

DMD #15578

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

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DMD #15578

## Running Title

*In vitro* Metabolites of 4-t-Nonylphenol

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## Outline of Section

Number of text pages	12
Number of figures	5
Number of references	32
Abstract	230 words
Introduction	772 words
Results and Discussion	1240 words

## List of Abbreviations

NP                      Nonylphenol

DMD #15578

4-nNP	4-normal nonylphenol
4-tNP	4-tert nonylphenol
4-tOP	4-tert octylphenol
NPEs	Nonylphenol ethoxylates
P363-NP	4-(3',6'-dimethyl-3'-heptyl) phenol
P363-NC	4-(3',6'-dimethyl-3'-heptyl) catechol
CDC	Centers for Disease Control and Prevention.
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
MRM	Multiple-reaction-monitoring

DMD #15578

## Abstract

Nonylphenol (NP) has been widely used for over 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 were 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.

DMD #15578

## Introduction

Nonylphenols (NPs) are widely used for the synthesis of nonylphenol ethoxylates (NPEs), which are one of the most important type of nonionic surfactants, with an annual worldwide production of approximately 650,000 tons (Guenther et al., 2002). Small quantities of NPs are also used as stabilizers or antioxidants in the manufacture of plastics, such as polystyrene and polyvinyl chloride. The major source of NPs contamination in the environment is the microbial degradation of NPEs (Datta et al., 2002; Ferrara et al., 2001; Lye et al., 1999), although contamination by NPs may also result from leaching of plastic products. NPs have been detected in river water, sewage sludge, and drinking water (Diaz et al., 2002; Meesters et al., 2002; Pryor et al., 2002; Bennie, 1999). NPs are somewhat lipophilic (Nimrod et al., 1996), and they have been reported to bioaccumulate in marine and aquatic wildlife, such as fish (Ekelund et al., 1990; Lewis et al., 1996).

NPs 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 NPs (Soto et al., 1991). The estrogenic potency of NPs was also confirmed from a series of *in vitro* (Flouriot et al., 1995; Jobling et al., 1995; Routledge et al., 1996; White et al., 1994) and *in vivo* studies (Colerangle, 1996; Harries et al., 1997; Lee et al., 1996). NPs could stimulate the synthesis of vitellogenin in rainbow trout hepatocytes (Jobling et al., 1993; White et al., 1994). NP displaced estradiol from fish and human cell line estrogen receptors at 1500–3000-fold concentrations or nominally at about  $10^{-5}$  M (White et al., 1994). *In vivo* data suggested that NPs could induce uterine growth in immature female

DMD #15578

rats, albeit estrogenic potency of NPs is many orders of magnitude lower than that of estradiol (Lee et al., 1996).

The potential adverse health effects of NPs in humans are unknown. However, the estrogenic activity of NPs has raised concerns about human exposure to these compounds; this exposure has been estimated to be approximately 2  $\mu\text{g}/\text{kg}$  body weight/day (Bolt et al., 2001). Exposure to NPs can occur through dermal absorption, ingestion, or inhalation. In particular, exposure to NPs through drinking water (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 NPs is necessary.

As a result of the method used to produce NPs industrially, NPs consist of predominantly branched alkyl chain isomers of 4-NP (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 NPs in mammalian species are very limited. In Sprague-Dawley rats, after oral or intravenous 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. 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 (Lee et al., 1998). Zalko et al. studied the metabolic fate of 4-n-nonylphenol (4-nNP) in Wistar rats and reported the identification of about 10 different metabolites, most of them formed by the  $\omega$ - or  $\beta$ -oxidation of the 9-carbon linear alkyl chain (Zalko et al., 2003). The metabolic profile for the branched 4-NPs may be different, because the  $\beta$ -oxidation pathway will

DMD #15578

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 may not be an adequate biomarker of exposure because 4-nNP represents only a very small percentage of the NPs 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 NPs. 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-high performance liquid chromatography-tandem mass spectrometry (on-line SPE/HPLC-MS/MS).

