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Letter

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Identification and Structural Characterization of Three New Metabolites of Bupropion in Humans

Jennifer E. Sager,[1] John R. Choiniere,[1] Justine Chang[2], Alyssa Stephenson-Famy[2], Wendel L. Nelson,[3] *Nina Isoherranen[1]

Department of Pharmaceutics[1], Obstetrics and Gynecology[2] and Medicinal Chemistry[3], University of Washington, Seattle, Washington, USA

KEYWORDS: Bupropion, structural characterization, metabolites, aromatic hydroxylation.

ABSTRACT: Bupropion is a widely used antidepressant and the recommended CYP2B6 probe drug. However, current understanding of bupropion elimination pathways is limited. Bupropion has three active circulating metabolites, OH-bupropion, threohydrobupropion and erythrohydrobupropion, but together with bupropion these metabolites and their conjugates in urine represent only 23% of the dose, and the majority of the elimination pathways of bupropion result in uncharacterized metabolites. The aim of this study was to determine the structures of the uncharacterized bupropion metabolites using human clinical samples and in vitro incubations. Three new metabolites, 4'-OH-bupropion, erythro-4'-OH-hydrobupropion and threo-4'-OH-hydrobupropion were detected in human liver microsome incubations and were isolated from human urine. The structures of the metabolites were confirmed via comparison of UV absorbance, NMR spectra and mass spectral data to those of the synthesized standards. In total, these metabolites represented 24% of the drug related material excreted in urine.

Bupropion is a norepinephrine and dopamine reuptake inhibitor approved as an antidepressant[1] and for smoking cessation[2] and weight loss therapy.[3] It is also the preferred sensitive in vivo CYP2B6 probe substrate recommended by the FDA.[4] Yet, despite its numerous clinical and research applications, the factors contributing to the observed inter- and intraindividual variability in bupropion disposition and in its safety and efficacy are poorly understood.[5–8]

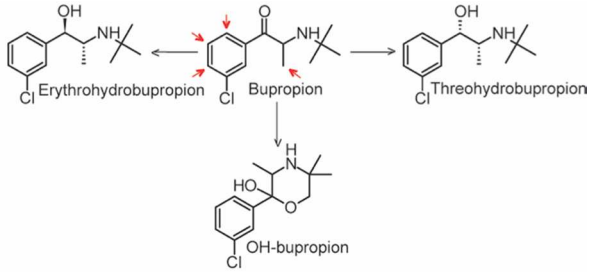

Figure 1. Bupropion and its active metabolites. Red arrows indicate potential additional hydroxylation sites

In vivo, bupropion undergoes extensive metabolism, with <1% of the dose excreted unchanged in urine after a single oral dose.[9–11] Much of the pharmacological activity and toxicity of bupropion is attributed to its three known primary metabolites, OH-bupropion, threohydrobupropion and erythrohydrobupropion (Figure 1),[12,13] which circulate at concentrations higher than bupropion.[1] OH-bupropion appears to be the main contributor to the efficacy of bupropion in smoking cessation, while both OH-bupropion and threohydrobupropion are suggested to be primarily responsible for the anti-depressant activity.[12,14,15] All three metabolites also have lower LD_{50} values than bupropion[11] and cause increased convulsion risk in mice.[16] Thus, it is possible that additional, yet unidentified metabolites of bupropion may also have pharmacological activity and contribute to the effects of bupropion in vivo. Such metabolites and novel metabolic pathways may also contribute to the interindividual variability in bupropion disposition, efficacy and toxicity. Thus better characterization of the elimination pathways of bupropion is necessary.

Current knowledge of bupropion metabolism is incomplete. Following a single oral radiolabeled dose, only 23 % of the dose was recovered in urine as bupropion, OH-bupropion, erythrohydrobupropion, threohydrobupropion and their conjugates (N- and O-glucuronides and sulfates[17,18]) while 22 % of the dose was excreted in urine as *m*-chlorohippuric acid and 36 % as uncharacterized polar metabolites.[11] Possible structures of some of the uncharacterized metabolites such as aromatic hydroylation products or products of hydroxylation of the methyl group (indicated by red arrows in Figure 1) have been proposed based on mass spectral data from in vitro incubations and urine samples from clinical subjects.[18] Furthermore, analysis of human urine samples revealed the presence of a reduced and hydroxylated metabolite.[18,19,20] However, the structures of these metabolites and their relative importance in circulation or elimination of bupropion are unknown. The goal of this study was to characterize the major unknown metabolites of bupropion and generate standards of these metabolites for quantitative characterization of bupropion elimination pathways. These metabolites can be evaluated for their potential pharmacological and toxicological activity.

