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Research paper

Benoxacor is enantioselectively metabolized by rat liver subcellular fractions

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ABSTRACT

This study investigated the enantioselective metabolism of benoxacor, an ingredient of herbicide formulations, in microsomes or cytosol prepared from female or male rat livers. Benoxacor was incubated for ≤30 min with microsomes or cytosol, and its enantioselective depletion was measured using gas chromatographic methods. Benoxacor was depleted in incubations with active microsomes in the presence and absence of NADPH, suggesting its metabolism by hepatic cytochrome P450 enzymes (CYPs) and microsomal carboxylesterases (CESs). Benoxacor was depleted in cytosolic incubations in the presence of glutathione, consistent with its metabolism by glutathione S-transferases (GSTs). The depletion of benoxacor was faster in incubations with cytosol from male than female rats, whereas no statistically significant sex differences were observed in microsomal incubations. The consumption of benoxacor was inhibited by the CYP inhibitor 1-aminobenzotriazole, the CES inhibitor benzil, and the GST inhibitor ethacrynic acid. Estimates of the intrinsic clearance of benoxacor suggest that CYPs are the primary metabolic enzyme responsible for benoxacor metabolism in rats. Microsomal incubations showed an enrichment of the first eluting benoxacor enantiomer (E_1-benoxacor). A greater enrichment occurred in incubations with microsomes from female (EF = 0.67 ± 0.01) than male rats (EF = 0.60 ± 0.01). Cytosolic incubations from female rats resulted in enrichment of E_1-benoxacor (EF = 0.54 ± 0.01), while cytosolic incubations from male rats displayed enrichment of the second eluting enantiomer (E_2-benoxacor; EF = 0.43 ± 0.01). Sex-dependent differences in the metabolism of benoxacor in rats could significantly impact ecological risks and mammalian toxicity. Moreover, changes in the enantiomeric enrichment of benoxacor may be a powerful tool for environmental fate and transport studies.

1. Introduction

Safeners, such as benoxacor, are co-formulated with herbicides to protect crops from herbicidal damage [1,2]. Benoxacor is a chiral dichloroacetamide safener typically co-applied with chloroacetanilide herbicides (e.g., metolachlor) [3]. It contains a 3-methyl-2,3-dihydro-1,4-benzoxazine moiety with a chiral center in the 3-position and, like other chiral agrochemicals, is produced as a racemate. Safeners promote the rapid biotransformation of herbicides to less toxic metabolites within cereal crops, but not weed species [1]. Under the United States' Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), active ingredients of pesticide preparations are compounds that prevent, destroy, repel, or mitigate pests. Since the biological activity of safeners, such as benoxacor, is directed towards crops, they are considered "inert" and, thus, are not held to the same scrutiny as the active ingredients of herbicides during the registration process under FIFRA [1]. However, inert ingredients may enhance the toxicity of their co-formulated pesticide or cause adverse effects themselves [4,5].

Benoxacor is co-applied with metolachlor, an herbicide extensively used in the Midwest of the United States; however, few peer-reviewed studies of its occurrence, environmental fate and transport, and toxicity are available. A study by the United States Geological Survey (USGS) detected benoxacor in surface water of Iowa and Illinois, United States [3]. In the USGS study, benoxacor detection was highly correlated with the planting of corn and was mostly detected between March–June

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Abbreviations

E_1-benoxacor	First Eluting Enantiomer of Benoxacor
EF	Enantiomeric Fraction
E_2-benoxacor	Second Eluting Enantiomer of Benoxacor
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
USGS	United States Geological Survey
GSTs	Glutathione S-Transferases
CYPs	Cytochrome P450 enzymes
CESs	Carboxylesterases
GTA	Astec CHIRALDEX GTA column
CB	Agilent CP-Chiralsil Dex CB column
k_{dep}	rate of depletion
$t_{1/2}$	half-life
CL_{int}	apparent intrinsic clearance
NADPH	Nicotinamide adenine dinucleotide phosphate
GSH	Glutathione
ANOVA	Analysis of Variance

at levels between 4 and 190 ng/L. This concentration is much lower than the experimental solubility of benoxacor (22 ± 1.7 mg/L) [6]. Studies also suggest that benoxacor moves through the environment, similarly to metolachlor [7]. Thus, wildlife, livestock, and individuals living in these areas may be exposed to safeners, e.g., via their drinking water. Therefore, studies are needed to assess potential human exposures to benoxacor and other safeners.

Benoxacor undergoes direct photolysis forming monochlorinated and fully dechlorinated transformation products [8]. It also undergoes abiotic hydrogenolysis in iron-reducing environments [9]. It has also been shown that benoxacor photoproducts serve as photosensitizers for metolachlor and that metolachlor on quartz is degraded 1.8 times faster than when benoxacor is not present [10]. Most metabolism studies of benoxacor are focused on its metabolism in plants [11]. In plants, several enzymes, including GSTs and CYPs, seem to play a major role in the detoxification of safeners and their co-formulated herbicides [11,12]. CESs are also major detoxifying enzymes found in microsomes and cytosol that do not require the addition of an external cofactor [13] and may metabolize benoxacor. The literature on the toxicity of benoxacor in animal models is limited to regulatory studies [14] or studies in insects, such as *Chironomus riparius* [1,15].

The metabolism of benoxacor may influence its environmental fate, transport, and toxicity, despite its inert classification [1]. Like other chiral environmental contaminants, its metabolism and toxicity may be enantioselective [16,17]. We used subcellular fractions, including cytosol and microsomes from female and male rat livers, to identify the classes of drug-metabolizing enzymes involved in the enantioselective metabolism of benoxacor in a toxicologically relevant mammalian model system. We focused on CYPs and GSTs based on results from structurally similar agrochemicals [18,19]. Determining which enzymes metabolize benoxacor and characterizing the enantioselectivity of these enzymes towards benoxacor addresses critical knowledge gaps for this common agrochemical.