## Experimental

### *Materials and Standards*

4-(3',6'-dimethyl-3'-heptyl) phenol (P363-NP), 4-(3',6'-dimethyl-3'-heptyl) catechol (P363-NC), and D<sub>3</sub>-P363-NC were obtained from CanSyn Chem Corp. (Toronto, 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

DMD #15578

%) and phosphoric acid (98%) were purchased from EM Science (Gibbstown, NJ). 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, and  $\beta$ -glucuronidase/sulfatase (*Helix pomatia*, H1) were purchased from Sigma-Aldrich (St. Louis, MO).  $^{13}\text{C}_4$ -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 NPs. 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  $\text{D}_3$ -P363-NC were prepared in methanol. In a 1.5 mL autosampler vial, 20  $\mu\text{L}$  of rat liver microsomes (or 40  $\mu\text{L}$  of human liver microsomes) were mixed with 100  $\mu\text{L}$  of P363-NP (10  $\mu\text{g}/\text{mL}$ ), 50  $\mu\text{L}$  of NADPH regenerating system solution A (29 mM  $\text{NADP}^+$ , 66 mM Glucose-6-phosphate, and 66 mM  $\text{MgCl}_2$  in  $\text{H}_2\text{O}$ ), 10  $\mu\text{L}$  of NADPH regenerating system solution B (40 U/ml Glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), and 820  $\mu\text{L}$  of pH 7.4 buffer (0.1 M phosphoric acid). The mixture was incubated at 37 °C for 1 hour. Then, 100  $\mu\text{L}$  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.,



DMD #15578

2005). Urine (100  $\mu\text{L}$ ) was spiked with 20  $\mu\text{L}$  of internal standard solution ( $\text{D}_3\text{-P363-NC}$ , 1  $\mu\text{g}/\text{mL}$ ), 50  $\mu\text{L}$  of  $\beta$ -glucuronidase/sulfatase (*H. pomatia*, 463 000U/g solid) solution in buffer (4 mg/mL, pH-5.0), and 20  $\mu\text{L}$  of 1 ppm 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/ $^{13}\text{C}_4$ -4-methylumbelliferone mixed standard. The 4-methylumbelliferone/ $^{13}\text{C}_4$ -4-methylumbelliferone peak area ratio was monitored to evaluate the extent of the deconjugation reaction. Samples were incubated for 4 hours at 37  $^\circ\text{C}$ , 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- $\mu\text{L}$  injection loop, and one column compartment with a 6-port switching valve (Ye et al., 2005). The SPE column was a LiChrosphere RP-18 ADS (25  $\times$  4 mm, 25  $\mu\text{m}$  particle size, 60  $\text{\AA}$  pore size, Merck KGaA, Germany), and the HPLC columns were two Chromolith<sup>TM</sup> Performance RP-18 (100  $\times$  4.6 mm; Merck KGaA). The mobile phase A and B were water and 100 % MeOH, respectively, for both SPE and HPLC. The gradient program for SPE was 0-4 min, 20 % B; 4.1-9 min, 100 % B; and 9.1-23 min, 20 % B. The gradient program for HPLC was 0-5 min, 50 % B; 5.1-10 min, from 50 % to 65 % B; 10-18 min, from 65 % to 100 % B; 18-20 min, 100 % B; 20.1-23 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<sup>TM</sup> mass spectrometer (Applied Biosystems, Foster

DMD #15578

City, CA). Typical settings were curtain gas (N<sub>2</sub>) flow: 20 arbitrary units (au); collision gas flow: 9 au; nebulizer gas (air) flow: 50 au; nebulizer gas temperature: 500 °C; and corona needle voltage: -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 ( $[C_9H_9O]^-$ ), and 147 ( $[C_{10}H_{11}O]^-$ ) for P363-NP; and 235 ( $[M-H]^-$ ), 149 ( $[C_9H_9O_2]^-$ ), and 163 ( $[C_{10}H_{11}O_2]^-$ ) for P363-NC. Under the experimental conditions described above, P363-NP and P363-NC eluted at 21.0 min 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 hour 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 (Figure 1A). By contrast, with the human microsomes, only one major compound H1 ( $m/z = 219$ , RT = 21.1 min) was identified (Figure 1C). Since 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 compound R3 ( $m/z = 251$ , RT=15.9 min) was observed (Figure 1B), while two major precursors H2 ( $m/z = 235$ , RT=19.9 min) and

