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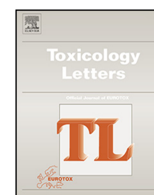

# Evaluation of the stereoselective biotransformation of permethrin in human liver microsomes: Contributions of cytochrome P450 monooxygenases to the formation of estrogenic metabolites

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## HIGHLIGHTS

- Permethrin undergoes stereoselective oxidase and esterase metabolism in human liver microsomes.
- Sequential metabolism from the esterase cleavage product PBOH to the oxidized product 3,4-PBOH is stereoselective for 1*R-trans* PM.
- 1*S-cis* PM consistently has the least efficient turnover.
- Cytochrome P450s catalyze both esterase and oxidase activities.
- CYP2C19 has greater catalytic activity than CYP3A4 and stereoselectively catalyzes the transformation of 1*R-trans* PM.

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## ABSTRACT

Permethrin (PM) is a pyrethroid insecticide that exists as 4 enantiomers. Biotransformation of PM to estrogen receptor agonists (3-phenoxybenzyl alcohol (PBOH) and 3-(4′-hydroxyphenoxy)-benzyl alcohol (3,4 PBOH)) has been shown to be stereoselective in other vertebrate species. This study evaluated the biotransformation of PM enantiomers in human liver microsomes and with recombinant CYP3A4 and CYP2C19. PBOH and 3,4 PBOH were the only metabolites detected from *in vitro* incubations including each of the 4 enantiomers of PM with 1*R-trans* PM having the most efficient NADPH-catalyzed biotransformation to both metabolites. Coincubation with the CYP inhibitor ketoconazole and time course experiments with liver microsomes and recombinant CYP2C19 and CYP3A4 indicated CYP-catalyzed stereoselective cleavage of the ester followed by 4-hydoxylation to 3,4′ PBOH. These data indicate potential dispositional differences may occur with PM enantiomers and a shift in putative molecular targets. While cleavage of pyrethroid esters lead to detoxification of the acute neurological effects, formation of the benzyl alcohol and hydroxylated metabolite may lead to estrogenic responses, since each of these metabolites are estrogen receptor ligands.

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## 1. Introduction

Permethrin [3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane 1 carboxylate] (PM) is a pyrethroid pesticide widely used throughout the world to control various insects for crop protection and prevention of insect-derived disease (Casida and Quistad, 1999). The acute neurological toxicity and metabolism of pyrethroids in animals have been well investigated and reported (Verschoyle and Barnes, 1972; Gaughan et al., 1977; Tomigahara et al., 1994). However, more recent studies have indicated some pyrethroids impair development in vertebrates through potential endocrine disrupting mechanisms (Sinha et al., 2006; Zhang et al., 2007). Pyrethroids can possess one to three chiral centers resulting in two to eight enantiomers. The centers of chirality are in the cyclopropane ring of the acid moiety and the α-carbon of the alcohol moiety. In the case of PM, the possession of two chiral centers results in two diastereoisomers (*cis*- and *trans*-) and four stereoisomers (1*R-cis*-, 1*S-cis*-, 1*R-trans*-, and 1*S-trans*-).

Evidence of stereoselective metabolism of some pyrethroids has been reported in rats (Gaughan et al., 1977) and mice (Soderlund and Casida, 1977) with rates of conversion of *trans* PM to hydrolytic metabolites being greater than *cis* PM. Microsomal studies in mice

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however indicated no significant difference between enantiomers of *trans* PM. In contrast, studies in rainbow trout liver microsomes indicated significant enantiomeric differences in the formation of ester cleavage and hydroxylated metabolites. In addition, ester cleavage and hydroxylation was NADPH dependent and inhibited by the CYP inhibitor, ketoconazole (Nillos et al., 2010).