In order to identify the primary bupropion metabolites formed in vitro, bupropion was incubated with human liver microsomes (HLM) and metabolite formation was measured using multiple reaction monitoring (MRM) (See Supporting Information for experimental procedures). Upon incubation of

bupropion with HLM, NADPH dependent formation of six metabolites was observed (Figure 2). In addition to OH-bupropion (Figure 2a), erythrohydrobupropion and threohydrobupropion (Figure 2b), three additional monohydroxylation products (M1-M3) were detected with a parent (M+H) mass of m/z 256 and a main MRM transition of m/z 256→ 182 (Figure 2c). On the basis of ion abundance, M1 and M2 appeared to have similar importance in bupropion metabolism, while M3 formation appeared minor. All three metabolites had similar fragmentation pathways (Figures 2c and d). The main fragmentation of loss of 56 Da indicates a loss of the *t*-butyl group as isobutylene corresponding to hydroxylation outside of the t-butyl group. However, the fragmentation patterns did not allow for the determination of the exact hydroxylation site.

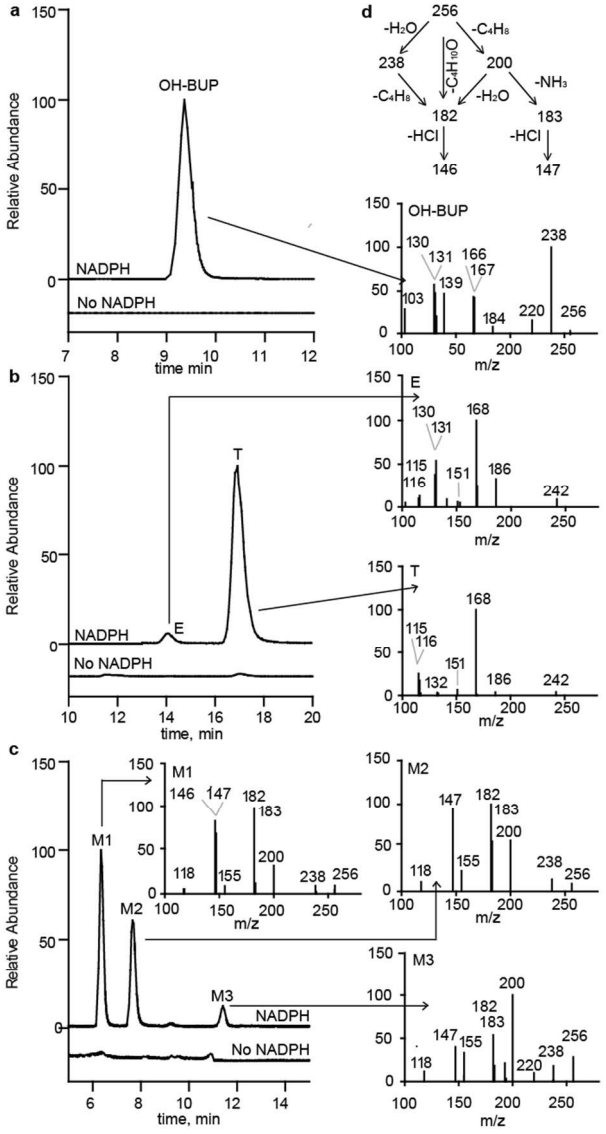

Figure 2. Characterization of metabolites formed from bupropion in HLM incubations. (Representative MRM chromatograms of m/z 256→238 (a), m/z 242→168 (b) and m/z 256→182 (c) depicting NADPH-dependent formation of OH-bupropion (a), erythro- (E) and threo- (T) hydrobupropion (b) and M1, M2 and M3 (c) and the MS/MS spectra of m/z 256 (a and c) and m/z 242 (b) for the detected metabolites. (d) Proposed fragmentation pathway of M1-M3.