DMD #15578

H3 ( $m/z = 251$ ,  $RT=16.0$  min) were present when using the human microsomes (Figure 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, on the basis of 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 (Figure 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. We therefore 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 (Figure 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

DMD #15578

by the on-line SPE-HPLC system. To increase the accuracy of the measurements, 20  $\mu\text{L}$  of D<sub>3</sub>-P363-NC (1  $\mu\text{g}/\text{mL}$ ) was added after incubation to all 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). Since 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 Figure 3. Interestingly, P363-NC was the only metabolite after incubation for 10 min with the rat microsomes (Figure 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 (Figure 3A). When using human microsomes, P363-NC was also the major metabolite after 10 min of incubation (Figure 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 (Figure 3B).

With the rat microsomes, over 90 % of P363-NP was oxidized to R2 and R3 within 30 min (Figure 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, after 2 hours, more than 80 % of P363-NP was oxidized to H2 and H3 (Figure 4B). These data suggest that the activity of the enzymes differs depending upon the species, although the same three metabolites were formed regardless of the enzymatic preparations used (Figure 5). The reason(s) for these

DMD #15578

differences in metabolic activity between rat and human microsomes are, at present, unknown,

We incubated P363-NP (0.1 ppm, 1.0 ppm or 10 ppm) with rat microsomes. In all cases, P363-NP was initially metabolized to P363-NC, but as the reaction progressed, metabolites with an oxidized alkyl side chain became dominant suggesting no P363-NP 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 et al., 2000). 4-tOP was first metabolized to a catechol, and both 4-tOP and its catechol 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-tOP 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 orally 4-tNP; 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 NPs 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 NPs. P363-NC

DMD #15578

was detected above the limit of detection (LOD) of 0.2 ng/mL in 20 % of the samples at concentrations ranging from <LOD to 1.1 ng/mL. The MRM transition of the hydroxylated catechol (251/149) was also monitored, and no significant signals were observed. The relatively low frequency and magnitude of detection of P363-NC may reflect low human exposure to NPs or the fact that P363-NC is not a major metabolite of NPs in humans *in vivo* (e.g., P363-NC could be further oxidized to an o-quinone). In order to link the urinary concentrations of P363-NC to NPs exposure, toxicokinetic data of P363-NP in humans are needed. Although our findings suggest that oxidative metabolism is important for 4-tNP, additional research is warranted to evaluate the suitability of these oxidative products as 4-tNP exposure biomarkers.

DMD #15578

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DMD #15578

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DMD #15578

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DMD #15578

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DMD #15578

### **Footnote section**

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DMD #15578

## Figure Legends

Figure 1. Total ion chromatograms of precursor ion scans ( $m/z$  133 and  $m/z$  149) for P363-NP incubated with rat liver microsomes (A and B), and with human liver microsomes (C and D).

Figure 2. Enhanced product ion (EPI) mass spectra of P363-NP incubated with rat microsomes. A) EPI of molecular ion 235 at  $RT=17.3$  min. B) EPI of molecular ion 251 at  $RT=15.9$  min.

Figure 3. Representative multiple reaction monitoring total ion chromatograms of P363-NP incubated with (A) rat and (B) human liver microsomes for different time intervals. (The Y-axis scales are different for all of the figures.)

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

Figure 5. Proposed *in vitro* metabolic pathways of P363-NP with (A) rat and (B) human liver microsomes. (The bold arrows refer to the major pathways.)