2. Materials and Methods

2.1. Subcellular fractions

Microsomal (lot numbers 0810450 and 1610290 for pooled liver microsomes from female and male Sprague Dawley rats, respectively) and cytosolic preparations (lot numbers 0810450 and 1410273 for pooled liver cytosol from female and male Sprague Dawley rats, respectively) were purchased from Sekisui XenoTech (Kansas City, KS, USA). Benoxacor (Lot # BCBT8607) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Chemicals

Benoxacor (98.4% purity; InChIKey: PFJJMJDEVDLPNE-UHFFFAOYSA-N) and trifluralin (99.3% purity; InChIKey: ZSDSQXJSNMTJDA-UHFFFAOYSA-N; recovery standard) were purchased from Sigma-Aldrich (St Louis, MO, USA). The internal standard, acenaphthene (99.3% purity), was purchased from Accustandard (New Haven, CT, USA). The nonspecific inhibitor of CYPs, 1-aminobenzotriazole [20], was obtained from TCI (≥98% purity; InChIKey: JCXKHYLLVKZPKE-UHFFFAOYSA-N; Portland, OR, USA). Benzil, classical CES inhibitor [21,22], was purchased from ACROS Organics (≥99% purity; InChIKey: WURBFLDFSFBTLW-UHFFFAOYSA-N; New Jersey, NJ, USA). Ethacrynic acid, a frequently used inhibitor of GSTs [23,24], was provided by Enzo Life Sciences (≥98%; InChIKey: AVOLMBLBETYQHX-UHFFFAOYSA-N; Farmingdale, NY, USA). Pesticide grade ethyl acetate used for liquid-liquid extractions was supplied by Fisher Chemical (Fair Lawn, NJ, USA). Chemicals for the preparation of buffers were obtained from commercial sources and were of ACS grade (≥98% purity).

2.3. Microsomal incubations with benoxacor

Microsomal incubations contained 0.1 mg/mL protein, 0.5 mM NADPH, and 5 μM benoxacor [19] in incubation buffer as described [25]. Samples were preincubated for 5-min and then spiked with 10 μL of 2.5 mM benoxacor in DMSO for a final concentration of 5 μ*M* benoxacor and a total volume of 5 mL. A 500 μL aliquot was taken every 5 min for 30 min and added to 2 mL of ice-cold 1% formic acid. Control samples were incubated in parallel and consisted of buffer blanks spiked with vehicle (DMSO) to assess chemical degradation in aqueous solution, inactivated microsomes with NADPH and benoxacor to control for chemical degradation by the constituents (e.g., metal ions) in microsomal preparations, NADPH and benoxacor without microsomes to determine if benoxacor reacts with NADPH, and active microsomes with benoxacor but no NADPH to evaluate if the degradation is due to oxidative metabolism by CYPs or other enzymes, such as CESs.

2.4. Cytosolic incubations with benoxacor

Cytosolic incubations contained 1 mg/mL protein, 5 mM glutathione (GSH), and 5 μM benoxacor in incubation buffer [19] and were carried out as described above for the microsomal incubations. Control incubations were identical as described above for microsomal incubation, except GSH was used as the cofactor instead of NADPH to assess if the loss of benoxacor was due to glutathione conjugation.

2.5. Microsomal inhibition studies

Inhibition studies for CYPs and CESs were prepared in the same way as the previous microsomal experiments except for the addition of 1-aminobenzotriazole in ethanol (vehicle volume 25 μL) to inhibit CYP activity or benzil in ethanol (vehicle volume 25 μL) to inhibit CES activity. Three concentrations of 1-aminobenzotriazole (35, 350, 3500 μ*M*) or benzil (5, 50, 500 n*M*), a vehicle control, and all other previously included controls were run in parallel. Concentrations of inhibitors were chosen based on k_i values reported previously [20–22]. Samples were collected at 0 and 15 min for CYPs, and 0 and 30 min for CESs, to keep the metabolism within the linear range of previous microsomal incubations. The reaction was quenched by adding a 500 μL aliquot of the incubation to 2 mL of 1% ice-cold formic acid.

2.6. Cytosolic inhibition studies

Inhibition studies for GSTs were prepared in the same way as

previous cytosolic incubation experiments with the exception of the addition of ethacrynic acid in ethanol (vehicle volume 25 μL) to inhibit GST activity. Three concentrations of ethacrynic acid (10, 100, 1000 μ*M*), a vehicle control, and all other previously included controls were performed in parallel. Inhibitor concentrations were chosen based on previously reported inhibition studies [23,24]. Samples were collected at 0 and 5 min to keep the metabolism within the linear range of previous microsomal incubations. The reaction was quenched by adding a 500 μL aliquot of the incubation to 2 mL of 1% ice-cold formic acid.

2.7. Extraction of benoxacor

All samples were heated to 110 °C for 10 min to denature the protein, allowed to cool to room temperature, and spiked with 25 μL of 20 μg/mL trifluralin in ethyl acetate before liquid-liquid extraction with 2 mL of ethyl acetate. The samples were then placed on a tube rotator for 1 min at 40 rpm and centrifuged at 1811 ***g*** for 5 min. The organic layer was transferred to a new tube, and the aqueous phase was re-extracted with ethyl acetate. The combined organic extract was washed with 2 mL of 1% KCl, dried with approximately 0.25 g of $MgSO_4$, and transferred to a GC vial.

2.8. Gas chromatographic analyses

We adapted a method for detecting dichloroacetamide safeners in surface water [3] to analyze the depletion of benoxacor in our metabolism studies. These analyses were performed on an Agilent 6890 N gas chromatograph coupled to an Agilent 5975 MS in the SIM mode to quantify benoxacor using acenaphthene as the internal standard. The inlet was set to splitless injection at a temperature of 280 °C. The column utilized was an Agilent SLB-5MS column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) with a constant helium flow rate of 1 mL/min. The oven conditions were as follows: Initial temperature, 50 °C, hold for 1 min, 15 °C/min to 240 °C, hold for 13 min, 15 °C/min to 300 °C, hold for 10 min. The temperatures of the MS source and the MS quad were 230 °C and 150 °C, respectively.

We used an Agilent 7890 A gas chromatograph coupled to a ^{63}Ni-μECD detector to determine the enantiomeric fraction (EF) of benoxacor. Enantioselective analyses of benoxacor were optimized on an Astec CHIRALDEX GTA column (GTA) (30 m length, 0.25 mm inner diameter, 0.12 μm film thickness) (Table S1) and an Agilent CP-Chiralsil Dex CB (CB) column (25 m length, 0.25 mm inner diameter, 0.25 μm film thickness) (Table S2). The CB column was used for all enantioselective analyses of benoxacor. The following temperature program was used: initial temperature, 50 °C, hold for 1 min, 10 °C/min to 145 °C, hold for 50 min, 15 °C/min to 200 °C, hold for 12 min. The helium flow rate was 2 mL/min. The injector temperature was set at 250 °C, and the detector temperature was set to 300 °C. The EF was calculated by the equation $EF = A_1/(A_1+A_2)$, where A_1 is the area for the first eluting enantiomer, and A_2 is the area for the second eluting enantiomer. Details regarding the optimization of the separation of benoxacor enantiomers are provided in the Supplementary Material.

