Metabolism of Bupropion by Carbonyl Reductases in Liver and Intestine

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Running Title

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Abbreviations:

ARK: aldo-keto reductase
CYP: Cytochrome P450
LC-MS/MS: Liquid Chromatograph-Mass Spectrometry/Mass Spectrometry
MRM: Multiple Reaction Monitoring
SDR: Short chain dehydrogenase/ reductase
Abstract

Bupropion’s metabolism to form hydroxybupropion in the liver by CYP450 2B6 (CYP2B6) has been extensively studied, however, the metabolism to form erythro/threohydrobupropion in the liver and intestine by carbonyl reductases (CR) has not been well characterized. The purpose of this investigation was to compare the relative contribution of the two metabolism pathways of bupropion (by CYP2B6 and CR) in the subcellular fractions of liver and intestine and to identify the CRs responsible for erythro/threohydrobupropion formation in the liver and the intestine. The results showed that the liver microsome generated the highest amount of hydroxybupropion ($V_{\text{max}}= 131$ pmol/min/mg, $K_m = 87 \mu M$). In addition, liver microsome and S9 fraction formed similar levels of threohydrobupropion by CR ($V_{\text{max}}= 98-99$ pmol/min/mg and $K_m = 186-265 \mu M$).

Interestingly, the liver has similar capability to form hydroxybupropion (by CYP2B6) and threohydrobupropion (by CR). In contrast, none of the intestinal fractions generate hydroxybupropion, suggesting that the intestine does not have CYP2B6 available for metabolism of bupropion. However, intestinal S9 fraction formed threohydrobupropion at an extent of 25% of the amount of threohydrobupropion formed by liver S9 fraction. Enzyme inhibition and western blots identified that 11β-dehydrogenase isozyme 1 in the liver microsome fraction is mainly responsible for the formation of threohydrobupropion, while AKR7 in the intestine may be responsible for the same metabolite formation. These quantitative comparisons of bupropion metabolism by CR in the liver and intestine may provide new insight on its efficacy and side effects by these metabolites.
Introduction

Bupropion is a norepinephrine/ dopamine reuptake inhibitor (Stahl et al., 2004), which is clinically used for treatment of major depressive disorder (MDD) and smoking cessation (Fava et al., 2005). Currently, over 11 million prescriptions annually of bupropion have been issued to over 40 million patients (Desmarais, Beauclair, & Margolese, 2011; Reese et al., 2008). Bupropion hydrochloride (HCl) extended release (ER) tablets, which is marketed as Wellbutrin XL by Biovail, has many generics manufacturers such as Teva/Impax, Mylan, Actavis, and Par Pharmaceuticals which used the current bioequivalence (BE) standard based on Cmax and AUC. The original approvals of the 300-mg generic bupropion HCl ER tablets were based on the demonstration of in vivo BE of the 150-mg generic bupropion HCl ER tablets to the brand name product and other in vitro criteria. However, a follow-up in vivo BE study on 300-mg Budeprion (bupropion HCl) ER tablets, manufactured by Impax Laboratories and distributed by Teva Pharmaceuticals, showed that the 300 mg strength failed to demonstrate BE (Woodcock, Khan, & Yu, 2012). It is not clear if the failure of extrapolating the BE conclusion from 150-mg to 300-mg tablets was related to changes of metabolism of bupropion in the liver and intestine between different formulations and different strengths of bupropion. Therefore, it is important to study in detail the bupropion metabolism mechanism in the liver and intestine.

Bupropion is rapidly (Tmax 1.3-1.9 hours) absorbed and extensively distributed throughout the body (Vd = 19 L/kg), and less than 1% of the parent compound is eliminated in urine (Jefferson, Pradko, & Muir, 2005; Schroeder, 1983). The majority of bupropion is eliminated by metabolism. It is well known that bupropion forms three primary metabolites: hydroxybupropion (by CYP2B6) and the diastereoisomers, threohydrobupropion and erythrohydrobupropion (by CR) (Loboz, Gross, Ray, & McLachlan, 2005) (Figure 1). Different metabolites of bupropion pose significant impact for its efficacy since these
metabolites have 25-50% potency as compared to bupropion based on antidepressant screening tests in an animal model (Bondarev, Bondareva, Young, & Glennon, 2003; Damaj et al., 2004). In addition, the plasma levels of hydroxybupropion are 5-10 fold higher than the parent drug bupropion after oral administration of bupropion HCl (Bondarev et al., 2003; Damaj et al., 2004; Damaj et al., 2010; Jefferson et al., 2005; Zhu et al., 2012).

The metabolism of bupropion by CYP2B6 in the liver to form hydroxybupropion, the major metabolite of bupropion, has been extensively studied. Studies have shown that the kinetic formation of hydroxybupropion in liver microsome can form at a high extent with a $V_{\text{max}}$ ranging from 85-254 pmol/mg/min and a $K_{\text{m}}$ ranging from 103-198 µM (Coles & Kharasch, 2008; Molnari & Myers, 2012; Skarydova et al., 2014). However, the metabolism of bupropion by CR has not been well characterized. For example, what metabolic pathways (CYP2B6 and CR) play a more important role in the liver and intestine for bupropion metabolism? Which subcellular fraction in the liver and intestine are responsible for metabolism of bupropion? Are there any differences in how bupropion is metabolized in the liver and in the intestines? Which CR is responsible for bupropion metabolism in the liver and the intestine?

