STEREOSELECTIVE DISPOSITION OF BUPROPION AND ITS THREE MAJOR METABOLITES: 4-HYDROXYBUPROPION, ERYTHRO-DIHYDROBUPROPION, AND THREO-DIHYDROBUPROPION

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Dedication:

In honor of Helen E. Grove

I did it.

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STEREOSELECTIVE PHARMACOKINETIC DISPOSTION OF BUPROPION AND ITS THREE MAJOR METABOLITES: 4-HYDROXYBUPROPION, ERYTHRO-DIHYDROBUPROPION, AND THREO-DIHYDROBUPROPION

Bupropion is a dual dopamine-norepinephrine uptake inhibitor and a nicotine receptor antagonist. Clinically, bupropion is given as a racemate for the management of depression, smoking cessation aid, and for the management of weight. Bupropion has also been targeted as a phenotypic probe of CYP2B6 activity. Bupropion metabolites are formed via oxidation (4-hydroxybupropion) through CYP2B6, and reduction (erythro- and threo-dihydrobupropion) through carbonyl reductases. These metabolites exhibit pharmacological activity, but little is known regarding their stereoselective disposition due to the lack of a chiral assay. A novel reversed phase chiral-HPLC-MS/MS method involving a simple liquid-liquid extraction procedure and a small plasma sample volume (50µL) was developed that allowed simultaneous separation and quantification of enantiomers of bupropion, 4-hydroxybupropion, and those of threo- and erythro-dihydrobupropion in human plasma. This method was successfully implemented to determine the unique stereoselective disposition of bupropion and its metabolites in 15 human volunteers administered a single 100 mg oral dose of racemic bupropion. Significant differences ($p<0.05$) in the stereoselective metabolism were observed for all of the enantiomers. The highest plasma exposure ($AUC_{0-\infty}$) was (2R, 3R)-4-hydoxybupropion, almost 65 fold higher, than (2S, 3S)-4-hydoxybupropion, and over 32 fold greater than the parent R-bupropion. The second highest plasma exposure was threo-dihydrobupropion A, which was almost 5 fold higher than threo-dihydrobupropion B. (Nomenclature of the enantiomers for erythro- and threo-dihydrobupropion was based on the chromatography of the first eluting peak as “A” and the second eluting peak as “B”.) Threo-dihydrobupropion A and B showed the most significant difference

between the racemic and enantiomer profiles. Although the AUC was greater for threo-dihydrobupropion B, threo-dihydrobupropion A had a significantly ($p<0.05$) higher C_{max}. The half-life for threo-dihydrobupropion A and erythro-dihydrobupropion A were the longest for all analytes, which could indicate accumulation in multiple dosing. The importance of this study was, for the first time, to be able to characterize the stereoselective metabolism of bupropion and its three major metabolites. This new method and subsequent pharmacokinetic data should enhance further research into bupropion stereoselective metabolism, drug interactions, and effect.

David R. Jones, Ph.D., Chair

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List of Abbreviations

APAP ..... acetaminophen
$AUC_{0-\infty}$ ..... area under the plasma concentration time curve from zero to infinity
BUP ..... bupropion
CL ..... apparent oral clearance
C_{last} ..... last measured concentration
C_{max} ..... maximal plasma concentration
CV% ..... percent coefficient of variation
CYP2B6 ..... cytochrome P450 2B6
EDTA ..... ethylenediaminetetraacetic acid
Ery A ..... erythro-dihydrobupropion A
Ery A glucuronide ..... erythro-dihydrobupropion β-D glucuronide A
Ery B ..... erythro-dihydrobupropion B
Ery B glucuronide ..... erythro-dihydrobupropion β-D glucuronide B
HLM ..... human liver microsomes
HPLC ..... High performance liquid chromatography
ICRC ..... Indiana Clinical Research Center
IRB ..... Institutional Review Board
k_{el} ..... elimination rate constant
λ_z ..... terminal elimination rate constant
LLOQ ..... Lower limit of quantification
MS/MS ..... Triple quadrupole mass spectrometer
NADPH ..... β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetra sodium salt hydrate
OH-BUP 4-hydroxybupropion
QC ..... quality control
R, R-OH BUP ..... (2R, 3R)-4-hydroxybupropion
R, R-threo glucuronide ..... R, R- dihydrobupropion β-D-glucuronide
S, S-OH BUP ..... (2S, 3S)-4-hydroxybupropion
S, S-threo glucuronide ..... S, S-dihydrobupropion β-D-glucuronide
$t_{1/2}$ ..... half-life
Threo A ..... threo-dihydrobupropion A
Threo B ..... threo-dihydrobupropion B
T_{max} ..... time of maximal plasma concentration
UGTs ..... uridine 5'-diphosopho-glucuronosyltransferase
V_d ..... volume of distribution calculated by clearance