To determine whether M1-M3 are present in vivo, urine and plasma were obtained from 5 subjects on bupropion therapy and analyzed for M1-M3 using MRM of m/z 256→ 182. Urine was analyzed before and after acid deconjugation since sulfate and glucuronide conjugates of bupropion metabolites have been reported.[18] Metabolite M1 was detectable in plasma and urine from all 5 subjects while M2 was not detected in any of the samples (Supplemental Figure S2). Metabolite M3 was detected in the urine of 3 subjects prior to acid deconjugation, and in the acid deconjugated urine of all 5 subjects, but at much lower abundance than M1. Acid deconjugation markedly increased M1 and M3 ion abundance (Figure 3a), suggesting an important role for conjugation in the elimination of these metabolites.

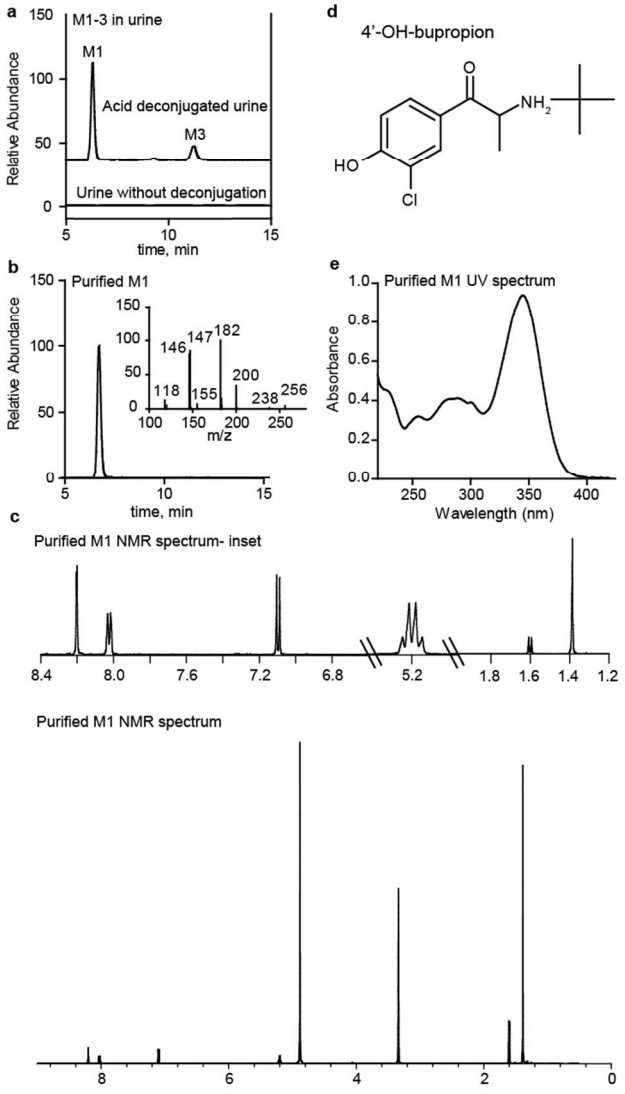

Figure 3. (a) A representative MRM chromatogram of m/z transition 256→182 from urine before or after acid deconjugation. (b) A representative MRM chromatogram of m/z transition 256→182 with an MS/MS spectrum of m/z 256 and (c) and NMR spectrum of M1 isolated from urine. (d) Structure of M1 (4'-OH-bupropion) and (e) UV spectrum for M1 isolated from acid deconjugated urine.

