isomers (~10%) in two commercial NP mixtures (Russ et al., 2005), as a substrate to identify in vitro metabolites of NP by rat and human liver microsomes by using on-line solid-phase extraction (SPE)/high-performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

Materials and Methods

Materials and Standards. P363-NP, 4-(3′,6′-dimethyl-3′-heptyl) catechol (P363-NC), and D$_2$-P363-NC were obtained from CanSyn Chem Corp. (Toronto, ON, Canada). Pooled female human liver microsomes, pooled female Sprague-Dawley rat liver microsomes, NADPH regenerating system solution A, and NADPH regeneration system solution B were purchased from BD Gentest (Woburn, MA). HPLC-grade methanol was obtained from Tedia (Fairfield, OH), and analytical-grade formic acid (98%) and phosphoric acid (98%) were purchased from EM Science (Gibbstown, NJ). 4-Methylumbelliferone glucuronide, 4-methylumbelliferone sulfate, and β-glucuronidase/sulfatase (Helix pomatia, H1) were purchased from Sigma-Aldrich (St. Louis, MO).

FIG. 1. Total ion chromatograms of precursor ion scans (m/z 133 and 149) for P363-NP incubated with rat liver microsomes (A and B) and with human liver microsomes (C and D).
13C4-4-methylumbelliferone was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Urine Collection. Forty urine samples were collected from a diverse group of male and female adult volunteers with no documented occupational exposure to NP. Samples were collected anonymously, so no personal or demographic data were available, and study participants did not sign an informed consent. A waiver of informed consent was requested under 45 CFR 46.116(d).

Sample Preparation. Stock solutions of P363-NP, P363-NC, and D3-P363-NC were prepared in methanol. In a 1.5-ml autosampler vial, 20 μl of rat liver microsomes (or 40 μl of human liver microsomes) was mixed with 100 μl of P363-NP (10 μg/ml), 50 μl of NADPH regenerating system solution A (29 mM NADP+, 66 mM glucose 6-phosphate, and 66 mM MgCl2 in H2O), 10 μl of NADPH regenerating system solution B (40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), and 820 μl of pH 7.4 buffer (0.1 M phosphoric acid). The mixture was incubated at 37°C for 1 h. Then, 100 μl was transferred into a new vial, and the enzyme reaction was stopped by diluting the sample to 1 ml with 0.1 M formic acid.

To measure the total (free plus conjugated species) concentrations of NP metabolites in human urine, we used an adaptation of our published method (Ye et al., 2005). Urine (100 μl) was spiked with 20 μl of internal standard solution (D3-P363-NC, 1 μg/ml), 50 μl of β-glucuronidase/sulfatase (H. pomatia, 463,000 U/g solid) solution in buffer (4 mg/ml, pH 5.0), and 20 μl of 1 ppm 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/13C4-4-methylumbelliferone mixed standard. The 4-methylumbelliferone/13C4-4-methylumbelliferone peak area ratio was monitored to evaluate the extent of the deconjugation reaction. Samples were incubated for 4 h at 37°C and then acidified with 0.1 M formic acid.

On-Line SPE/HPLC/MS/MS. The metabolites of P363-NP were concentrated and separated from other components in the in vitro preparation or human urine by on-line SPE/HPLC. The on-line SPE/HPLC system consisted...
of several Agilent 1100 modules (Agilent Technologies, Wilmington, DE),
namely, two binary pumps with degassers, an autosampler with a 900-μl
injection loop, and one column compartment with a six-port switching valve
(Ye et al., 2005). The SPE column was a LiChrosphere RP-18 ADS (25 × 4
mm, 25-μm particle size, 60 Å pore size) (Merck Biosciences, Darmstadt,
Germany), and the HPLC columns were two Chromolith Performance RP-18
(100 × 4.6 mm; Merck Biosciences). The mobile phases A and B were water
and 100% MeOH, respectively, for both SPE and HPLC. The gradient program
for SPE was 0 to 4 min, 20% B; 4.1 to 9 min, 100% B; and 9.1 to 23 min, 20%
B. The gradient program for HPLC was 0 to 5 min, 50% B; 5.1 to 10 min, from
50 to 65% B; 10 to 18 min, from 65 to 100% B; 18 to 20 min, 100% B; and
20.1 to 23 min, 50% B. The flow rates were 1 ml/min (SPE) and 0.75 ml/min
(HPLC). Detection was performed by negative ion-atmospheric pressure
chemical ionization tandem mass spectrometry on an API 4000 Q trap mass
spectrometer (Applied Biosystems, Foster City, CA). Typical settings were
curtain gas (N2) flow, 20 arbitrary units (au); collision gas flow, 9 au; nebulizer
gas (air) flow, 50 au; nebulizer gas temperature, 500°C; and corona needle
temperature, −3 V. The mass spectrometer and Agilent modules were programmed
and controlled by use of the Analyst 1.4.1 software (Applied Biosystems); the
acquisition method was built in “LC sync” mode.