To date, there are 11 known CR enzymes, which are categorized into two superfamilies: short-chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) (Matsunaga, Shintani, & Hara, 2006; Rosemond & Walsh, 2004). SDR family has 5 CR enzymes: CBR1, CBR3, 11 β-dehydrogenase isozyme 1 (11β-HSD), DHRS4, and L-Xylosulose reductase. AKR family has 6 CR enzymes: AKR7A2, AKR7A3, AKR1C1, AKR1C2, AKR1C3, and AKR1C4. The subcellular locations of most CR enzymes are in the cytoplasm, except for 11β-HSD which is localized in the microsomes (Matsunaga et al., 2006).

In this study we investigated the metabolism of bupropion in subcellular fractions (microsome, cytosolic, and S9 fractions) of the liver and intestine to compare the extent of
formation of all three metabolites in the different subcellular fractions of the liver and intestine. In addition, we conducted inhibition studies with these subcellular fractions to determine which CR enzymes are important for bupropion metabolism. These results confirm that CYP2B6 in microsome is mainly responsible for hydroxybupropion. In comparison, the liver microsome and S9 fraction formed similar levels of threohydrobupropion by CR, which was similar to hydroxybupropion’s formation. This suggests that the metabolism of bupropion by CYP2B6 and CR in the liver is equally important. In contrast, none of the intestinal fractions detected hydroxybupropion, which suggests that the intestines didn’t contribute to the CYP2B6 metabolism of bupropion. Intestinal S9 fraction indeed generated threohydrobupropion. In fact, the formation of threohydrobupropion in the intestinal S9 fraction is 25% the amount that formed from liver S9 fraction. Furthermore, the enzyme inhibition studies and Western blotting assay suggest that 11β-HSD is responsible for the formation of threohydrobupropion in the liver microsome, while ARK7 may be responsible for the same metabolite in the intestine. These results quantitatively compare bupropion metabolism by CR in the liver and intestine, which may provide new insight on its efficacy by these metabolites.
Materials and Methods

Chemicals/ Reagents:
Bupropion HCl and venlafaxine HCl (internal standard; IS) were purchased from Sigma (St. Louis, MO). Hydroxybupropion was purchased from Caymen Chemicals (Ann Arbor, MI) and a racemic mixture of both erythrohydrobupropion and threohydrobupropion were purchased from Toronto Research Chemicals (Toronto, Canada). β-Nicotinamide adenine dinucleotide 2’phosphate (NADPH) was also purchased from Sigma. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified with a Milli-Q water system (Bedford, MA). Mix Gender Pooled Human microsomes, cytosolic and S9 fractions for both liver and intestines (duodenum and jejunum) were purchased from Xenotech (Lenexa, KS). The following CR inhibitors were purchased from Sigma (St. Louis, MO); rutin, fluefenamic acid, and carbenozolone. The following antibodies were used in the western blot; AKR1C1/2 (Abcam (Cambridge, England) Cat # ab131375), carbonyl reductase 1/2/3 (Santa Cruz Cat # sc-292143), 11β-Hydroxysteroid Dehydrogenase (Type 1) (Cayman Chemical Cat # 10004303), AKR7A antibody (Santa Cruz Cat # sc-32944), CYP2B6 antibody (Santa Cruz Cat # sc-67224), Goat Anti-Mouse secondary antibody (Santa Cruz Cat # sc2005), and Anti-rabbit IgG antibody (Cell Signal Cat # 7074).

LC-MS/MS Method
The LC-MS/MS analysis was conducted using an Agilent 1200 HPLC system coupled to an API 3200 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. Quantitative analysis was accomplished on a Supelco C18 (150 x 4.6 mm I.D., 5 µm). The mobile phases used were purified water + 0.04% formic acid (A) and methanol + 0.04% formic acid (B). The LC was ran at isocratic at
35% methanol +0.04% and a flow rate of 0.8 mL/min. The LC-MS/MS was operated at positive ESI ionization. The MRM transitions and collision energies were determined for bupropion, hydroxybupropion, threohydrobupropion, erythrohydrobupropion and venlafaxine. The analytical data was processed by Analyst software (version 1.2; Applied Biosystems, Foster City, CA, USA). The quantitation of bupropion, hydroxybupropion, threohydrobupropion, and erythrohydrobupropion in these in vitro assay were performed by multiple reaction monitoring (MRM) of the [M-H]⁺ ion, using an internal standard (IS) to establish peak area ratios. The method development was derived and optimized from previous studies that monitored bupropion and metabolites by HPLC or LC-MS/MS (Borges, Yang, Dunn, & Henion, 2004; Cooper, Suckow, & Glassman, 1984; Loboz et al., 2005; Wang et al., 2012).

Subcellular Kinetic Assay:
Liver and intestinal microsome, cytosolic, and S9 fraction were conducted with concentrations of bupropion as the substrate from 1-4000 µM dissolved in PBS (3.3 mM MgCl₂ + 100 mM K₂HP0₄+ 100 mM KH₂HPO₄ buffer pH=7.4) (no organic solvent was used in this system). The master mix consisted of microsome, cytosolic, or S9 fraction at a final concentration of 1 mg/mL, 4 µL of corresponding substrate, and PBS (3.3 mM MgCl₂ + 100 mM K₂HP0₄+ 100 mM KH₂HPO₄ buffer pH=7.4). A fresh sample of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) was prepared at 16.7 mg/mL in PBS buffer. Both master mix and NADPH was heated for 3 minutes at 37ºC. Following, NADPH was added to master mix to initiate reaction. Sample was collected at 30 minutes; sample was spiked into ice cold methanol containing 500 nM of internal standard (venlafaxine).