# Figure 1

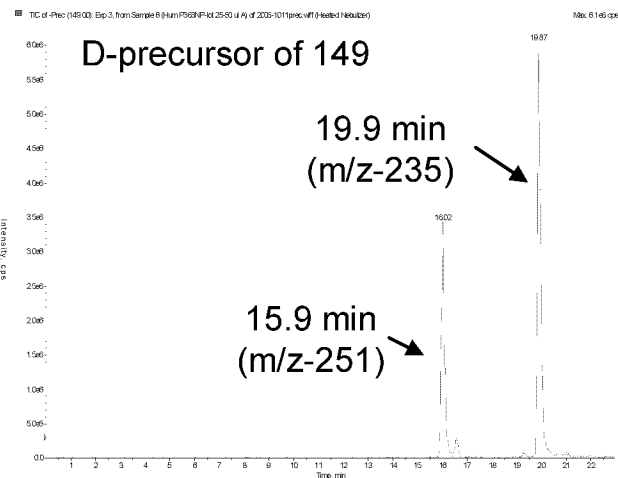
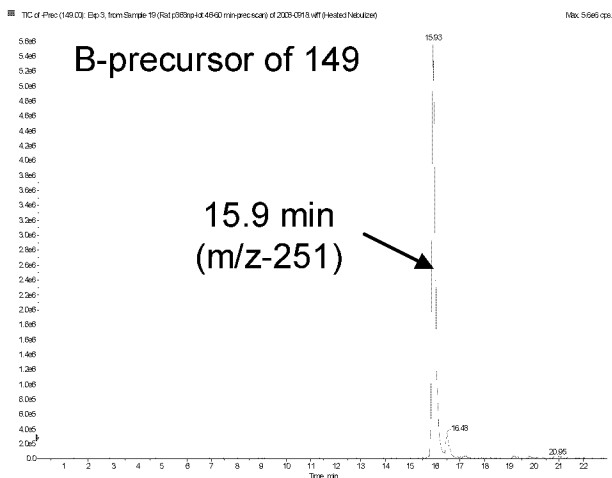
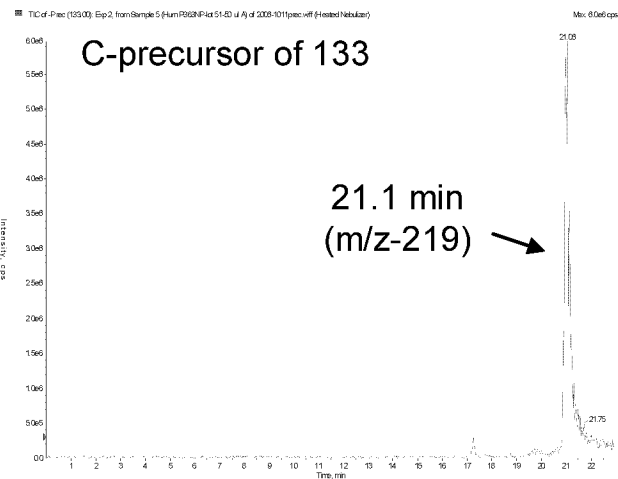
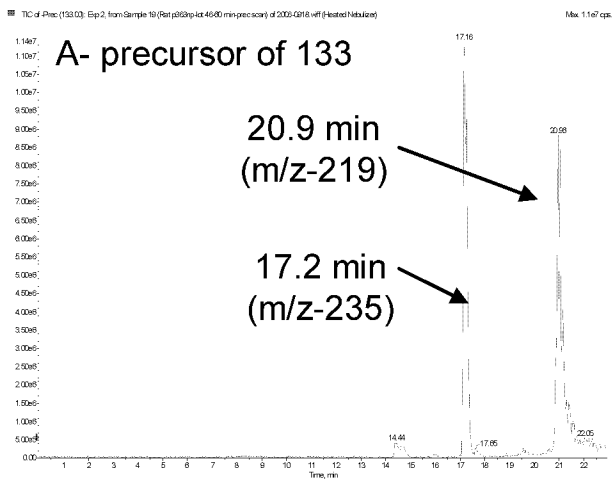


Figure 2.