2.9. Intrinsic clearance calculations

Apparent intrinsic clearance and scaled intrinsic clearance of benoxacor were calculated for incubations with rat liver microsomes and cytosol from previously reported methods [26]. Briefly, initial rates of benoxacor metabolism (k_{dep}) were determined by plotting the natural logarithm of the percentage of benoxacor remaining against time. Linear regression analysis was applied to determine the time when 50% of benoxacor would be depleted ($t_{1/2}$). Apparent intrinsic clearance was calculated by equation (1), where V is the incubation volume, and $t_{1/2}$ is the *in vitro* half-life of the substrate [26]. For microsomal incubations, depletion rate (k_{dep}) values were corrected for endogenous CES activity.

$$CL_{int} = \sum_{i}^{n} \frac{V_{max,i}}{K_{m,i}} = \frac{0.693 \times V}{\text{in vitro } t_{\frac{1}{2}}} \quad (1)$$

After the CL_{int} was calculated, it was corrected for the total microsomal or cytosolic protein present within the incubation. The corrected values were then scaled to total hepatic clearance (scaled CL_{int}) using previously reported scaling factors for rat microsomes and cytosol (Table 1) [26].

2.10. Statistical analyses

Graphpad Prism 8.1.2 was used to explore statistical differences between both sexes and controls. Unpaired t-tests were used to compare time points and rates between preparations of microsomes and cytosol from female and male rats. Paired t-tests were employed to compare zero-minute time points of a specific sex to later time points. One-way Analysis of Variance (ANOVA) was used to compare 30-min time points for both depletion and change in enantiomeric analyses. Tukey's test for multiple comparisons was employed to confirm statistical significance in active incubations compared to control samples. For all analyses, $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Oxidative metabolism of benoxacor by rat liver microsomes

Benoxacor was depleted in incubations with microsomes from both female and male rats over a 30-min time-course in the presence of NADPH (Fig. 1). The percentage of benoxacor decreased linearly for 20 and 30 min in experiments with liver microsomes from female and male rats, respectively (Fig. 1A, Table S3). At the 30-min time point, 48 ± 1% and 45 ± 1% of benoxacor remained in incubations with microsomes from female and male rats, respectively. The depletion of benoxacor was not significantly different between female and male microsomes; however, the rate of benoxacor consumption, calculated at the 20-min time point, was lower in experiments using microsomes from male rats (Fig. 1B). This observation, while not statistically significant, was unexpected as the total CYP content was lower in microsomal preparations from female than male rats (0.595 nmol/mg protein vs. 0.679 nmol/mg protein, respectively). Differences in the metabolism between sexes are quite common and occur with a wide range of xenobiotics and drugs [27,28]. Control incubations with heat-inactivated microsomes and without microsomes showed minimal disappearance of benoxacor (Fig. 1C and D). This observation is consistent with benoxacor decay resulting from oxidative, CYP-mediated metabolism in incubations with active liver microsomes.

In incubations with microsomes from male and female rats containing 1-aminobenzotriazole, a classical CYP inhibitor [20], the depletion of benoxacor was inhibited in a concentration-dependent manner (Fig. 2A and B), indicating that the depletion of benoxacor was from the metabolism of CYPs and not from protein binding. In

Table 1
Predicted intrinsic and scaled intrinsic clearance values for CESs, CYPs, and GSTs estimated based on a previously presented method [26].

Value	Male	Female
CES CL_{int} (mL/min/mg protein)	0.15	N/A
CES Scaled CL_{int} (mL/min/g liver)	6.74	N/A
CYP CL_{int} (mL/min/mg protein)	1.11	1.28
CYP Scaled CL_{int} (mL/min/g liver)	50.13	57.80
GST CL_{int} (mL/min/mg protein)	0.44	0.28[a]
GST Scaled CL_{int} (mL/min/g liver)	40.18	25.87[a]

[a] Significant difference between female and male clearance using an unpaired *t*-test ($p = 0.0038$).
N/A, not applicable.