Carbonyl Reductase Inhibition Study
For inhibition studies, bupropion substrate was used at the corresponding $K_m$ for threohydrobupropion (since this was the dominant metabolite formed by CR) determined from the subcellular kinetic analysis (liver microsome = 186 µM, liver S9 = 265 µM, liver cytosolic = 90 µM, intestinal microsome = 150 µM, intestinal cytosolic = 5.6 µM, and intestinal S9 = 573 µM). The master mix consisted of microsome, cytosolic, or S9 at a final concentration of 1 mg/mL, bupropion, PBS (3.3 mM MgCl$_2$ + 100 mM potassium phosphate buffer pH=7.4), and CR inhibitor at 3 fold higher than the IC$_{50}$ (the 3XIC$_{50}$ values were; rutin 6.1 µM, flufenamic acid 60 µM, and carbenoxolone 250 nM). A fresh sample of the cofactor NADPH was prepared at 16.7 mg/mL in PBS buffer. Both master mix and NADPH was heated for 3 minutes at 37ºC. Following, NADPH was added to master mix to initiate reaction. Sample was collected at 0, 30, 60, and 90 minutes; sample was spiked into ice cold methanol containing 500 nM of internal standard (venlafaxine).

**Standards and Sample Preparation**

Stock solutions of bupropion, hydroxybupropion, threohydrobupropion, or erythrohydrobupropion at 2 mg/mL were prepared in methanol to generate a working solution of 100 µg/mL. An aliquot of this solution was diluted in 1(MeOH):1(Milli-Q water) to get a series of working standard solutions of 5, 10, 25, 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL. Internal standard (IS) solution was prepared by diluting the stock solution of venlafaxine to yield a final concentration of 500 nM in 1(MeOH):1(Milli-Q water). After preparation of working standards, 50 µL of the appropriate concentrations of analyte was added to 150 µL of IS solution (500 nM of venlafaxine in in 1(MeOH):1(Milli-Q water)), and 50 µL of PBS. Fifty µL of sample from microsome, cytosolic, or S9 reaction at each time point was spiked into 150 µL of IS solution (500 nM of venlafaxine in in 1(MeOH):1(Milli-Q water)) and 50 µL 1(MeOH):1(Milli-Q water). Samples were vortex for 1 min, followed by
centrifugation for 15 min at 14,000 rpm in an Eppendorf centrifuge. The supernatant was transferred to vials and 5 µl was injected for LC-MS/MS analysis.

**Western Blot**

Subcellular fraction; liver and intestinal microsome, S9, and cytosolic fractions were lysed using radioimmunoprecipitation assay buffer ((RIPA: 50 mM Tris-HCL, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4± 0.2) (Boston BioProducts, BP-115)) with 1% protease inhibitor and 1% EDTA. Approximately 200 µL of RIPA buffer was used to resuspend each subcellular fraction which was incubated on ice for 30 minutes. Following, each sample was centrifuged at 14,000 rpm for 15 minutes at 4ºC. The protein concentration of the supernatant of each sample was quantified using Pierce BCA Protein Assay Kit (23225). All samples were diluted to have a protein concentration of 750 µg/mL. Laemmli sample buffer (BIO-RAD 161-0737) was used according to protocol; mix 950 µL of sample buffer with 50 µL of β-mercaptoethanol. Each sample was prepped by using 50 µL of protein sample with 25 µL of sample buffer and boiled samples at 95 ºC for 10 minutes. All samples were loaded on an SDS-PAGE gel (25 µL) and ran at 200 V for about 2 hours. The ladder see-blue (Life Technologies LC5625) was used to determine the proteins molecular weight. The running buffer for the SDS-Page gel consist of 3.0 g of tris base, 14.4 g of glycine, and 1 g of SDS with ddH2O to 1 L. A wet transfer was performed using transfer buffer (3.03 g of tris base, glycine 14.4 g, 200 µL of methanol, and ddH2O to 1 L). The transfer was done using a PVDF immune-blot membrane (BIO-RAD 162-0177) at 250 mV for 3 hours. The membrane was blocked for 1.5 hours using 5% of milk in Tris-Buffered Saline and Tween 20 (TBST buffer) (TBST buffer: 2.4 g of tris, 8 g of NaCl adjust pH to 7.6 with HCl, 0.1% tween 20 (v/v), and 1L of ddH2O). Primary antibody was added to membrane at various dilutions according to manufacturer’s protocol; AKR1C1/2 antibody (dilution 1:500), CRB1/2/3 (dilution 1:500), 11β hydroxysteroid dehydrogenase (dilution 1:200), AKR7
(dilution 1:200), and CYP2B6 (1:200) (dilutions were used as suggested by manufactures) and incubated at 4ºC overnight. Membrane was washed with TBST (3X) before the corresponding secondary antibody was added; AKR1C1/2 (dilution 1:2000), CRB1/2/3 (dilution 1:2000), 11β hydroxysteroid dehydrogenase (dilution 1:5000), AKR7 (dilution 1:5000), and CYP2B6 (dilution 1:5000) for 1.5 hours at room temperature. The membrane was washed again with TBST (3X). Stripping buffer (Thermo Scientific 21059) was used to remove previous antibody, we confirmed that the antibody was washed out each time. Proteins were detected using X-ray development; 5 mL of substrate (2.5 mL of reagent 1 and 2.5 mL of reagent 2) was added to the membrane before detection (thermo cat #’s 1859701 and 1859698).