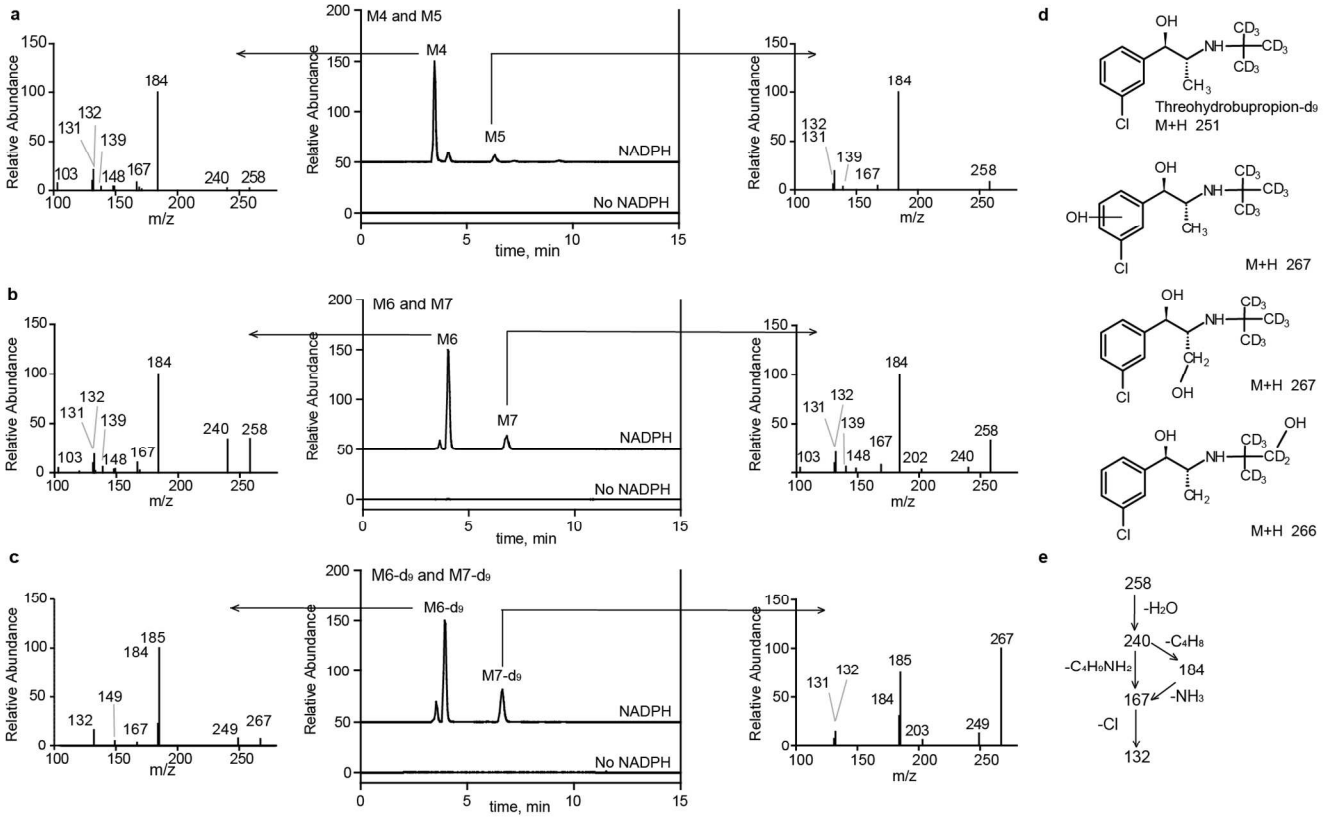

Figure 4. Characterization of metabolites formed in erythrohydrobupropion and threohydrobupropion incubations. Representative MRM chromatogram of m/z transition 258→184 depicting NADPH-dependent formation of M4 and M5 from erythrohydrobupropion (a) and M6 and M7 from threohydrobupropion (b) in HLM. The insets show the MS/MS spectra of m/z 258 for M4 and M5 (a) and M6 and M7 (b). (c) A representative MRM chromatogram of m/z transition 267→184 depicting NADPH-dependent formation of M6-d_9 and M7-d_9 following threohydrobupropion-d_9 incubation in HLM and MS/MS spectra of m/z 267 for M6-d_9 and M7-d_9. (d) Structures of threohydrobupropion-d_9 and its potential hydroxylation products. (e) Proposed fragmentation pathways of M4-M7.