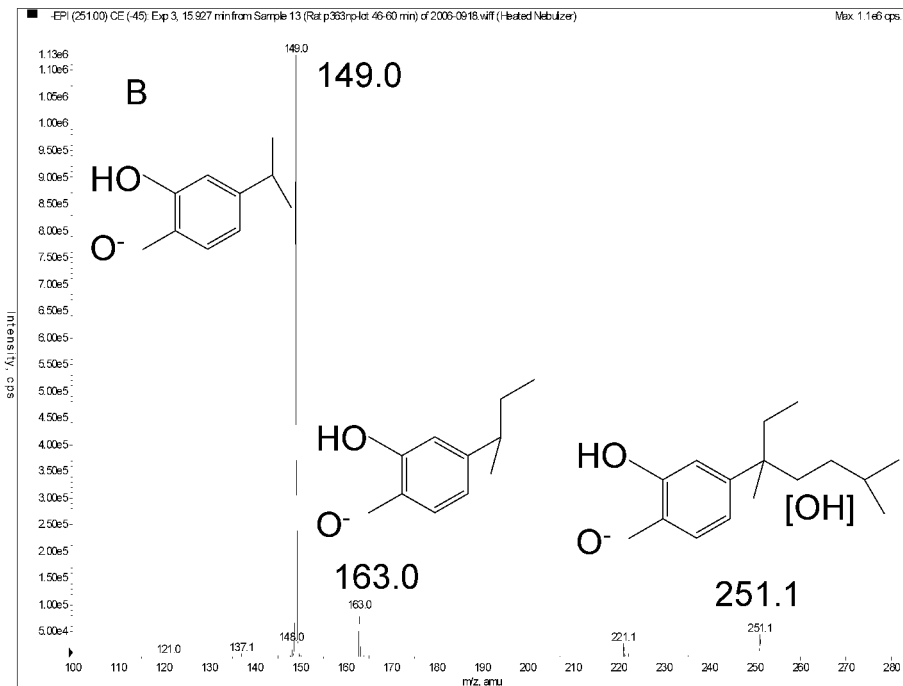
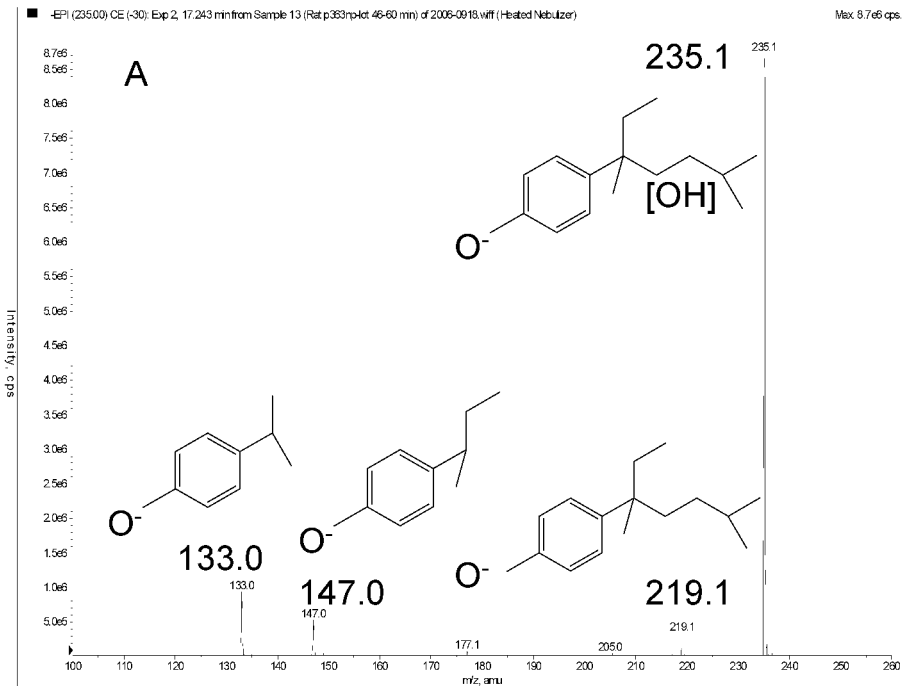