Data Analysis

For microsome, cytosolic, and S9 kinetics, all data was converted into pmol/min/mg and plotted against concentration of bupropion. Graphpad Prism5 was used to simulate the $K_m$ and $V_{max}$ using the Michaelis-Menten model using the following equation.

$$\gamma = \frac{V_{max} \times [substrate]}{(K_m + [substrate])}$$

The intrinisic clearance for S9 formation of each metabolite was calculated using the following equation.

$$CL_{int} = \left(\frac{V_{max}}{K_m}\right)$$

For statistical analysis, R version 3.0.3 was ran with a t-test.
Results

LC-MS/MS development for bupropion and metabolites

An LC-MS/MS method was developed in order to quantify bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion. Since the fragmentations of the diastereoisomers (threohydrobupropion and erythrohydrobupropion) are the same and bupropion also had very similar fragmentation, it was necessary for all analytes to be separated by LC. Figure 2A shows the MRM chromatograms of the successful separation of all analytes. The MS parameters are highlighted in figure 2B for each analyte.

Calibration curves for each analyte were performed in order to quantify samples in later studies. A wide linear range was achieved for each analyte (figure 2C). In addition, the coefficient of determination for each analyte was greater than or equal to 0.99. The lower limit of detection was either 5 or 10 ng/mL (noted in figure 2C) depending on which analyte was being monitored.

Metabolism in Liver Subcellular fractions

To begin with, we used liver microsome, cytosolic, and S9 fractions to look at bupropion's metabolism. Bupropion was used as the substrate at concentrations ranging from 1-4000 µM. Samples were analyzed by LC-MS/MS to establish the kinetics, and bupropion, hydroxybupropion, threohydrobupropion, and erythrohydrobupropion were monitored. Figure 3A-C shows the formation of hydroxybupropion in liver microsome, S9 fraction, and cytosolic fraction respectively. Hydroxybupropion was formed at the highest extent in liver microsome ($K_m = 87.98 \pm 20.2$ µM and $V_{max} = 131.2 \pm 5.6$ pmol/min/mg), which was expected since microsomes typically contain concentrated amounts of CYP’s. In the S9 fraction, hydroxybupropion formation was still apparent but the formation occurred at a lower of extent.
(\(K_m = 99.53 \pm 18.91 \mu M\) and \(V_{max} = 51.45 \pm 1.9\) pmol/min/mg). Hydroxybupropion formation in the cytosolic fraction was almost negligible (\(K_m = 71.35 \pm 127 \mu M\) and \(V_{max} = 1.594 \pm 0.52\) pmol/min/mg). These results suggest that CYP enzymes which are subcellularly localized in microsomes are responsible for the formation of hydroxybupropion in the liver.

Threohydrobupropion was also formed in all subcellular liver fractions (Figure 4A-C). The extent of formation in both microsome and S9 fractions were about the same; however, the affinity differed slightly (microsome: \(K_m = 186.3 \pm 53.48 \mu M\) and \(V_{max} = 98.37 \pm 6.6\) pmol/min/mg; S9: \(K_m = 265.7 \pm 77.79 \mu M\) and \(V_{max} = 99 \pm 7.5\) pmol/min/mg). In the cytosolic fraction, threohydrobupropion was formed at a lesser extent (\(V_{max} = 14.56 \pm 0.714\) pmol/min/mg and \(K_m = 89.82 \pm 22 \mu M\)). These results suggested that the CR enzyme which is subcellularly localized in the microsomes, 11\(\beta\) hydroxysteroid dehydrogenase, plays a major role in the conversion of bupropion to threohydrobupropion. In addition, since the cytosolic fraction still forms threohydrobupropion to some extent, this suggested there may be multiple CR enzymes responsible for this metabolism.

Finally, we saw that erythrohydrobupropion was also formed in liver microsome, S9, and cytosolic fractions (figure 5A-C, respectively); however, the extent of formation was very small in all subcellular fractions (microsome: \(K_m = 41.45 \pm 26.62 \mu M\) and \(V_{max} = 2.649 \pm 0.3\) pmol/min/mg; cytosolic: \(K_m = 274.4 \pm 254 \mu M\) and \(V_{max} = 3.654 \pm 1.2\) pmol/min/mg; S9: \(K_m = 107 \pm 32.14 \mu M\) and \(V_{max} = 4.23 \pm 0.286\) pmol/min/mg). These results suggest that hydroxybupropion and threohydrobupropion is the dominant metabolite in the liver subcellular fraction. Yet, similar to threohydrobupropion, since formation of erythrohydrobupropion occurred in both the microsome and cytosolic fractions, this again suggested that multiple CR enzymes may be involved in erythrohydrobupropion’s formation. A summary of the liver kinetics are summarized in Table 1A.
Using the Michaelis-Menten kinetic parameters (Vmax and Km) we were able to calculate the intrinsic clearance for each metabolite using the S9 fraction (since this contains both microsome and cytosolic fractions) in the liver (Table 2). After adding each metabolite intrinsic clearance, we calculated the liver contributes a CLint of 931.8 µL/min/mg.