CHAPTER 1-INTRODUCTION

Bupropion ([(±)-1-(3-chlorophenyl)-2-[(1,1-dimethylethyl) amino]-1-propanone], a dual dopamine-norepinephrine uptake inhibitor and a nicotine receptor antagonist, is widely used in the management of depression and as a smoking cessation aid under the tradename Wellbutrin®, since the early 1970s. In 2014, under the trade name Contrave®, a combination of bupropion and naltrexone was approved by the FDA for the management of weight. It is also being assessed, in clinical trials, as a candidate for the treatment of drug abuse and attention-deficit hyperactivity disorder [1-4]. Clinically, bupropion is administered orally as a racemate. The metabolism of bupropion is of major importance since human studies conducted at the end of 1970 and beginning of 1980's have reported that bupropion undergoes extensive hepatic metabolism, with <1% of the administered dose excreted as unchanged in urine over 48 hours after bupropion dosing. Despite its proven efficacy, the use of bupropion is associated with large interpatient variability in clinical response [5-8], while some patients are at increased risk for adverse effects such as dose-dependent seizure [9] and often fatal outcomes [10] among drug users abusing bupropion.

Previous studies have identified three primary metabolites: 4-hydroxybupropion (a morpholinol) formed via hydroxylation of the t-butyl moiety; and two amino alcohols (erythro- and threo-dihydrobupropion) formed via reduction of the aminoketone group of bupropion [11-17]. These metabolites exhibit pharmacological and toxicological activity in different preclinical depression, behavioral and biochemical models as well as in humans [17-25]. A recent study found that approximately 10% of the administered dose of bupropion and metabolites were found in the urine after 48 hours. Of the 10% recovered, glucuronide metabolites of 4-hydroxybupropion, threo-dihydrobupropion, and erythro-dihydrobupropion accounted for over 62% of total excretion [26]. The plasma exposure of 4-hydroxybupropion and threo-dihydrobupropion at steady-state is

much higher (17- and 7-fold respectively) than the parent drug bupropion [14-15, 27, 28]. Thus, it has been suggested that bupropion metabolites contribute significantly to the beneficial and/or adverse effects of bupropion. Although the primary racemic metabolites have been identified for over three decades, only R-bupropion (R-BUP), S-bupropion (S-BUP), (2R, 3R)-4-hydroxybupropion (R, R-OH BUP), and (2S, 3S)-4-hydroxybupropion (S, S-OH BUP) have been stereoselectively profiled, due to lack of a chiral assay for erythro- and threo-dihydrobupropion. Therefore, the mechanisms underlying large interpatient variability in the exposure of these active metabolites are not fully understood.

Bupropion's primary metabolite, 4-hydroxybupropion (OH-BUP), is generated through cytochrome P450 2B6 (CYP2B6). CYP2B6 is mainly expressed in the liver. Since the end of the 1990s, approximately 70 structurally diverse drugs and other chemicals have been identified as substrates of this enzyme. Recent studies indicate that CYP2B6 plays an important role in human drug metabolism [25]. An imperfect understanding of the mechanisms responsible for the extensive inter-individual variability in CYP2B6-mediated drug metabolism and associated drug interactions exist, which hampers the goal of effective and safe therapy. In order to gain more of an understanding of CYP2B6 metabolism, bupropion has been targeted as a phenotypic probe for CYP2B6 activity [28]. However, bupropion metabolism is complex, and chiral, making bupropion not a good choice as a marker. When chiral bupropion is hydroxylated by CYP2B6 an additional chiral center is created. The result is a possibility of generating four diastereomers (RR-, SS-, RS- and SR-4-hydroxybupropion), but only (2R, 3R)-4-hydroxybupropion and (2S, 3S)-4-hydroxybupropion have been quantified in human plasma [29-31] (Figure 1). The reported plasma exposure of R, R-OHBUP is over 20-fold higher than S, S-OHBUP [28], but there is evidence to support that most of the pharmacological activities of bupropion reside in S, S-OHBUP [18,22,24].