Results and Discussion

First, we obtained the full-scan mass spectra of P363-NP and P363-
NC. The mass to charge ratio (m/z) of the molecular ion and the two most
abundant fragment ions were 219 ([M-H])−, 133 ([C9H9O]−), and 147
([C10H11O2]−) for P363-NP and 235 ([M-H])−, 149 ([C9H9O2]−), and
163 ([C10H11O2]−) for P363-NC. Under the experimental conditions
described above, P363-NP and P363-NC eluted at 21.0 and 19.9 min,
respectively. Because the metabolites of P363-NP could potentially fragment
like the parent compound, we obtained a precursor ion mass spectrum of m/z = 133, the most abundant fragment ion of P363-NP, after incubation with the microsome homogenates for 1 h and on-line
SPE/HPLC separation. Using the rat microsomes, two major compounds,
R1 (m/z = 219, retention time (RT) = 21.0 min) and R2 (m/z = 235, RT = 17.2 min), were observed (Fig. 1A). By contrast, with the human
microsomes, only one major compound, H1 (m/z = 219, RT = 21.1
min), was identified (Fig. 1C). Because NP catechol has been reported to
be a metabolite of NP in rats (Doerge et al., 2002), we obtained a
precursor ion mass spectrum of m/z = 149, the most abundant fragment
ion of P363-NC. With the rat microsomes, one major precursor com-
 pound, H2 (m/z = 235, RT = 15.9 min), was observed (Fig. 1B), whereas
 two major precursors, H2 (m/z = 235, RT = 19.9 min) and H3 (m/z = 251, RT = 16.0 min), were present when using the human microsomes
(Fig. 1D).

To identify these metabolites, we obtained the enhanced product ion (EPI) mass spectra of the microsome homogenates after on-line
SPE/HPLC separation. First, based on EPI data and retention times of the authentic standards, we unequivocally identified H1 and R1 as
P363-NP and H2 as P363-NC. The EPI scan of R2 showed fragments at m/z = 133, 147, and 219 (Fig. 2A). R2’s earlier elution than R1’s
strongly suggested that R2 was a hydroxylated P363-NP. Furthermore,
the fragmentation pattern of R2 suggested that the hydroxyl group resided on the alkyl side chain because if the −OH was on the
ring, the fragments should have been at m/z = 149 and 163. Therefore,

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Representative multiple reaction monitoring total ion chromatograms of P363-NP incubated with rat (A) and human liver microsomes (B) for different time intervals. (The y-axis scales are different for all the figures.)
we tentatively identified R2 as an alkyl side chain hydroxylated P363-NP. For both R3 and H3, the EPI scans showed fragmentation patterns with ions at $m/z$ 149 and 163 (Fig. 2B), similar to that of P363-NC. The identical chromatographic behavior and EPI mass spectra data suggested that R3 and H3 were the same metabolite, the most hydrophilic of those identified, as illustrated by eluting first from the HPLC system. These data strongly suggest that R3 (and H3) is a hydroxylated catechol, a further oxidation metabolite from R2 and H2, with one hydroxyl group on the ring and another one on the alkyl side chain. The exact oxidation site on the branched alkyl side chain could not be unequivocally determined with the available data; additional NMR experiments are needed to fully characterize the chemical structures of these metabolites.