Figure. 3

A. Rat Microsomes

B. Human Microsomes

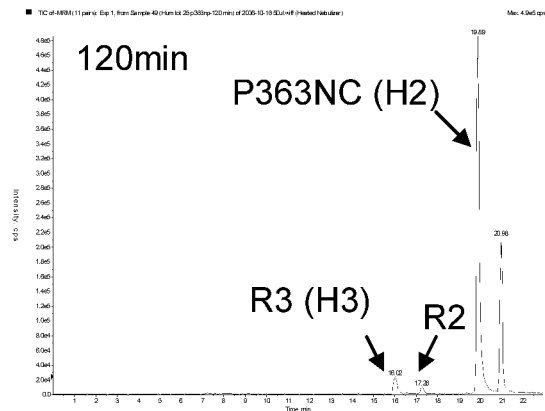
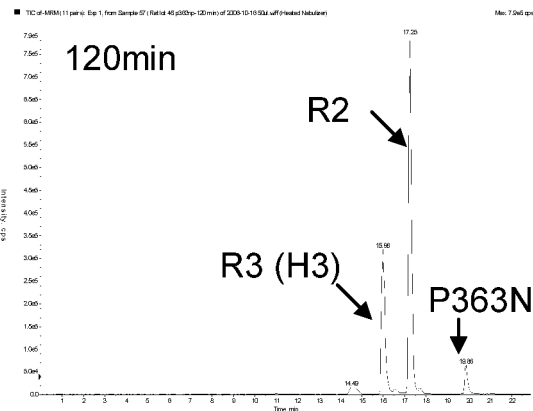
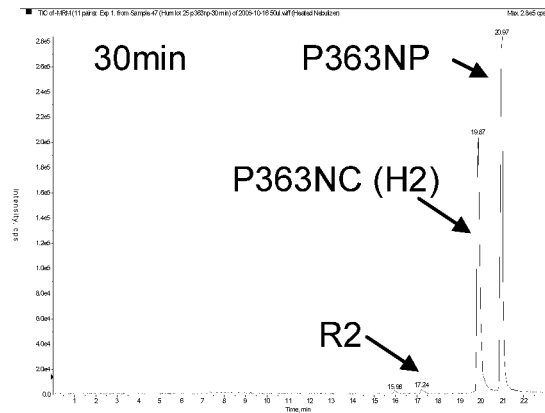
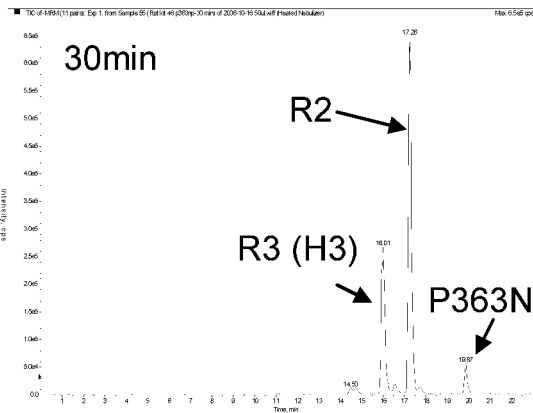
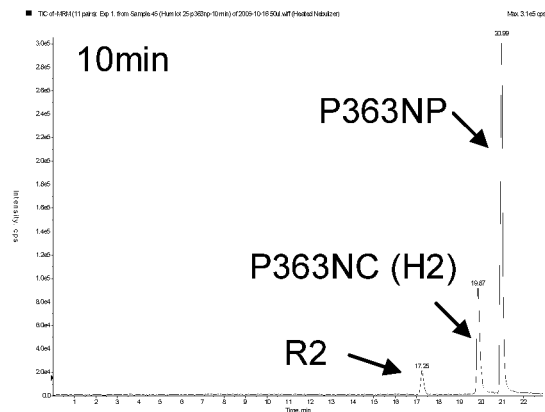
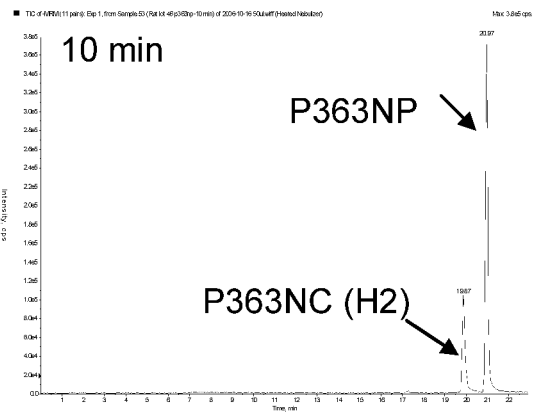