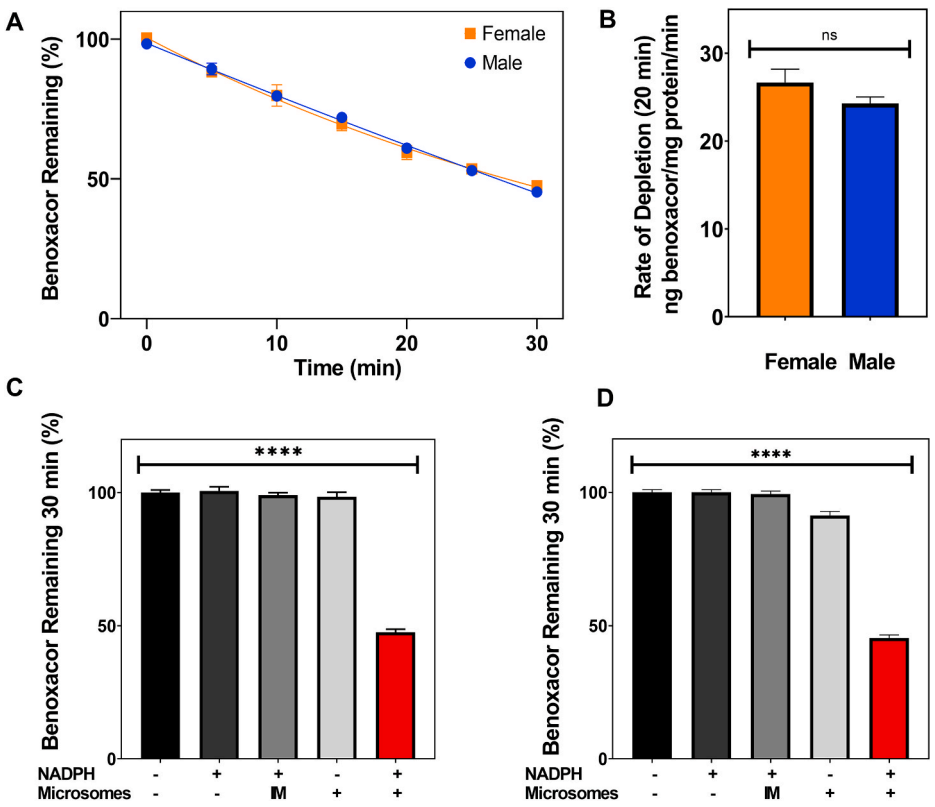

Fig. 1. Benoxacor is metabolized by CYPs and CESs in incubations with rat liver microsomes. (A) Depletion of benoxacor over a 30-min time-course in female and male rat liver microsomes. Comparison of (B) the protein adjusted rate of depletion of benoxacor at 20 min for female and male rat liver microsomes; (C) the percentage of benoxacor in active female rat liver microsomal incubations and control incubations at 30 min, and (D) the percentage of benoxacor in active male rat liver microsomal incubations and control incubations at 30 min, see text for more details. An unpaired *t*-test was used to determine significance in (B). One-way ANOVA was used to determine significance in (C&D). Tukey's multiple comparisons test confirmed a significant decrease in active incubations in (C) ($p < 0.0001$) and a significant decrease in both the microsomes without NADPH ($p < 0.0001$) and active incubations ($p < 0.0001$) at the 30-min time point. The values of benoxacor depletion are shown in Table S1. Data are the mean ± standard deviation for three independent replicates; for some data points, the standard deviation is smaller than the size of the symbols. ns, not significant; NADPH, nicotinamide adenine dinucleotide phosphate; IM, heat-inactivated microsomes; ****$p < 0.0001$ (one-way ANOVA).

microsomal preparations from female rats, the percent of benoxacor remaining at 15-min was 71 ± 1% without inhibitor and 98 ± 1% with 3500 μ*M* of inhibitor present. In microsomal preparations from male rats, the percent of benoxacor remaining at 15-min was 72 ± 1% without inhibitor and 95 ± 1%. These experiments provide further evidence that benoxacor, unlike other dichloroacetamide safeners [19], is metabolized by CYPs.

Levels of benoxacor were significantly lower in incubations with active microsomes from male rats without NADPH compared to control incubations with inactive microsomes ($p = 0.0002$; Fig. 1D). No significant depletion of benoxacor was observed in experiments with liver microsomes from female rats (Fig. 1C). Moreover, benoxacor decreased over time in incubations with rat microsomes from male rats in the absence of NADPH (Fig. S1). This finding suggests that drug-metabolizing enzymes other than CYPs are involved in the depletion of benoxacor in male but not female rats. Because no cofactors (e.g., NADPH) are involved in this metabolism, it is likely that CESs metabolize benoxacor in a sex-dependent manner. Multiple studies have reported higher protein levels and activities of CESs in male than female rats [29,30], which likely explains why we observed CES-mediated metabolism only with microsomal preparations from male rats. While benoxacor does not contain an ester, CESs are capable of metabolizing amide groups [31]. For example, the drug selexipag is hydrolytically cleaved by CESs at a sulfonamide moiety to yield its pharmacologically active metabolite [32]. We hypothesize that benoxacor is similarly cleaved at its amide moiety to form dichloroacetic acid and its 1,4-benzoxazine moiety.

We performed additional inhibition studies to confirm the role of CESs in the hepatic metabolism of benoxacor in male rat microsomal preparations. The loss of benoxacor was significantly inhibited by benzil, a classical CES inhibitor [21,22], in metabolism studies with microsomes prepared from male rat liver and without NADPH (Fig. 2C). Briefly, 92 ± 2% of benoxacor remained after 30 min in incubations without benzil. In contrast, benoxacor was not depleted in experiments with benzil at concentrations ranging from 5 to 500 n*M*. While the inhibition of benoxacor metabolism was independent of the benzil concentration, as with the inhibition of CYPs, this finding is not unexpected as only a relatively small amount of benoxacor was depleted in these studies. Analogous inhibition studies were not carried out with microsomal preparations from female rats since no significant depletion was seen in the previous incubations (Fig. 1C). These observations demonstrate that microsomal CESs play a role in the metabolism of benoxacor in male but not female rats.

Work to identify products of benoxacor metabolism is ongoing, with complete metabolite elucidation beyond the scope of the current work focused on enantioselective metabolism. Generally, little is known in the peer-reviewed literature about benoxacor metabolites, with most microsome or whole organism work to date exploring other dichloroacetamide herbicide safeners (e.g., dichlormid) [19]. Our group has recently structurally elucidated abiotic transformation products of benoxacor generated via reaction with sunlight [8], and similar tools are being used to probe the nature of metabolites produced herein.

3.2. Enantiomeric enrichment in microsomal incubations

Rat liver microsomes enantioselectively metabolized the first eluting enantiomer of benoxacor (E_1-benoxacor) in the presence of NADPH (Fig. 3). Over the 30-min time-course, the EF values of benoxacor increased from 0.48 to 0.67 in studies with microsomes from female rats and from 0.48 to 0.60 in incubations with microsomes from male rats (Fig. 4A, Table S4). EF values were significantly different at 30-min for incubations using microsomes from female vs. male rats (Fig. 4B). These differences are consistent with sex-dependent differences in the expression of the CYPs responsible for benoxacor metabolism.

Incubations of active microsomes without NADPH showed a slight, but significant decrease in the EF value from 0.48 to 0.47 (unpaired *t*-test, $p = 0.0012$) in incubations with microsomes from female rats and from 0.48 to 0.46 (unpaired *t*-test, $p = 0.0021$) in studies with microsomes from male rats (Fig. 4C and D). No significant change in the EF value of benoxacor was observed in the other control incubations. This