Despite the identification of threo- and erythro-dihydrobupropion for more than three decades, the disposition, pharmacological, and toxicological effect of these metabolites is less investigated when compared with 4-hydroxybupropion, due to the lack of a chiral assay. Reduction of the aminoketone group of bupropion by carbonyl reductases [32, 33] creates an additional chiral center adjacent to that of the bupropion chiral center (Figure 1).

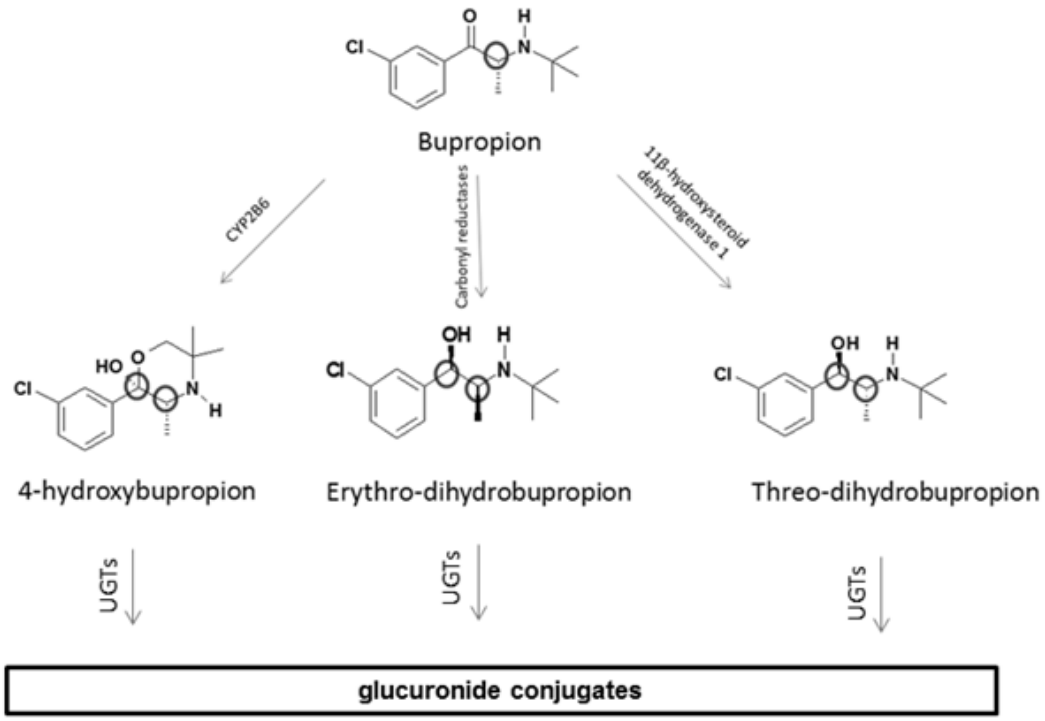

Figure 1. Chemical structures (with chiral centers circled), Phase I, and Phase II metabolic pathways for bupropion, 4-hydroxybupropion (cytocromeP450 2B6), erythro-dihydrobupropion (carbonyl reductases), and threo-dihydrobupropion (11β-hydroxysteroid dehydrogenase 1). Phase II glucuronidation occurs through uridine 5'-diphosopho-glucuronosyltransferase (UGTs).