**Metabolism in Intestinal Subcellular fractions**

We continued to evaluate the metabolism of bupropion using intestinal microsome, cytosolic, and S9 fractions. Similar to the liver metabolism, we used bupropion at concentrations from 1-4000 µM and analyzed samples by LC-MS/MS to establish the kinetics of hydroxybupropion, erythrohydrobupropion, and threohydrobupropion. However, unlike the liver fractions where all metabolites were detected, the only metabolite that formed through the intestinal metabolism was threohydrobupropion. Both hydroxybupropion and erythrohydrobupropion were undetectable in both intestinal microsome, cytosolic, and S9 fraction. This suggested that CYP2B6 metabolism does not occur in the intestines since hydroxybupropion was unable to form.

The extent in which threohydrobupropion was formed was smaller compared to its formation in the liver (Figure 6 A-C) (microsome: K_m = 149.9 ± 28.8 µM and V_max = 5.55 ± 0.4 pmol/min/mg; cytosolic: K_m = 569 ± 64.89 µM and V_max = 5.649 ± 0.214 pmol/min/mg and S9: K_m = 573.4 ± 188.9 µM and V_max = 25.87 ± 2.8 pmol/min/mg). The formation of threohydrobupropion was 25% of the formation that occurred in the liver S9 fraction. Similar to the formation in the liver, these data suggest that multiple CR enzymes are involved in the formation of this metabolite. A summary of the intestinal S9 kinetics formation of each metabolite is summarized in Table 1B.

Similarly to the liver, using the Michaelis-Menten kinetic parameters (V_max and K_m) we were able to calculate the intrinsic clearance for threohydrobupropion using the S9 fraction in
the intestines (Table 2). This was about 20 fold lower than the liver CL\textsubscript{int} clearance since two of the metabolites did not form in the intestines and threohydrobupropion formations was 25% of the formation in the liver S9 fraction. Nevertheless, the CL\textsubscript{int} in the intestines S9 fraction was estimated to be 45 µl/min/mg.

Metabolite inhibition by Carbonyl Reductase Inhibitors

Next we went on to evaluate which CR enzymes are important for the reduction of bupropion and whether there might be multiple enzymes involved in this process. Using the microsome, cytosolic, and S9 fraction assay, we added various CR inhibitors and analyzed the reduction of metabolite formation. The inhibitors that were chosen were the following: rutin, which has been shown to target the CRB family of CR at an IC\textsubscript{50} of 2.1 µM; flufenamic acid, which has been reported to inhibit AKR family 67% at concentrations of 20 µM; and carbenoxolone, which targets the microsomal CR 11β hydroxysteroid dehydrogenase at an IC\textsubscript{50} values in the nM range (Carlquist, Frejd, & Gorwa-Grauslund, 2008; Rosemond, St John-Williams, Yamaguchi, Fujishita, & Walsh, 2004; Su et al., 2007). We monitored all metabolite formation with each inhibitor and compared these results to a control with no inhibitor.

For the formation of hydroxybupropion, none of the three inhibitors had a significant effect compared to control on any liver subcellular fraction (Figure 7A), as expected since formation of this metabolite occurs via CYP2B6. In addition, this metabolite was again not detected in any intestinal fraction. These results suggested no CRs are involved in formation of hydroxybupropion.

However, for threohydrobupropion formation, inhibition was observed in both the liver and intestinal subcellular fractions. In the liver, carbenoxolone, the inhibitor of 11β hydroxysteroid dehydrogenase, showed as much as 82.4% inhibition compared to control in the liver
microsome. Flufenamic acid was shown to have about a 40% inhibition on threohydrobupropion formation in liver cytosolic fraction (Figure 7B). These results suggested that in the liver, 11β hydroxysteroid dehydrogenase was the dominant enzyme in the liver for reduction of bupropion to threohydrobupropion.

However, in the intestinal subcellular fractions, carbenoxolone seemed to have no significant effect on inhibiting threohydrobupropion formation. Furthermore, flufenamic acid showed inhibition on intestinal fractions ranging from 57.8-78.7% of threohydrobupropion formation (Figure 7B). Minor inhibition was seen with rutin, implicating minor involvement of the CRB family of CR enzymes in the formation of threohydrobupropion. All together, the liver and intestinal data for the formation of threohydrobupropion suggest that both 11β hydroxysteroid dehydrogenase and the AKR family of CR enzymes are the major CR enzymes responsible for threohydrobupropion formation.

In the same way, erythrohydrobupropion formation was inhibited by both carbenoxolone and flufenamic acid (Figure 7C). Carbenoxolone inhibited the formation of erythrohydrobupropion by 95% in liver microsome and 91.6% in liver S9 fraction yet had no effect on liver cytosolic fraction. Flufenamic acid showed about 67-88% inhibition in the liver S9 and cytosolic fractions. These results suggested that 11β hydroxysteroid dehydrogenase and AKR family are the dominant enzymes that form erythrohydrobupropion in the liver.