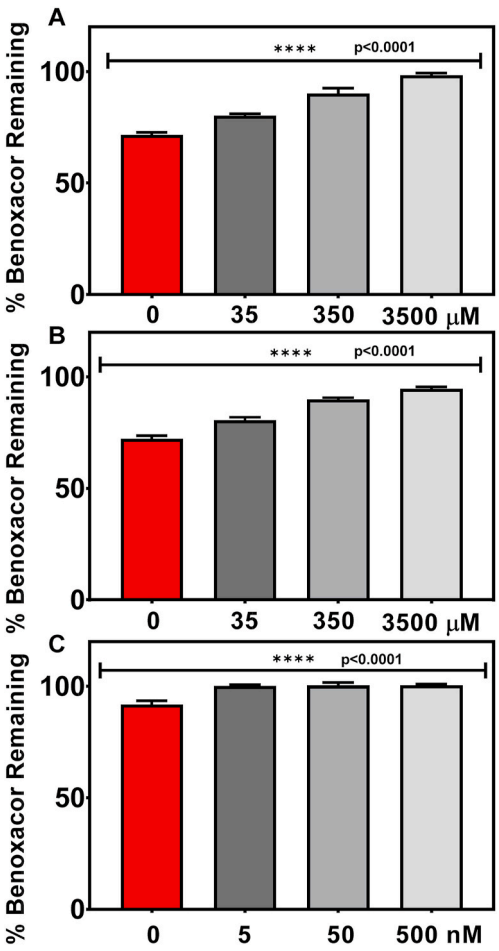

Fig. 2. The depletion of benoxacor in metabolism studies with liver microsomes from female and male rats is inhibited in a concentration-dependent manner by classical inhibitors of (A) microsomal CYPs from female rats, (B) microsomal CYPs from male rats, and (C) microsomal CESs from male rats. Incubations with liver microsomes from (A) female and (B) male rats in the presence of NADPH were performed for 15 min with the addition of 1-aminobenzotriazole (0, 35, 350, and 3500 μ*M*) to inhibit benoxacor metabolism by CYPs. (C) Incubations with liver microsomes from male rats without NADPH were performed for 30 min with the addition of benzil (0, 5, 50, and 500 n*M*) to inhibit metabolism by CESs; see text for more details. One-way ANOVA with Tukey's multiple comparisons test was used to determine significance in (A–C). Data are the mean ± standard deviation for three independent replicates. Control incubations showed minimal benoxacor depletion. For 1-aminobenzotriazole controls, the benoxacor remaining in the incubations was 101 ± 1% for 35 μM, and 100 ± 1% for 350 and 3500 μM controls. For benzil controls, the benoxacor remaining was 100 ± 1% for 5, 50, and 500 nM.

observation is consistent with an enzymatic conversion of benoxacor, for example, by CESs. Consistent with our results, CESs can be highly enantioselective [33]. For instance, propranolol derivatives, cocaine, and cypermethrin analogs are enantioselectively metabolized by human CESs [34].

3.3. Metabolism of benoxacor by rat liver cytosol

In the presence of GSH, benoxacor was depleted in incubations with liver cytosol from both female and male rats over a 30-min time-course (Fig. 5A, Table S5). At the 30-min time point, 18 ± 2% and 9 ± 2% of benoxacor remained in incubations with cytosol from female and male rats, respectively. The depletion of benoxacor was significantly lower in experiments with cytosol from female rats compared to male rats at the 5-min time point (*t*-test, p = 0.0350) (Fig. 5B). Control incubations with

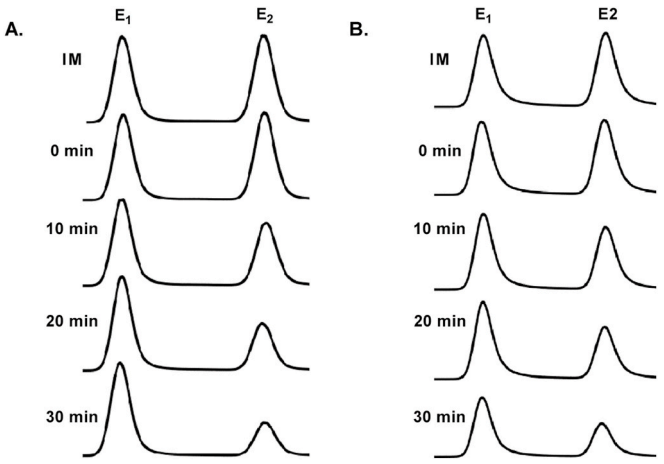

Fig. 3. Representative gas chromatograms show time-dependent changes in the enantiomeric enrichment of benoxacor in metabolism studies with rat liver microsomes from (A) female and (B) male rats. Incubations with female and male rat liver microsomes showed an enrichment of the benoxacor enantiomer eluting first on the enantioselective column. Microsomal incubations contained 0.1 mg/mL protein, 0.5 mM NADPH, and 5 μM benoxacor in incubation buffer. All samples were incubated for 30-min, and an aliquot was taken every 5-min for measurement. After extraction, a CB column was utilized to separate the enantiomers of benoxacor on an Agilent 7890 A gas chromatograph coupled to a ^{63}Ni-μECD detector as described under Materials and Methods.

heat-inactivated cytosol and without cytosol showed no significant disappearance of benoxacor. This observation is consistent with a GST-mediated metabolism of benoxacor in incubations with active liver cytosol. In incubations containing different concentrations of the classical GST inhibitor ethacrynic acid [24,35], the depletion of benoxacor was inhibited in a concentration-dependent manner compared to control incubations without the inhibitor (Fig. 6A and B). This observation indicates that the depletion of benoxacor was from the metabolism of GSTs and not due to the binding of benoxacor to cytosolic proteins. In cytosolic preparations from female rats, the percent of benoxacor remaining at 5-min was 71 ± 3% without inhibitor and 98 ± 2% with 1000 μ*M* of inhibitor present. In microsomal preparations from male rats, the percent of benoxacor remaining at 5-min was 64 ± 1% without inhibitor and 96 ± 2% with inhibitor.

3.4. Enantiomeric enrichment in cytosolic incubations

Benoxacor was enantioselectively metabolized by rat liver cytosol in the presence of GSH. E_1-benoxacor was enriched in cytosol preparations from female rats, whereas E_2-benoxacor was enriched in cytosol preparations from male rats (Fig. 7). Over the 30-min time-course, the EF values of benoxacor increased from 0.48 to 0.55 in incubations with cytosol from female rats but decreased from 0.47 to 0.43 in experiments with cytosol from male rats (Fig. 8A, Table S6). The enrichment of different enantiomers in females and males is likely due to sex-specific differences in the expression of GST isoforms [36]. This finding could impact sex-dependent toxicities as typically only one enantiomer of a pharmaceutical or pesticide shows activity towards a specific cellular target [16]. EF values at the 30-min time point were significantly higher (*t*-test, p = 0.0002) in experiments with cytosol from female than male rats (Fig. 8B). Control incubations with heat-inactivated cytosol and without cytosol showed no statistical change in EF value (Fig. 8C and D).