The formation of threo-dihydrobupropion appears to be catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase 1 [32], while other carbonyl reductases appear to catalyze the formation of erythro-dihydrobupropion [33]. The steady-state plasma exposure of racemic threo-dihydrobupropion is much higher than racemic erythro-

dihydrobupropion [27], probably due to enantioselective reduction by microsomal 11β-hydroxysteroid dehydrogenase type 1 [32]. Previous in vivo studies in mice have shown that the activity of 4-hydroxybupropion is approximately 50% of the parent drug, while threo-dihydrobupropion and erythro-dihydrobupropion both exhibit about 20% activity of bupropion [31]. There have been documented cases, in humans, which show the amount of threo-dihydrobupropion plasma exposure to be higher than that of 4-hydroxybupropion; in cerebrospinal fluid, after over-dose, or abuse of bupropion [34]. The activity and high plasma exposure indicate threo- and erythro- dihydrobupropion may have important stereoselective pharmacological activities and disposition, but have only been studied as racemic mixtures. It is possible, as with 4-hydroxybupropion, these metabolites show marked stereoselective disposition and effect, but detailed studies on their stereoselective metabolism and disposition are particularly hampered by the lack of a chiral assay. These metabolites are of interest because: 1) they exhibit pharmacological activity [21] and threo-dihydrobupropion' s plasma exposure is 7-fold higher than bupropion [27]; and 2) in vitro and in vivo evidence suggests that reduction of bupropion to threo-dihydrobupropion is a major clearance mechanism of bupropion [27].

The aims of the present study were to: a) develop a novel chiral LC-MS/MS assay that allows simultaneous separation and quantification of enantiomers of bupropion, 4-hydroxybupropion, threo-dihydrobupropion, and erythro-dihydrobupropion, b.) obtain and evaluate enantioselective pharmacokinetics of bupropion and its three major metabolites in 15 healthy volunteers administered a single 100 mg oral dose of racemic bupropion, c.) show data that demonstrate bupropion is a poor marker for CYP2B6 activity due to complex, chiral metabolism.

CHAPTER 2-MATERIALS AND METHODS

R-Bupropion (R-BUP), S-bupropion (S-BUP), (2R,3R)-4-hydroxybupropion (R, R-OH BUP), (2S,3S)-4-hydroxybupropion (S, S-OH BUP), racemic erythro-dihydrobupropion (Ery A and Ery B), racemic threo-dihydrobupropion (Threo A and Threo B), racemic erythro-dihydrobupropion β-D glucuronide (Ery A glucuronide, Ery B glucuronide), R, R- dihydrobupropion β-D-glucuronide (R, R-threo glucuronide), and S, S-dihydrobupropion β-D-glucuronide (S, S-threo glucuronide) were purchased from Toronto Research Chemicals (Toronto, Ontario). Optically pure standards for rac-erythro-dihydrobupropion and rac-threo-dihydrobupropion are not commercially available. The lack of synthetic standards precludes accurate identification of enantiomers of erythro- and threo-dihydrobupropion. Therefore, nomenclature of the enantiomers for erythro- and threo-dihydrobupropion was based on the chromatography of the first eluting peak as "A" and the second eluting peak as "B". Thus enantiomers of erythro-dihydrobupropion (Ery) were denoted as Ery A and Ery B and enantiomers of threo-dihydrobupropion (Threo) are denoted as Threo A and Threo B. The internal standard acetaminophen (APAP), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetra sodium salt hydrate (NADPH), magnesium chloride, and ammonium bicarbonate were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). HPLC grade ammonium hydroxide, methanol, acetonitrile, sodium phosphate monobasic certified dehydrate, and ethyl acetate were purchased from Fisher Scientific (Fairlawn, NJ). Mixed gender pooled 20-donor HLM S9 fractions (20mg/mL protein concentration) were purchased from Corning (Woburn, MA). All solvents were HPLC grade. Deionized water was purified using a Barnstead Nanopure Infinity ultrapure water system (Boston, Massachusetts). Plasma from human whole blood (tri-K EDTA, male, drug free, non-smoker) for standard and quality control preparations was purchased from Biological Specialty Corp. (Colmar, PA).

STUDY PROTOCOL

A total of 15 non-smoking, healthy female and male volunteers (18 to 79 years old), who were within 32% of their ideal body weight, and who agreed to refrain from taking any prescription medications, over-the counter medications, hormonal agents, and herbal, dietary, and alternative supplements that may interact with the metabolism of bupropion at least 2 weeks prior to the start of the study and until study completion, were chosen to participate in the study. Approval to conduct the study was obtained, in advance, from the Institutional Review Board (IRB) of Indiana University and all participants signed an IRB-approved informed consent prior to enrollment. The trial is registered at ClinicalTrials.gov (Identified # NCT02401256).