Based on ion abundance, M1 appeared to be the predominant unknown hydroxylation product of bupropion in vitro and in vivo. To determine the structure of M1, it was extracted from acid deconjugated urine and purified using preparative chromatography. A sufficient quantity of M1 was isolated to allow structural characterization via NMR, UV-Vis and MS/MS spectroscopy (Figure 3b-e). The presence of only 3 protons in the aromatic region of the NMR spectrum suggested that M1 was a product of aromatic hydroxylation (Figure 3c). The hydroxylation site was assigned to the 4' position based on NMR chemical shifts and coupling constants (Figure 3c and supporting information). Reference standard of 4'-OH-bupropion was then synthesized and the structure of M1 confirmed by comparison of the LC retention time (6.42 min), and MS/MS, NMR and UV (λ_{max} 350 nm) spectra between the isolated metabolite and the synthesized compound (Figure 3 and Supplemental Figures S3 and S4).

Previous studies of bupropion metabolism have reported the presence of reduced and hydroxylated bupropion metabolites, yet the precursor(s) for the metabolite(s) have not been identified. To characterize the sequential metabolism, OH-bupropion, threohydrobupropion erythrohydrobupropion and 4'-OH-bupropion were incubated in HLM or human liver cytosol to determine which substrate(s) and subcellular fractions are involved in the formation of the reduced and hydroxylated metabolite(s). NADPH dependent formation of two hydroxylated metabolites from both erythrohydrobupropion (M4 and M5) and threohydrobupropion (M6 and M7) was detected with an M+1 ion of m/z 258 and MRM transition of 258→184, following incubation of erythro- and threohydrobupropion in HLM (Figure 4). No NADPH dependent formation of metabolites with m/z transitions of 258→184 was observed in incubations of threohydrobupropion, erythrohydrobupropion, OH-bupropion or 4'-OH-bupropion in cytosol or following OH-bupropion incubation in HLM (Supplemental figure S5). Based on ion abundance, metabolites M4 and M6 were the major hydroxylation products of erythro- and threohydrobupropion, respectively, while M5 and M7 appeared to be minor metabolites. Chromatograms and representative spectra for M4-M7 are shown in Figure 4. While the [M+H] m/z 258 ion of all four metabolites (M4-M8) suggested that these are hydroxylated metabolites of threo- and erythrohydrobupropion ([M+H] 242), the MS/MS fragmentation patterns (Figure 4a and b) did not allow determination of hydroxylation sites. Due to the hydroxylation, these metabolites lack the loss of 56 Da alone and loose H_2O or $C_4H_8 + H_2O$, as shown in the proposed fragmentation pathway in Figure 4e. To identify the site of hydroxylation in these compounds, threohydrobupropion-d_9, containing deuteriums in the *t*-butyl group, was incubated in HLM to determine whether M6 and M7 were hydroxylated in the *t*-butyl group, as was previously proposed.[18] Depending on the hydroxylation site, the products were expected to differ by one Da in the M+H ion, as shown in Figure 4d, allowing

for detection of *t*-butyl hydroxylation. Parent masses of m/z 267 for both M6 and M7 suggested no loss of *t*-butyl deuteriums (Figure 4c), confirming that the hydroxylation was either in the aromatic ring or in the methyl group (Figure 4d).