**Western Blot**

Finally, we went on to confirm whether these enzymes of various CR enzymes are expressed in the different subcellular fraction. Analysis of protein expression was performed using an immunoblot after separation by SDS-Page gel (Figure 8). Both liver and intestinal microsome, cytosolic, and S9 fraction was examined for CYP2B6, 11β hydroxysteroid dehydrogenase, CRB1/2/3, AKR7 family, and AKR1A family.
It was observed that CYP2B6 was primarily only expressed in liver microsome with minor expression in the liver S9 fraction (lane 1 & 2). CYP2B6 was absent in liver cytosolic (lane 3) and all intestinal fractions (lane 4-6). This is consistent with the metabolite formation data suggested that hydroxybupropion predominantly formed in liver microsome and S9 and does not participate in intestinal metabolism of bupropion. 11β hydroxysteroid dehydrogenase was highly expressed in liver microsome and S9 fraction (lanes 1 & 2); its expression in the intestines was almost non-existing, supporting our results with the inhibition data that 11β hydroxysteroid dehydrogenase activity is dominant in the liver. The CRB1/2/3 enzymes were primarily found to be expressed in both liver and intestinal S9 and cytosolic fractions (lanes 2, 3, 5, 6); however, these enzymes may not be important in bupropion's metabolism, as suggested by the inhibition data. The AKR1A family had very little expression in any of the subcellular fractions except minor expression in liver S9 and liver cytosolic (lanes 2 & 3). Finally, the AKR7 family enzymes were found to be expressed in all subcellular fractions. This supported the CR inhibition data seen by flufenamic acid in both liver and intestines. All together, the enzyme expression data verified the results seen in the formation and inhibition studies as those enzymes were expressed in the corresponding subcellular fraction.
Discussion

In our studies, we show that the contribution of hydroxybupropion and threohydrobupropion formation in the liver (microsome and S9 fractions) occur at similar levels. In addition, we showed that no CYP2B6 expression or metabolism to form hydroxybupropion in the intestines occurs. However, the only metabolite that forms in the intestines is threohydrobupropion. Its formation in the intestinal S9 fraction is 25% of that seen in the liver S9 fraction. Furthermore, inhibition studies prove that there are multiple CR enzymes that are involved in the metabolism of bupropion to threohydrobupropion; and the CR activity may have a gastrointestinal (GI) regional dependency which influences the metabolism of the parent compound. Western blots confirmed that the CR enzymes important for metabolizing bupropion are expressed in the corresponding subcellular fractions.

Previous studies have shown that CYP450 2B6 metabolism of bupropion forms hydroxybupropion (Benowitz, Zhu, Tyndale, Dempsey, & Jacob, 2013; Coles & Kharasch, 2008; Ilic et al., 2013; Kharasch, Mitchell, & Coles, 2008; Kirchheiner et al., 2003). In addition, studies have also suggested that other CYPs such as CYP2C19, CYP2E1, and CYP3A4 might have a minor roles in the hydroxybupropion formation but still need to be confirmed (Chen et al., 2010). Therefore, based off of liver microsome stability assays, it was thought that hydroxybupropion was the major metabolite. Several studies failed to realize that the CR pathway to form threohydrobupropion and erythrohydrobupropion may not occur extensively in liver microsomes since most of these enzymes are subcellularly located within the cytosol (Coles & Kharasch, 2008; Meyer et al., 2013).

Therefore, examining all subcellular fractions; microsome, cytosolic, and S9 fraction will help to explain more broadly which enzymes are responsible for bupropion's metabolism.
and at what rate these metabolites are formed. Typically, CYP enzymes are localized in the microsomes. On the other hand, most other CR are subcellularly located in cytosolic fractions, except for 11β-HSD, one of the only CR enzymes that is subcellularly localized in microsomes. Using an S9 fraction, which contains both cytosolic and microsomes, allowed us to compare metabolite formations across the three metabolites. We found that in the S9 fractions threohydrobupropion has a 2-fold higher formation compared to hydroxybupropion suggesting that many CR enzymes have been under looked for bupropion's metabolism. Although hydroxybupropion formation in microsomes is slightly higher than S9 fraction, this difference in activity between microsome and S9 fraction is normal since microsomes are concentrated CYP, where S9 fraction contain both CYP and cytosolic fraction (Brandon, Raap, Meijerman, Beijnen, & Schellens, 2003; Jia & Liu, 2007).

In Molnari et al [14], the authors found that threohydrobupropion was the major metabolite in liver microsome, which disagrees with many previous studies that identified hydroxybupropion as the major metabolite and the results presented here. Although, in our studies, threohydrobupropion was the major metabolite formed in liver S9 fraction, hydroxybupropion still forms at the highest extent in liver microsomes. Moreover, the authors saw no change with flufenamic acid in inhibition studies in the liver where we did. However, this inhibitor seemed to have a larger effect on intestinal fraction, which was not pursued in the Molnari study. Likewise, in Meyers et al. 2013 (Meyer et al., 2013) the authors showed the 11β-HSD was the CR enzyme important for metabolizing bupropion to form threohydrobupropion. While our data agrees with this, a more thorough analysis of subcellular fractions could have been explored. In this study, the authors only examine liver microsomes and in which case only 11β-HSD activity would be observed. Therefore, cytosolic fractions were needed to be examined to see if there were multiple CR enzymes that contribute to bupropion metabolism.
Our results suggest that both hydroxybupropion and threohydrobupropion are important metabolites to consider for the metabolism of bupropion. This is consistent with in vivo studies which looked at the pharmacokinetic levels of bupropion and metabolites and that both hydroxybupropion and threohydrobupropion showed higher plasma concentration than the parent drug, bupropion (erythrohydrobupropion concentration was minor or undetectable) (Laizure, DeVane, Stewart, Dommiss, & Lai, 1985).