STUDY DESIGN

Prior to dosing with bupropion, baseline blood (~10 mL) and urine samples (~50 mL) were collected. Subjects received a single 100 mg dose of slow release racemic bupropion in a tablet form (Sandoz NDC 0781-1064-01 LotEM0855) by mouth, after an overnight fast, with ~250 mL water. A standard meal was served 3 hours after drug dosing. Subjects were admitted to admitted in the Indiana Clinical Research Center (ICRC) inpatient setting then the following samples were collected after dosing: 1.) blood (~10 mL) was collected from the intravenous catheter at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, and 24 hours for pharmacokinetic studies. 2.) All urine voided during the 24 hour ICRC stay was collected in fractions of 0 to 12 and 12 to 24 hours. Urine from each of these intervals was collected in a different container. After the 24 hour blood draw and urine collection, subjects were discharged home and given instructions and two urine containers to save urine for one day. One container was used for the 24-36 hour void, and the second for the 36-48 hour void. Subjects returned to the ICRC on day 3 and day for collection of a 48 and 72 hour blood samples. Plasma was separated from whole

blood by centrifugation at 3000 rpm for 20 minutes. Plasma and urine samples were immediately stored at -80˚C until analysis.

STANDARD CURVE AND QUALITY CONTROL SAMPLES

Standard stock solutions (1mg/mL each) of R-bupropion, S-bupropion, (2R,3R)-4-hydroxybupropion, (2S,3S)-4-hydroxybupropion, were prepared separately in polypropylene tubes by adding methanol. Erythro- and threo-dihydrobupropion are commercially available only as 50:50 racemic mixtures. Therefore, the adjusted concentration of the stock solution was 0.5 mg/mL each for Ery A, Ery B, Threo A, and Threo B in methanol. Separate stock solutions were created for all analytes for quality control (QC) samples. All solutions were stored at -20°C. Human plasma (total volume of 50 µL) was used to prepare the standard curve and quality control (QC) samples. The standard curves for R-BUP, S-BUP, R, R-OH BUP, and S, S-OH BUP had the following concentrations: 0.3, 1, 3, 10, 30, 100, and 300 ng/mL. The standard curves for Ery A, Ery B, Threo A, and Threo B had the following concentrations: 0.15, 0.5, 1.5, 5, 15, 50, and 150 ng/mL. Quality control samples were prepared in duplicate. The QC samples had the following concentrations for R-BUP, S-BUP, R, R-OH BUP, S, S-OH BUP: 1 ng/mL (low QC), 30 ng/mL (medium QC), and 200 ng/mL (high QC). The QC samples had the following concentrations for Ery A, Ery B, Threo A, and Threo B: 0.5 ng/mL (low QC), 15 ng/mL (medium QC), and 100 ng/mL (high QC) .

SAMPLE PREPARATION

Frozen plasma samples (stored in a -80º freezer) were thawed to ambient temperature. After the addition of 20 µL of 0.1 ng/µL of APAP (internal standard) and 2 mL of ethyl acetate to the tube, the sample was vortex mixed for 30 seconds. After centrifugation at 3000rpm at ambient temperature for three minutes, the upper organic phase was transferred to a clean 12X75 polypropylene tube and evaporated to dryness. A

50 µL volume of methanol was added to each tube and the tube was mixed for 20 seconds on a vortex mixer. A 10 µL aliquot of each sample was injected into the HPLC.