3.5. Apparent hepatic clearance of benoxacor

The intrinsic clearance of benoxacor by different drug-metabolizing enzymes, such as CYPs, GSTs, and CESs, was estimated using the data from rat microsomal and cytosolic incubations (Table 1) [26]. The

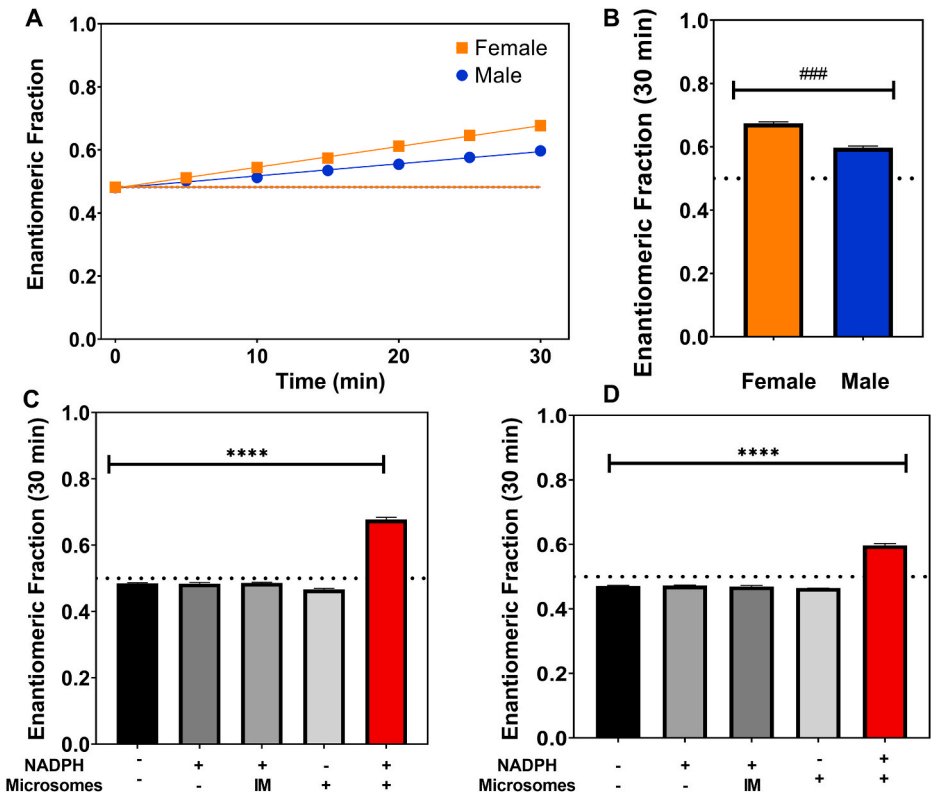

Fig. 4. Benoxacor is enantioselectively metabolized by rat liver microsomes, with E_2-benoxacor being enriched in incubations with microsomes from both female and male rats. (A) Change in the enantiomeric fraction of benoxacor in experiments with female and male rat liver microsomes over a 30-min time-course. Comparison of the enantiomeric fraction of benoxacor at 30 min between (B) incubations with female vs. male rat liver microsomes; (C) experiments with active female rat liver microsomes and controls, and (D) incubations with active male rat liver microsomal incubations and controls, see text for more details. The EF values at all time points were significantly different from the 0-min timepoint. EF values were also significantly different between incubations with microsomes from female and male rats at all time point ≥5 min ($p = 0.0379$). An unpaired *t*-test was used to determine significance in (B). One-way ANOVA was used to determine significance in (C&D). Tukey's multiple comparisons test confirmed a significant increase in the EF values in active preparations from female ($p < 0.0001$) and male rats ($p < 0.0001$). EF values are reported in Table S2. Representative chromatograms are shown in Fig. S2. Data are the mean ± standard deviation for three independent replicates; for some data points, the standard deviation is smaller than the size of the symbols. The dotted lines represent EF = 0.5. NADPH, nicotinamide adenine dinucleotide phosphate; IM, heat-inactivated microsomes; ****p < 0.0001 (one-way ANOVA); ###p < 0.001 (unpaired *t*-test).