To the best of our knowledge, no authors have studied the formation of bupropion's metabolites in any intestinal fractions. The intestines have been shown to be involved in both phase I and phase II metabolism, which might influence the metabolism of bupropion. Although CYP enzyme expression is typically less in the intestines compared to the liver (20 pmol/mg of microsome compared to 300 pmol/mg of microsome) (Peters & Kremers, 1989), metabolism in this region of the GI tract should still be investigated. Likewise, the expression of CR enzymes has been found to be highly concentrated in both liver and small intestines (Gervot et al., 1999; Peters & Kremers, 1989). We did not observe any hydroxybupropion (CYP2B6 metabolism) in the intestines, this finding is consistent with another study that looked for various CYP expression in intestinal microsomes and similarly saw no CYP2B6 present (Paine et al., 2006). However, in this study threohydrobupropion metabolized by CR was able to form in all three subcellular intestinal fractions (microsome, S9, and cytosolic) again showing how studies have discounted the CR pathway for metabolism of bupropion. The intestinal subcellular fractions used in these studies were taken from the duodenum and jejunum. The intestinal metabolism is an important concept to understand since these metabolites are active. One hypothesis for the failure of extrapolating BE from lower strength to higher strength, as discussed in the introduction, could be that we have discounted the CR metabolism particularly in the intestines and these enzymes were saturating. However, a
more thorough analysis would be needed to disapprove/approve the hypothesis and this is also true *in vivo*.

In conclusion, these results suggest that depending on the subcellular localization of the bupropion and tissue type, the metabolism can be different forming different metabolites by multiple enzymes (both CYP and CR).
Acknowledgements

I would like to thank Ruijuan Luo and Ting Zhao for their advice on the method development.
**Authorship Contributions**

Participated in research design: Jamie Connarn, Xinyuan Zhang, Andrew Babiskin, and Duxin Sun.

Conducted experiments: Jamie Connarn.

Performed data analysis: Jamie Connarn.

Wrote or contributed to the writing of the manuscript: Jamie Connarn.
References


Footnotes

Disclaimer: The views expressed in this article are those of the authors and not necessarily those of the Food and Drug Administration (FDA).
Figure Legends

Figure 1. **Bupropion and Metabolism.** Bupropion is metabolized by Cytochrome P450 2B6 to form hydroxybupropion and by Carbonyl Reductase to form the diasterisomers, threohydrobupropion and erythrohydrobupropion.

Figure 2. **Method Development for Bupropion and Metabolites.** (A) Separation of Bupropion and metabolite for detections using LC-MS/MS. (B) LC/MS parameters for bupropion and metabolites. (C) Validation with Standards for bupropion and metabolites. All analytes had a good linear range with acceptable coefficient of determination.

Figure 3. **Hydroxybupropion Metabolite Formation in Liver Subcellular Fractions.** Hydroxybupropion formation is indicated in (A) Liver Microsome (B) Liver S9 (C) Liver Cytosolic. Data are presented as mean ± S.D. (n = 3).

Figure 4. **Threohydrobupropion Metabolite Formation in Liver Subcellular Fractions.** Threohydrobupropion formation is indicated in (A) Liver Microsome (B) Liver S9 (C) Liver Cytosolic. Data are presented as mean ± S.D. (n = 3).

Figure 5. **Erythohydrobupropion Metabolite Formation in Liver Subcellular Fractions.** Erythohydrobupropion formation is indicated in (A) Liver Microsome (B) Liver S9 (C) Liver Cytosolic. Data are presented as mean ± S.D. (n = 3).

Figure 6. **Threohydrobupropion Metabolite Formation in Intestinal Subcellular Fractions.** Threohydrobupropion formation is indicated in (A) Intestinal Microsome (B) Intestinal S9 (C) Intestinal Cytosolic. Data are presented as mean ± S.D. (n = 3).

Figure 7. **Metabolite Inhibition by Carbonyl Reductase Inhibitors.** (A) Hydroxybupropion’s direct measured concentration. (B) Threohydrobupropion’s direct
measured concentration. (C) Erythrohydrobupropion’s direct measured concentration. FA=
flufenamic acid. Data are presented as mean ± S.D. (n = 3). *p<0.05, **p<0.01.