CONDITIONS FOR HPLC-MS/MS

Chromatographic separation was achieved using an Agilent 1290 series HPLC coupled with a PAL HTC-XT Leap autosampler using reverse phase chromatography, at 40°C, with a Lux 3µ Cellulose-3 250X4.6 mm chiral column. The buffer for mobile phase consisted of: 5mM ammonium bicarbonate and 0.1% ammonium hydroxide prepared as one solution in HPLC grade water. Mobile phase A was methanol: acetonitrile: 5mM ammonium bicarbonate plus 0.1% ammonium hydroxide (25:15:60, v/v/v). Mobile phase B was methanol: acetonitrile: 5mM ammonium bicarbonate plus 0.1% ammonium hydroxide (60:30:10, v/v/v). The mobile phase was delivered as a gradient with a constant flow rate of 400 µL/min. The gradient began after 6 minutes at 100% of mobile phase A. From 6-12 min mobile phase A was decreased to 95% in a linear fashion. From 12-30 min mobile phase A was decreased from 95% to 40% in a linear fashion. Next, mobile phase A was decreased from 40% to 0% in a linear fashion from 30-37 min. At 37.1 min the gradient was stepped to 100% A until 40 min, which was the end of the run. The column effluent was monitored using an ABSciex 5500 QTRAP triple-quadrupole mass spectrometer (Foster City, CA) equipped with an electrospray ionization probe in positive mode. The mass spectrometer was controlled by Analyst software (version 1.6.2) in conjunction with Windows 7®. A flow injection analysis was performed on each analyte to maximize sensitivity. The analytes were optimized at a source temperature of 650° C, under unit resolution for quadrupole 1 and 3. In addition, the analytes were given a dwell time of 200 msec and a settling time of 10 msec. The ion spray voltage was 5500 V and the interface heater was on. Optimal gas pressures for all of the analytes were: collision gas medium, curtain gas 10, ion source gas (1) 25, ion source gas (2) 25. Multiple reaction monitoring was used to measure Q1/Q3 transitions for: R-BUP and S-

BUP at 240.1/184.0; R, R-OH BUP and S, S-OH BUP at 255.9/139.0; Ery A, Ery B, Threo A, and Threo B at 241.9/116.0; and APAP at 152.0/109.9. Mass spectrometry settings for the analytes are listed in Table 1.

Analyte	Q1 (m/z)	Q3 (m/z)	Declustering Potential (volts)	Entrance Potential (volts)	Collision Energy (volts)	Exit Potential (volts)
R- and S-bupropion	240.1	184.0	66	8	17	10
(2R,3R)- and (2S,3S)-4-hydroxybupropion	255.9	139.0	66	6	35	10
Erythro- and threo-dihydrobupropion	241.9	116.0	51	10	45	8
Acetaminophen (internal standard)	152.0	109.9	86	4	21	4

Table 1. MS/MS settings (ABSciex 5500) for R-bupropion, S-bupropion, (2R,3R)-4-hydroxybupropion, (2S,3S)-4-hydroxybupropion, erythro-dihydrobupropion A and B, threo-dihydrobupropion A and B, and acetaminophen (internal standard).

INTRA-DAY VARIABILITY

Standards (in singular) and QC samples (in duplicate) were prepared with additional samples at the following concentrations (n=6 for each): the limit of quantification (LOQ) at 0.3 ng/mL for R-BUP, S-BUP, R, R-OH BUP, S, S-OH BUP and 0.15ng/mL for Ery A, Ery B, Threo A, and Threo B, low QC, medium QC, and high QC concentrations. The mean and standard deviation were estimated for each concentration from the six samples. Precision was assessed using percent coefficient of variation (% C.V.) and accuracy was estimated using percent accuracy. A single measurement was excluded from the analysis if that measurement was greater than three standard deviations from the mean of the remaining samples per published FDA Guidelines [35].

INTER-DAY VARIABILITY

Standards (in singular) and QC samples (in duplicate) at the low QC, medium QC and high QC concentrations were prepared each day for 5 days. The duplicates for each

QC sample of each day were averaged. The mean and standard deviation were estimated for each average of the 5 days. Accuracy and precision were assessed from the mean and standard deviation.

EXTRACTION EFFICIENCY

The efficiency of the extraction procedure was estimated by comparing the response of extracted analyte (in duplicate) to the response of the non-extracted analyte.

STABILITY

Stability of the analytes, at ambient temperature, over 48 hours was assessed. A non-extracted standard was diluted in methanol and injected into the HPLC approximately every 2 hours for over 48 hours. Precision was then assessed. Acceptable precision was less than 10%.

SPECIFICITY

Samples, containing internal standard only, were prepared with five different non-supplemented plasma samples following the sample preparation previously stated. Acceptable specificity occurred when all five samples exhibited an undetectable (below LOQ) concentration of the anaylte.