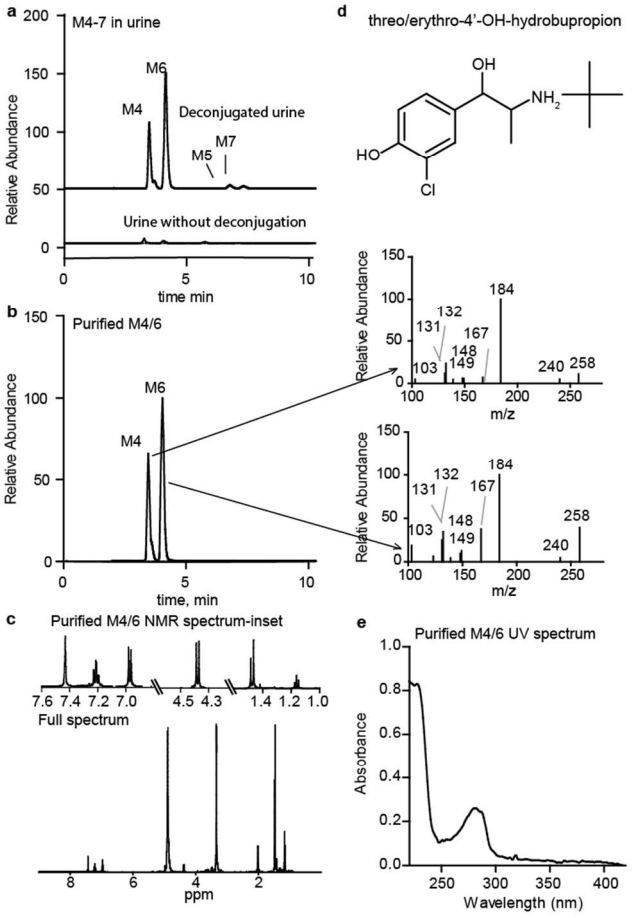

Figure 5. (a) A representative MRM chromatogram of m/z transition 258→184 from urine before or after acid deconjugation. (b) A representative MRM chromatogram of m/z transition 258→184 with the MS/MS spectra of M4 and M6 for m/z 258 and (c) NMR spectrum of M4+M6 isolated from urine. (d) Structure of threo/erythro-4'-OH-hydrobupropion (M4 and M6) and (e) UV spectrum for M4+M6 isolated from acid deconjugated urine.

Metabolites M4, M6 and M7 were all detectable in plasma and urine from 5 subjects (Figure 5 and Supplemental Figure S6). Acid deconjugation resulted in substantially greater ion abundance for M4, M6 and M7 than in untreated urine (Figure 5). Metabolite M5 was only detectable in urine following acid deconjugation (Figure 5a, Supplemental Figure S6). Based on the ion abundances from incubations and deconjugated urine, M4 and M6 were considered to be major contributors to the elimination of threo and erythrohydrobupropion. The metabolites M4 and M6 were isolated and purified from deconjugated urine as a mixture of the erythro and threo products (Figure 5b) to allow for characterization via NMR, UV-Vis, and MS/MS. The presence of three protons in the aromatic region of the NMR spectrum indicated that M4 and M6 were products of aromatic ring hydroxylation. The hydroxylation site was proposed to be at the 4'-position based on NMR chemical shifts and coupling contants. A reference standard was synthesized (Supplemental information) and the structures of M4 (erythro-4'-OH-hydrobupropion) and M6 (threo-4'-OH-hydrobupropion) were confirmed by comparison of LC retention times, NMR, UV and MS/MS spectra between the isolated and synthesized compounds (Figure 5, Supplemental Figures S7 and S8).

To establish the relative importance of the new metabolites in vivo, their concentrations were quantified in plasma and urine. The dose-normalized concentrations of 4'-OH-bupropion (M1), threo-4'-OH-hydrobupropion (M4) and erythro-4'-OH-hydrobupropion (M6) in plasma were low, 12 ± 6 nM for 4'-OH-bupropion, 7 ± 1 nM for threo-4'-OH-hydrobupropion (M4) and 33 ± 20 nM for erythro-4'-OH-hydrobupropion (M6). Concentrations of all three compounds were below the lower limit of quantification (3.3 nM) in one subject. On average, 4'-OH-bupropion (M1) concentrations were less than 0.01 % of those of bupropion. Circulating concentrations of threo-4'-OH-hydrobupropion (M4) and erythro-4'-OH-hydrobupropion (M6) were 1.8 and 2.2 % of the average concentration of bupropion at steady state. Metabolite safety is typically considered to be a concern when steady state concentrations are greater than 10% of the total drug related material.[21] Thus, it is likely unnecessary to screen these metabolites for toxicity. However, despite the low circulating concentrations of these compounds, they were abundant in urine. The overall recovery of bupropion metabolites is shown in Table 1. On average, 39 % of the total oral dose was recovered in urine over the dosing interval. The amount of threohydrobupropion and its metabolites represented 26 % of the dose, while only 5 % and 6 % of the dose was excreted as OH-bupropion and erythrohydrobupropion or their metabolites. On average, 4'-OH-bupropion, threo-4'-OH-hydrobupropion and erythro-4'-OH-hydrobupropion and their conjugates accounted for 0.4 %, 14 % and 11 % of the total oral bupropion dose excreted in urine, respectively (Table 1). Based on the amounts of threo- and erythrohydrobupropion excreted unchanged, as conjugates and as 4'-hydroxylation products, 4'hydroxylation accounts for about 70 % and 20 % of the elimination of erythro- and threohydrobupropion, respectively, and changes in the formation of the 4'OH- metabolites could have a substantial impact on the circulating levels of threo- and erythrohydrobupropion. To identify the enzymes responsible for the 4'-hydroxylation of bupropion, threohydrobupropion and erythrohydrobupropion each of the three compounds were incubated with a panel of recombinant CYP enzymes (see supplemental information for experimental details). Of the recombinant CYP enzymes only CYP2C19 formed 4'-OH-bupropion from bupropion and threo-4'-OH-hydrobupropion and erythro-4'-OH-hydrobupropion from threo and erythrohydrobupropion, respectively.