Figure 4.

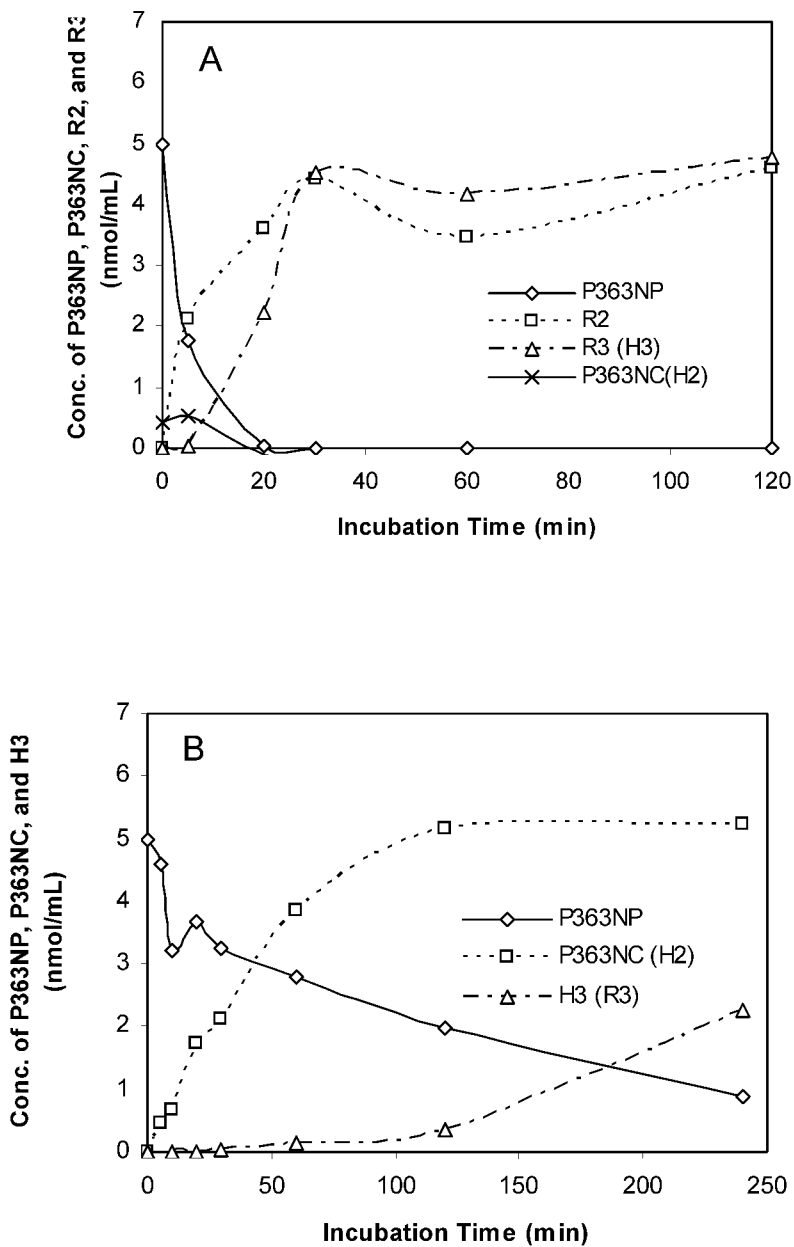


Figure 5.