IDENTIFICATION OF ENANTIOMERS OF BUPROPION METABOLITES

Synthetic standards of 4-hydroxybupropion enantiomers (R, R-OH BUP, S, S-OH BUP) were available commercially and thus positively confirmed (Figure 1). However, no authentic standards of enantiomers of erythro- and threo-dihydrobupropion were available. In addition, the erythro-dihydrobupropion is catalyzed through unknown carbonyl reductases and threo-dihydrobupropion is catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase 1 [32, 33] but no information is available regarding their stereoselective formation. To gain preliminary structural insight into the formation of these enantiomers, R- and S-bupropion (10 μM) were incubated with human liver S-9 fraction (containing cytosol and microsomes) in the presence and absence of NADPH.

The S-9 fraction was used to capture oxidation to 4-hydroxybuproion (microsome) and reduction to dihydrobupropion (cytosol). R- or S-bupropion (10 μM), in methanol, was added to polypropylene tubes and evaporated to dryness. Then, 1 mg of the S9 mixture were added to the tubes along with incubation buffer (0.1M Na_2HPO_4 plus 5 mM $MgCl_2$, pH 7.4).The reaction was initiated with 1 mM NADPH and incubated at 37°C for 30 minutes. The reaction was terminated by adding 3mLs of ice-cold ethyl acetate and mixing. Next, the samples were centrifuged at 3000 rpm for 5 minutes; the organic layer was transferred to clean polypropylene tubes, and evaporated to dryness. The dried samples were reconstituted with 100μL of methanol, and a 10μL aliquot was injected into the HPLC-MS/MS. Metabolite peaks were detected using the LC-MS/MS method described above.

URINE SAMPLE ANALYSIS

R-Bupropion (R-BUP), S-bupropion (S-BUP), (2R,3R)-4-hydroxy bupropion (R, R-OH BUP), (2S,3S)-4-hydroxy bupropion (S, S-OH BUP), rac-erythro-dihydrobupropion, and rac-threo-dihydrobupropion were quantified from urine samples by high performance liquid chromatography tandem mass spectrometry, HPLC-MS/MS (3200® AB Sciex, Framingham, MA). The Q1/Q3 transitions were: 241.2/ 185.2 for R- and S- BUP, 256.0/ 139.1 for R, R- and S, S-OH BUP, 242.2/116.2 for Ery A, Ery B, Threo A, Threo B, and 152.3/110.0 for acetaminophen. The HPLC conditions were as previously described above. For the extraction, 200μL of urine was transferred to clean polypropylene tubes, acetaminophen was added as the internal standard (20μL of 1ng/μL), and 4mL of ethyl acetate was added. The samples were vortexed, centrifuged, and the organic layer was transferred to clean polypropylene tubes, evaporated to dryness, and then reconstituted with 50μL methanol. A 10μL aliquot was then injected to the HPLC-MS/MS. The standard curve range for R-BUP, S-BUP, R, R-OH BUP, and S,

S-OH BUP was: 1, 3, 10, 30, 100, 300, and 1000 ng/mL. The standard curve range for Ery A, Ery B, Threo A, and Threo B was: 0.5, 1.5, 5, 15, 50, 150, and 500 ng/mL.

GLUCURONIDE URINE SAMPLE ANALYSIS

Racemic erythro-dihydro bupropion β-D glucuronide (Ery A glucuronide, Ery B glucuronide), R, R- dihydrobupropion β-D-glucuronide (R, R-threo glucuronide), and S, S-dihydrobupropion β-D-glucuronide (S, S-threo glucuronide), (2R, 3R)-4-hydroxy bupropion β-D glucuronide (R, R-OH BUP glucuronide), (2S, 3S)-4-hydroxy bupropion β-D glucuronide (S, S-OH BUP glucuronide) were quantified from urine samples by high performance liquid chromatography tandem mass spectrometry, HPLC-MS/MS (3200® AB Sciex, Framingham, MA). The Q1/Q3 transitions were: 418.0/168.0 for Ery A glucuronide, Ery B glucuronide, R, R-threo glucuronide, and S, S-threo glucuronide, 432.0/184.0 for S, S-OH BUP glucuronide and R, R-OH BUP glucuronide and 152.3/110.0 for acetaminophen. The R, R- and S, S-OH BUP glucuronides are not commercially available. For the quantification of these analytes, R, R-threo glucuronide standard curve was used to quantify R, R-OH BUP glucuronide and S, S-threo glucuronide was used to quantify S, S-OH BUP glucuronide. The HPLC column was a Phenomenex® C18(2) 150X4.6 mm 5 micron. The mobile phase consisted of methanol and 0.1% acetic acid (Mobile phase A was 2:98; v/v and mobile phase B was 90:10; v/v). The mobile phase was delivered as a linear gradient from 0 minutes (100%A) to 10 minutes (100%B) at a flow rate of 800µL/min, at 10.1 minutes the mobile phase was immediately stepped to 100%A and the column equilibrated until 14 minutes.