Table 1. The percent of the oral bupropion dose recovered in urine as bupropion and its metabolites over a steady state dosing interval

Compound	Total as free + conjugated (% of dose)	Free (µmole)*	Conjugated (µmole)*
Bupropion	1.4 ± 1.1	59 ± 71	6.5 ± 7.3
OH-bupropion	5.0± 3.2	77 ± 69	110 ± 63
Threo-hydrobupropion	21±14	610 ± 550	250 ± 140
Erythro-hydrobupropion	1.9±1	63 ± 65	22 ±12
4'-OH-bupropion	0.8±1.0	0.3 ± 0.2	29 ± 36
Threo-4'-OH-hydrobupropion	5.3±3.5	0.9 ± 0.9	200 ± 140
Erythro-4'-OH-hydrobupropion	4.1±2.7	3.3 ± 3.2	160 ± 100
m-chloro-hippuric acid	3.0 ±0.7	167 ±130	-
Total	39±15	980 ± 850	770 ± 220

*The molar amounts are normalized to the administered dose in each subject.

In conclusion, we report the identification and characterization of three new metabolites of bupropion, the 4'OH-bupropion, erythro-4'-OH-hydrobupropion and threo-4'-OH-hydrobupropion. Our findings are in agreement with previous tentative proposals of metabolite structures based on MS data but provide exact identification of the hydroxylation sites and methods for synthesizing these metabolites. The presence of hydroxylated metabolites of erythro- and threohydrobupropion, and conjugates of an aromatic hydroxylation product(s) were previously proposed based on MS data of human urine samples.[18] Similarly multiple monohydroxylation products of bupropion were proposed based on incubation data of bupropion in human liver S9 fractions and HLM.[19,20] This data shows that 4'hydroxylation is the predominant hydroxylation pathway for threohydrobupropion. This is in contrast to previous reports suggesting hydroxylation of the methyl group in addition to the aromatic hydroxylation products.[19,20] Quantitative analysis revealed low circulating concentrations for all three new metabolites, suggesting the risk for toxicity resulting from these metabolites is likely low. However, threo-4'-OH-hydrobupropion (M4) and erythro-4'-OH-hydrobupropion (M6) were important elimination pathways for the two active metabolites of bupropion, erythro- and threohydrobupropion. As these metabolites were exclusively formed by CYP2C19, genetic variability in CYP2C19 activity may have large impact on bupropion activity and toxicity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. The supporting information includes full descriptions of all experimental procedures, analytical assays, compound synthesis and characterization, and supporting results and NMR spectra.

Supplemental information (pdf)

AUTHOR INFORMATION

Corresponding Author

* Nina Isoherranen, E-mail: ni2@uw.edu, Phone: 206-543-3204.

Author Contributions

The manuscript was written through contributions of all authors. All authors participated in research design. J.E.S, J.R.C. and N.I. performed experiments, J.E.S, J.R.C., N.I. and W.L.N. performed data analysis and wrote or contributed to the writing of the manuscript.

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ABBREVIATIONS

HLM, Human liver microsomes. MRM, multiple reaction monitoring.

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