P363-NP was incubated with rat or human liver microsomes for different time intervals. Multiple-reaction monitoring (MRM) was used to determine the concentration of the different metabolites present in the enzyme homogenates after they were separated by the on-line SPE/HPLC system. To increase the accuracy of the measurements, 20 μl of D$_3$-P363-NC (1 μg/ml) was added after incubation to all the samples as the internal standard. The ion transitions used for quantitation were 219/133 (R1 and H1), 235/149 (H2), 235/133 (R2), and 251/149 (R3 and H3). Because authentic standards of R2 and R3/H3 were not available, we estimated the concentrations of R2 and R3/H3 by using the calibration curve of P363-NC. A P363-NP solution without any microsomes, but with all the other reagents, was also incubated and used as a blank. The representative MRM total ion chromatograms of P363-NP incubated with rat and human microsomes for 10, 30, and 120 min are shown in Fig. 3. Interestingly, P363-NC was the only metabolite after incubation for 10 min with the rat microsomes (Fig. 3A), as confirmed by comparing its fragmentation pattern and retention time with that of the authentic standard. After 30 min, two other metabolites, R2 and R3, were the major products (Fig. 3A). When using human microsomes, P363-NC was also the major metabolite after 10 min of incubation (Fig. 3B); a small peak ($m/z$ 235/133) appearing at the same retention time of R2 was identified as R2. P363-NC remained as the major metabolite for the duration of the incubation, with R2 and R3 as minor products (Fig. 3B).

![Fig. 4](image1.png)

**Fig. 4.** The kinetics of the in vitro metabolism of P363-NP by rat liver microsomes (A) and human liver microsomes (B). In the absence of true standards, the concentrations of R2, R3, and H3 were estimated from the calibration curve of P363-NC.

![Fig. 5](image2.png)

**Fig. 5.** Proposed in vitro metabolic pathways of P363-NP with rat (A) and human (B) liver microsomes. (The bold arrows refer to the major pathways.)
With the rat microsomes, more than 90% of P363-NP was oxidized to R2 and R3 within 30 min (Fig. 4A). Although P363-NC formed initially, its concentration decreased with the incubation time, suggesting further metabolism of this compound. By contrast, with the human microsomes, more than 80% of P363-NP was oxidized to H2 and H3 after 2 h (Fig. 4B). These data suggest that the activity of the enzymes differs depending on the species, although the same three metabolites were formed regardless of the enzymatic preparations used (Fig. 5). The reason(s) for these differences in metabolic activity between rat and human microsomes is, at present, unknown.

We incubated P363-NP (0.1 ppm, 1.0 ppm, or 10 ppm) with rat microsomes. In all the cases, P363-NC was initially metabolized to P363-NC, but as the reaction progressed, metabolites with an oxidized alkyl side chain became dominant, suggesting no P363-NC concentration dependence. Furthermore, when using technical grade t-NP instead of P363-NP or male instead of female pooled rat liver microsomes, very similar metabolite profiles were obtained (data not shown). Interestingly, the metabolic profiles at various incubation times of P363-NP by rat microsomes presented here were similar to those reported for another alkylphenol, 4-t-octylphenol (4-tOP), with primary rat hepatocytes (Pedersen and Hill, 2000). 4-tOP was first metabolized to a catechol, and both 4-iOP and 4-tOP were further metabolized by hydroxylation of the alkyl chain to two major metabolites, similar to R2 and R3. Because of the structural similarities between 4-iOP and 4-tNP, these metabolic similarities are not surprising. However, only the catechol was identified as the main metabolite in blood, liver, and other tissues in Sprague-Dawley rats administered 4-tNP p.o.; side chain hydroxylation metabolites of NP were not detected (Doerge et al., 2002). Further studies to evaluate in parallel both in vivo and in vitro metabolism of NP isomers using the same analytical methodology for detecting and identifying the metabolic products are warranted.

To check the usefulness of P363-NC, the major in vitro metabolite of P363-NP with human microsomes, as a biomarker of human exposure to NP for biomonitoring purposes, we measured the total concentrations of P363-NC in 40 urine samples collected anonymously from persons with no documented occupational exposure to NP. P363-NC was detected above the limit of detection of 0.2 ng/ml in 20% of the samples at concentrations ranging from less than the limit of detection to 1.1 ng/ml. The MRM transition of the hydroxylated catechol (251/149) was also above the limit of detection of 0.2 ng/ml in 20% of the samples at P363-NC concentrations.

The MRM transition of the hydroxylated catechol (251/149) was also above the limit of detection of 0.2 ng/ml in 20% of the samples at P363-NC. For biomonitoring purposes, we measured the total concentrations of alkylphenolic compounds in fish tissue using pressurized fluid extraction, solid phase cleanup, and high-performance liquid chromatography fluorospectrophotometry. J Agric Food Chem 55:1350–1354.


References