Figure 8. **Enzyme Expression in Human Subcellular Fractions.** Subcellular fractions were
ran on an SDS-polyacrylamide gradient (4-12% W/V) gel to detect various carbonyl
Lane 3: Liver Cytosolic (LC). Lane 4: Intestinal Microsome (IM). Lane 5: Intestinal S9 (IS9).
Lane 3: Intestinal Cytosolic (IC).
<table>
<thead>
<tr>
<th></th>
<th>Liver Microsome</th>
<th>Liver S9</th>
<th>Liver Cytosolic</th>
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<tr>
<td><strong>Vmax</strong></td>
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<td>(pmol/min/mg)</td>
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<td><strong>Km</strong></td>
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<tr>
<td>(µM)</td>
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<tr>
<td>HBUP</td>
<td>131.2 ± 5.8</td>
<td>51.4 ± 1.9</td>
<td>1.5 ± 0.5</td>
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<td></td>
<td>87.9 ± 20.2</td>
<td>99.5 ± 18.9</td>
<td>71.3 ± 127.1</td>
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<tr>
<td>TBUP</td>
<td>98.4 ± 6.6</td>
<td>99 ± 7.5</td>
<td>14.5 ± 0.7</td>
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<tr>
<td></td>
<td>186.3 ± 53.5</td>
<td>265.7 ± 77.7</td>
<td>89.8 ± 22.0</td>
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<tr>
<td>EBUP</td>
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<td>4.2 ± 0.28</td>
<td>3.65 ± 1.2</td>
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<td></td>
<td>41.4 ± 26.6</td>
<td>107 ± 32.1</td>
<td>274 ± 254.0</td>
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<thead>
<tr>
<th></th>
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<th>Intestinal Cytosolic</th>
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<tr>
<td><strong>Vmax</strong></td>
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<td><strong>Km</strong></td>
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<td>(µM)</td>
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<tr>
<td>HBUP</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>TBUP</td>
<td>5.55 ± 0.3</td>
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<td>149.9 ± 28.8</td>
<td>573.4 ± 188</td>
<td>569 ± 64.8</td>
</tr>
<tr>
<td>EBUP</td>
<td>NF</td>
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Table 1. **Summary of Subcellular Kinetics.** (A) The Vmax and Km are highlighted for each metabolite in liver microsome, S9, and cytosolic fraction. (B) The Vmax and Km are highlighted for threohydrobupropion metabolite in intestinal microsome, S9, and cytosolic fraction. If no metabolite formation occurred in the corresponding fraction, this was denoted by NF. HBUP: hydroxybupropion, TBUP: threohydrobupropion, EBUP: erythrohydrobupropion.
<table>
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<th>Subcellular Fraction</th>
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<tr>
<td><strong>Liver S9 Fraction</strong></td>
<td>Hydroxybupropion</td>
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<tr>
<td></td>
<td>Threohydrobupropion</td>
<td>372 µl/min/mg</td>
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<tr>
<td></td>
<td>Erythrohydrobupropion</td>
<td>39 µl/min/mg</td>
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<tr>
<td><strong>Total Cl&lt;sub&gt;int&lt;/sub&gt; from liver</strong></td>
<td></td>
<td>931.8 µl/min/mg</td>
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<tr>
<td></td>
<td><strong>Intestinal S9 Fraction</strong></td>
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</tr>
<tr>
<td>Hydroxybupropion</td>
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<tr>
<td>Threohydrobupropion</td>
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<tr>
<td>Erythrohydrobupropion</td>
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<tr>
<td><strong>Total Cl&lt;sub&gt;int&lt;/sub&gt; from Intestines</strong></td>
<td></td>
<td>45 µl/min/mg</td>
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<tr>
<td>Total contribution from liver and Intestines S9 Fraction</td>
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<td>976 µl/min/mg</td>
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Table 2. **Estimated Intrinsic Clearance.** Using equation (2) the intrinsic clearance for both liver and intestines S9 fraction was calculated based off the Michaelis Menten equation assuming the linear portion of the curve. The relative contribution for each metabolite in S9 liver or intestines is indicated in the table.
Figure 1.
Peaks | Compound | Retention time (minutes) | Q1 Mass | Q3 Mass | CE | DP | EP | CXP
---|---|---|---|---|---|---|---|---
(B) | Bupropion | 8.8 | 240.1 | 184 | 10 | 50 | 10 | 3
(A) | Hydroxybupropion | 7.3 | 256 | 238 | 12 | 50 | 10 | 3
(D)/(C) | Threo/Erythrohydrobupropion | 9.96/11.2 | 242 | 168.1 | 5 | 50 | 10 | 3
(E) | Venlafaxine | 12.5 | 278 | 260 | 10 | 50 | 10 | 3

Analyte | Linear Range | Linear equation | R^2 | LLOQ
---|---|---|---|---
Bupropion | 5-5000 ng/mL | Y=0.0136X+0.0194 | 0.9975 | 5 ng/mL
Hydroxybupropion | 10-5000 ng/mL | Y=0.0085X+0.0975 | 0.9900 | 10 ng/mL
Threohydrobupropion | 5-5000 ng/mL | Y=0.0546X+0.0012 | 0.9948 | 5 ng/mL
Erythrohydrobupropion | 10-5000 ng/mL | Y=0.0286+0.0652 | 0.9966 | 10 ng/mL
Figure 3.

(A) Liver Microsome Hydroxybupropion

(B) Liver S9 Hydroxybupropion

(C) Liver Cytosolic Hydroxybupropion
Figure 4.
Figure 5.
Figure 6.
Figure 7.

(A) Hydroxybupropion

(B) Threoxybupropion

(C) Erythroxybupropion

Legend:
- Control
- Rutin
- FA
- Carbenoxolone

Subcellular Fractions:
- Liver Microsome
- Liver S9
- Liver Cytosolic
- Intestinal Microsome
- Intestinal S9
- Intestinal Cytosolic

Measured Concentration (ng/mL)
Figure 8.