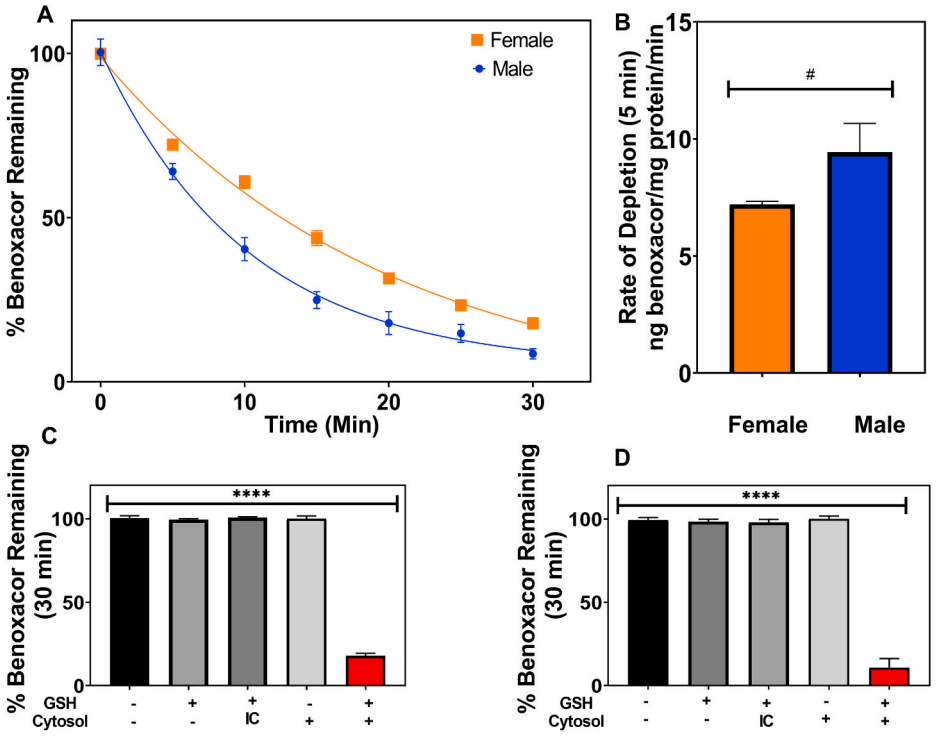

Fig. 5. Benoxacor is metabolized in the presence of GSH in incubations with rat liver cytosol, consistent with its metabolism by GSTs. (A) Depletion of benoxacor over a 30-min time-course in female and male rat liver cytosol. Comparison of: (B) the protein-adjusted rate of depletion of benoxacor at 5 min for female and male rat liver cytosol; (C) the percentage of benoxacor in active female rat liver cytosolic incubations and controls at 30 min, and (D) the percentage of benoxacor in active male rat liver cytosolic incubations and controls at 30 min, see text for more details. The percentage of benoxacor at all time points were significantly different from the 0-min time point, and changes in EF by preparations of cytosol from female and male rats were significantly different after 5 min ($p = 0.0071$). An unpaired *t*-test was used to determine significance in (B). One-way ANOVA was used to determine significance in (C&D). Tukey's multiple comparisons test confirmed a significant decrease in benoxacor in active preparations from female ($p < 0.0001$) and male rats ($p < 0.0001$) at the 30-min time point. Values for benoxacor depletion are reported in Table S3. Data are the mean ± standard deviation for three independent replicates; for some data points, the standard deviation is smaller than the size of the symbols. GSH, glutathione; IC, heat-inactivated cytosol; ****p < 0.0001 (one-way ANOVA); #p < 0.05 (unpaired *t*-test).

scaled hepatic CL_{int} of benoxacor decreased in the order CYP > GST ≫ CES in male rats. Similarly, the scaled hepatic CL_{int} followed the rank order CYP > GST in female rats. However, CYPs appear to play a more important role in the clearance of benoxacor in female than male mice, with CESs making no contribution to the clearance of benoxacor in female rats. No significant difference was seen between the clearance of CYPs in female or male rats. The clearance by GSTs, however, was significantly higher in male than female rats (unpaired *t*-test, $p = 0.0038$). Overall, CYP and GST metabolism are both major players in the sex-dependent metabolism of benoxacor in rats. While this is the first

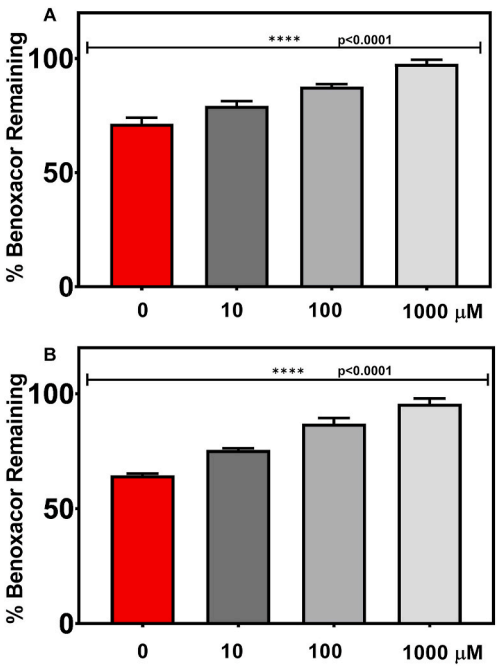

Fig. 6. The depletion of benoxacor in metabolism studies with liver cytosol from female and male rats is inhibited in a concentration-dependent manner by classical inhibitors of cytosolic GSTs from female and male rats. Incubations with liver cytosol prepared from (A) female and (B) male rat liver at 5 min with the addition of ethacrynic acid (0, 10, 100, and 1000 μ*M*) to inhibit GST metabolism, see text for more details. One-way ANOVA with Tukey's multiple comparisons test was used to determine significance in (A–C). Data are the mean ± standard deviation for three independent replicates. Control incubations showed minimal benoxacor depletion (100 ± 1%, 102 ± 1%, and 100 ± 1% for 10, 100, and 1000 μM ethacrynic acid, respectively).

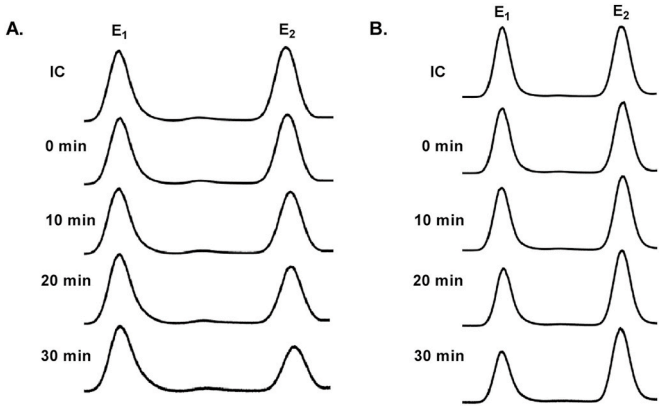

Fig. 7. Representative gas chromatograms show time-dependent changes in the enantiomeric enrichment of benoxacor in metabolism studies with rat liver cytosol from (A) female and (B) male rats. Incubations with female rat liver cytosol showed an enrichment of the benoxacor enantiomer eluting first on the enantioselective column (E_1-benoxacor). In contrast, an enrichment of the benoxacor enantiomer eluting second on the enantioselective column (E_2-benoxacor) was observed in experiments with male rat liver cytosol. Cytosolic incubations contained 1 mg/mL protein, 5 mM glutathione (GSH) and 5 μM benoxacor incubation buffer. All samples were incubated for 30-min, and an aliquot was taken every 5-min for measurement. After extraction, a CB column was utilized to separate the enantiomers of benoxacor on an Agilent 7890 A gas chromatograph coupled to a ^{63}Ni-μECD detector as described under Materials and Methods.

estimation of the intrinsic clearance of benoxacor, there are clear limitations to these estimations. First, the simplicity of the incubation system cannot mimic the complexity of the liver. This system does not consider liver transporters or the interplay between the enzymes capable of metabolizing benoxacor. Second, the scaling factors are approximations of the amount of liver protein within a rat and may not fully reflect the protein/gram liver in the rats used to prepare the microsomes used. Despite these limitations, these estimations provide a valuable approximation of the clearance of benoxacor for future toxicity studies.