In contrast to fish which have limited esterase activities, *in vitro* human metabolism studies showed that PM is hydrolyzed by hCE-1 (human carboxylesterase 1) and hCE-2 to 3-phenoxybenzyl alcohol (PBOH) (Ross et al., 2006; Yang et al., 2009) which is subsequently oxidized to by alcohol dehydrogenase and aldehyde dehydrogenase to phenoxybenzoic acid (PBCOOH) (Choi et al., 2002). PBOH is also hydroxylated to 3-(4′-hydroxyphenoxy)-benzyl alcohol (3,4-PBOH) which is an estrogen receptor agonist (McCarthy et al., 2006; Nillos et al., 2010). While carboxylesterase-catalyzed hydrolysis has been shown to be the predominant detoxification pathway of PM metabolism in mammals (Crow et al., 2007), subsequent hydroxylation by CYP isoforms (Scollon et al., 2009) may also contribute to the formation of other metabolites that have endocrine disrupting activities (Takaku et al., 2011). Recent studies by Scollon et al. (2009) showed that several CYP isoforms catalyze the oxidation of PM, but specific metabolites were not identified and enantiomeric differences were not evaluated.

Since little is known regarding the stereoselectivity of CYP-catalyzed biotransformation of pyrethroids in humans, this study investigated the stereoselective metabolism and conversion of the four different PM enantiomers using human liver microsomes. Metabolites were identified and temporal studies were conducted with liver microsomes as well as CYP2C19 and CYP3A4 which were previously shown to catalyze oxidative metabolism of PM.

## 2. Material and methods

### 2.1. Chemicals

Pure standards of permethrin diastereoisomers (*cis*- and *trans*-PM) enantiomers (chemical purity > 99%) were provided by Prof. Jay Gan (University of California Riverside). 3-Phenoxybenzyl alcohol (PBOH; 97% purity), 3-phenoxybenzoic acid (PBCOOH; 98% purity) and ketoconazole were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents (hexane, isopropanol, acetonitrile and methanol), used in this study were of analytical or HPLC grade. Human CYP2C19 and CYP3A4 Supersomes were purchased from BD Biosciences (San Jose, CA).

### 2.2. Synthesis of 3-(4′-hydroxyphenoxy)benzyl alcohol (3,4-PBOH)

3-(4′-Hydroxyphenoxy)-benzyl alcohol (3,4′-PBOH), >99% purity, was synthesized using the procedure described in Nillos et al. (2010). Briefly, the $Ag_2O$ oxidation of commercially available 3-(4-methoxyphenoxy)benzaldehyde afforded 3-(4-methoxyphenoxy)benzoic acid in 80% yield, which was deprotected and subsequently reduced according the standard method (Nillos et al., 2010) yielding the desired metabolite 3-(4-(hydroxymethyl)phenoxy-phenol.

### 2.3. Permethrin's enantiomer separation and analysis

Enantiomers of PM were resolved on an Agilent 1100 Series HPLC equipped with an online laser polarimeter detector (PDRChiral, Lake Park, FL, USA). Enantiomer resolution and identification were carried out at room temperature on a Chiralcel OJ column (250 mm, cellulose tris-(4-methyl benzoate) on a silica gel substrate, Daicel Chemical Industries, Tokyo, Japan) using hexane/isopropanol (96:4, v/v) as the mobile phase. The flow rate of the mobile phase was 0.8 mL/min. The UV detection wavelength was set at 254 nm for all analyses. The specific rotation of the resolved stereoisomers was determined at 675 nm and using a 50 mm cell path. The rotation sign (±) was directly indicated by a positive or negative peak on the polarimeter. Pure enantiomers used for metabolic transformation experiments were manually collected with a SCL-10AVP Shimadzu HPLC system equipped with the same Chiralcel OJ column and by using the same normal phase of hexane/isopropanol (96/4, v/v) system. Enantiomers were evaporated to dryness under a stream of pure nitrogen, followed by dissolution in acetone (carrier solvent). The purity of the derived stereoisomers was checked with re-analysis on HPLC and was found to be >99% in all cases.

### 2.4. Microsomal preparation

Human liver microsomes were provided by Dr. Allan Rettie of the University of Washington. Samples were pooled from six individuals and included 2 males (21–39 yr) and 4 females (36–63 yr). Each individual sample was previously characterized with regard to specific CYP content (mRNA), genotype, and catalytic activities (Lin et al., 2002). Protein concentrations were determined by the Coomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL, USA) using bovine serum albumin as a standard.

### 2.5. Microsomal biotransformation

Hepatic microsomal biotransformation of permethrin enantiomers was determined on the basis of the method by Godin et al. (2007) and Nillos et al. (2010) with some modifications. Supersome incubations were conducted in an identical manner. A 250 μL reaction volume containing 300 μg of microsomal protein, 100 μM substrate (PM enantiomers: 1*S*-*cis*-, 1*R*-*cis*-, 1*S*-*trans*-, and 1*R*-*trans*-permethrin-added in 5 μL of methanol), 1 mM NADPH, and 100 mM Tris–HCl buffer pH 7.4 was incubated at 37 °C for 90–120 min. The reaction was quenched by the addition of equal volumes of ice-cold acetonitrile following the addition of the internal standard *cis*-bifenthrin. Following centrifugation, the supernatant was transferred to HPLC glass vials and analyzed for metabolic products by HPLC/UV at λ = 254 nm. Negative controls omitted NADPH or included boiled microsomal protein. Cytochrome P450 inhibition experiments were conducted with co-incubation with 1 mM ketoconazole added (in 5 μL of methanol) immediately after NADPH addition and incubated for 5 min prior to the addition of individual PM enantiomers. For kinetic measurement, concentrations of substrate (individuals enantiomers) ranged from 1 μM to 500 μM. For time-dependent measurements, incubations of 30, 60, 90 and 120 min were utilized.

### 2.6. HPLC analysis

Reverse-phase HPLC-UV analysis of the products was based on the method of Nillos et al. (2010). Analysis was performed on an SCL-10AVP Shimadzu HPLC system equipped with a reverse-phase C-18 HPLC column (Agilent Hypersil ODS (4.0 mm × 250 mm) C18 (5 μm)). The mobile phase consisted of solvents A (90% acetonitrile and 10% water) and B (100% water adjusted to pH 1.7 with 85% phosphoric acid). The analytes were eluted using the following gradient program: 0 min (50% A; 50% B), 6 min (75% A; 25% B), 7 min (100%A; 0% B), 11 min (100% A; 0% B), 12 min (50% A; 50%B), and 20 min (50% A; 50% B) at a flow rate of 1 mL/min. Metabolites were detected at 254 nm and quantified using external standards. Retention times of metabolites were: 3.5 min for 3,4-PBOH; 4.3 min for 3,4-PBCOOH; 13.1 min for PBOH; 14.0 min for PBCOOH; 18.8 min for 4-OH-*trans*-PM; 19.3 min for 4-OH-*cis*-PM; 21.1 for *trans*-PM; and 22.9 min for *cis*-PM.

### 2.7. Data analysis

Statistical significance was assessed using one-way ANOVA test to evaluate differences between groups, with the use of GraphPad Prism version 5.00 for MacOS X (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's and Bonferroni's multiple range tests were performed. Samples showing levels below the detection limits were considered as having 50% of the minimal values detectable for statistical comparisons. All data was analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests. Kinetic parameters (Lineweaver–Burk plots) were calculated using Graphpad Prism v5.0 software package using non-linear regression (Graphpad Software Inc., La Jolla, CA).

## 3. Results

The detection parameters for the current study focused on metabolites absorbing Ultraviolet light at 254 nm thus relegating detection to metabolites possessing conjugated or aromatic moieties. Only two metabolites were consistently detected in microsomal or CYP-supersomal incubations with the 4 enantiomers of PM: PBOH and 3,4-PBOH. When evaluating the microsomal catalytic efficiency of metabolite formation using the 4 enantiomers, formation of each metabolite was different depending upon which enantiomer of PM was incubated with the microsomal mixture after 30 min (Table 1). Overall, there was greater catalytic efficiency ($V_{max}/K_m$) for 3,4-PBOH formation compared to PBOH. For both metabolites, microsomal transformation of *trans* PM had higher catalytic efficiency ($V_{max}/K_m$) than *cis* PM. The *R*-enantiomers of both *cis* and *trans* PM had slightly higher efficiencies of PBOH formation than the corresponding *S* enantiomers.

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