3.6. Comparative metabolism of benoxacor and other structurally related compounds

CYPs did not play a substantial role in an earlier study of the metabolism of N,N-diallyl-2,2-dichloroacetamide, another dichloroacetamide safener [19]. In contrast, our research indicates that hepatic CYPs readily metabolize benoxacor. CYPs also metabolize metolachlor, a herbicide structurally related to benoxacor [37]. The oxidation of metolachlor by CYPs, especially CYP3A and CYP2B isoforms, can lead to the formation of DNA-reactive metabolites like 2-methyl-6-ethylbenzoquinoneimine [38,39]. *In vitro* studies with rat and human hepatocytes suggest that, not surprisingly, humans are more sensitive to the toxicity of metolachlor than rats [40]. Analogously, oxidation of the 1,4-benzoxazine moiety of benoxacor by CYPs may produce reactive and toxic metabolites. Furthermore, the amidase activity of CESs could produce dichloroacetic acid, a known hepato- and immunotoxicant in mice [41]. Based on limited metabolism studies with dichloroacetamide safeners with cytosol and plants [11,19], cytosolic GSTs were expected to be a major metabolic and, ultimately, detoxication pathway for benoxacor. For example, benoxacor is dechlorinated by GSTs and forms a formylcarboxamide metabolite in plants [11,42]. Moreover, GSH protects cells in culture from the cytotoxicity of chloroacetanilide herbicides structurally related to benoxacor [43]. Overall, these findings indirectly indicate that conjugation with GSH by GSTs may be a detoxification route for benoxacor and its metabolites.

4. Conclusions

To our knowledge, this is the first paper reporting on the metabolism of benoxacor in mammalian subcellular fractions. Our results demonstrate that the enantioselective metabolism of benoxacor is complex. Specifically, benoxacor is enantioselectively metabolized by enzymes present in both the microsomal (e.g., CYPs and CESs) and cytosolic fractions (e.g., GSTs) from the rat liver. These results will be helpful for further in-depth studies aimed at characterizing the metabolites formed by different drug-metabolizing enzymes. Our results also revealed sex-dependent differences in the metabolism of benoxacor, which could be a significant factor with regards to their ecological risks and mammalian toxicity. Although further studies with other species are needed, our results also suggest that changes in the enantiomeric enrichment of benoxacor may be useful for environmental fate and transport studies. Moreover, the identification of the benoxacor metabolites formed by different enzymes is needed to aid in environmental and human biomonitoring studies assessing the environmental fate and transport, and exposures of wildlife, livestock, and humans to this overlooked agrochemical.

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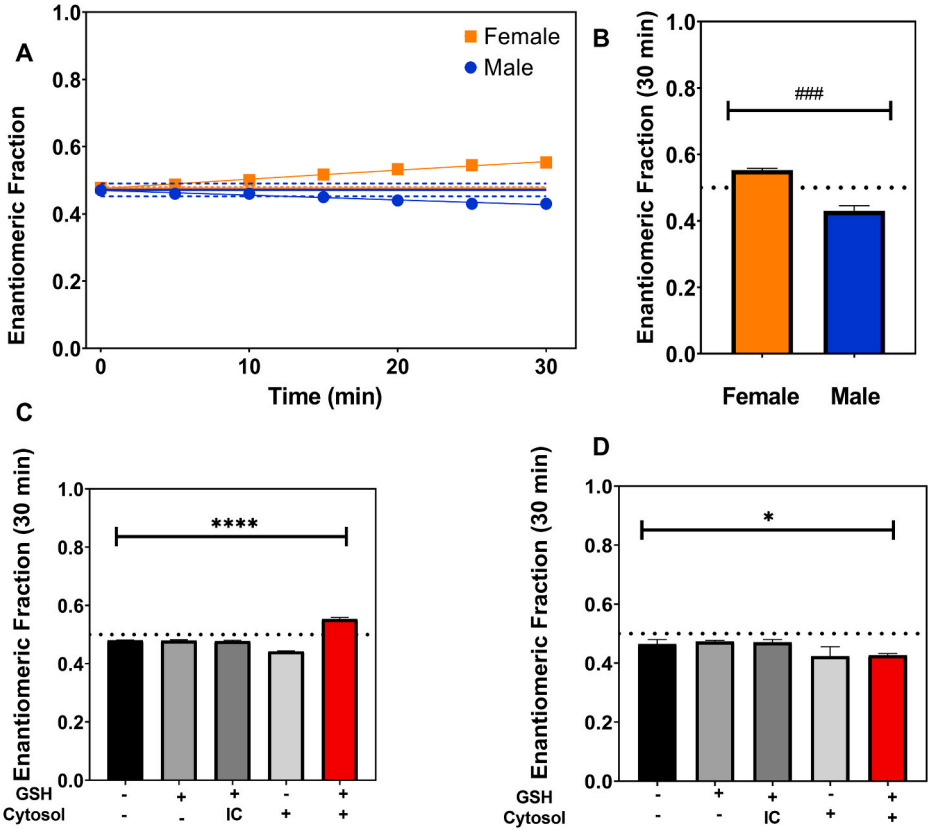

Fig. 8. Benoxacor is enantioselectively metabolized by rat liver cytosol. E_1-benoxacor was enriched in incubations with cytosol from female rats, and E_2-benoxacor was enriched in incubations with cytosol from male rats. (A) Change in the enantiomeric fraction of benoxacor in experiments with female and male rat liver cytosol over a 30-min time-course. Comparison of the enantiomeric fraction of benoxacor between (B) incubations with female vs. male rat liver cytosol at 30 min; (C) experiments with active female rat liver cytosol and controls at 30 min, and (D) incubations with active male rat liver cytosol and controls at 30 min, see text for more details. An unpaired *t*-test was used to determine significance in (B). One-way ANOVA was used to determine significance in (C&D). Tukey's multiple comparisons test confirmed a significant increase in the EF values in active preparations from female rats ($p < 0.0001$) and a significant decrease in active preparations from male rats ($p < 0.0221$) compared to heat-inactivated cytosol. EF values are reported in Table S4. Representative chromatograms are shown in Fig. S2. Data are the mean ± standard deviation for three independent replicates; for some data points, the standard deviation is smaller than the size of the symbols. The dotted lines represent EF = 0.5. GSH, glutathione; IC, heat-inactivated cytosol; ****$p < 0.0001$ (one-way ANOVA); ###$p < 0.001$ (unpaired *t*-test); *$p <$ 0.05 (One-way ANOVA).

Author contribution

Derek Simonsen: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/ Writing - original draft; Writing - review & editing. David M. Cwiertny: Conceptualization; Funding acquisition; Project administration; Supervision; Roles/ Writing - original draft; Writing - review & editing. Hans-Joachim Lehmler: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Supervision; Validation; Visualization; Roles/ Writing - original draft; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2020.109247.

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