The standard curve range was: 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000ng/mL for all glucuronides. Urine samples and standards (50µL) were transferred to micro-centrifuge tubes and 0.1ng/µL of acetaminophen was added as the internal standard. Then 100µL of water: methanol; 50:50; v/v was added to precipitate out some of the

salts. The samples were then vortex, centrifuged, and the supernatant was transferred to an HPLC vial. A 10 μL aliquot was injected into the HPLC-MS/MS.

PHARMACOKINETIC AND DATA ANALYSIS

Non-compartmental analysis of data was performed using Phoenix® WinNonlin® (version 6.4, Pharsight Corp., Cary, NC). Pharmacokinetic outcomes for analysis included: $t_{1/2}$ (half-life, $t_{1/2}=0.693/k_{el}$), C_{max} (maximal plasma concentration), T_{max} (time of maximal plasma concentration), $AUC_{0-\infty}$ (area under the plasma concentration time curve from zero to infinity), V_d (volume of distribution calculated by clearance/k_{el}), apparent oral clearance (calculated by (CL/F)= dose/$AUC_{0-\infty}$). The maximum observed concentration (C_{max}), time to reach C_{max} (T_{max}), and last measured concentration (C_{last}) was recovered directly from the concentration-time profile. Area under the curve from time zero to C_{last} (AUC_{0-last}) was determined using the trapezoidal rule with linear up/log down interpolation, then, the AUC from time zero to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-last} and the ratio of C_{last} to λ_z. $AUC_{0-\infty}$ not reported for analytes where the extrapolation percentage was in excess of 30%. Results are expressed as geometric mean and percent coefficient of variation (%CV). Urinary excretion kinetics were analyzed both as absolute and molar (nmol) amounts (based on individual urine collection volumes) of each metabolite in relation to bupropion using Phoenix® WinNonlin® (version 6.4, Pharsight Corp., Cary, NC). Statistical data comparison between the pharmacokinetic outcomes of the enantiomers were evaluated using a paired two-tailed Student's t-test on log-transformed data (Cl/F, AUC, $t_{1/2}$, C_{max}) or Wilcoxon signed-rank test (T_{max}) as appropriate. A p-value <0.05 was considered statistically significant. All statistical analyses were conducted using IBM® SPSS® software (version 23, Armonk, New York).

CHAPTER 3-RESULTS

A chromatogram at the LLOQ of R-BUP, S-BUP, R, R-OH BUP, S, S-OH BUP, Ery A, Ery B, Threo A, Threo B, and APAP (internal standard) shows successful separation of enantiomers of bupropion and metabolites (Figure 2). Intra-day and inter-day accuracy and precision for R-BUP, S-BUP, R, R-OH BUP, S, S-OH BUP, Ery A, Ery B, Threo A, and Threo B were calculated. The intra-day precision for all analytes ranged from 3.4% to 15.4% and the intra-day accuracy ranged from 80.6% to 97.8%. The inter-day precision for all analytes ranged from 6.1% to 19.9% and the inter-day accuracy ranged from 88.5% to 99.9%. The extraction efficiency was calculated by comparing peak areas of non-extracted analyte to the peak areas of extracted analyte. The extraction efficiency of all analytes was 70% or greater. The stability at a single non-extracted concentration for over 48 hours at ambient temperature resulted in less than 9.8% variability for all analytes. The method exhibited acceptable specificity because all five untreated plasma samples, purchased from Biological Specialty Corporation, had undetectable (below LOQ) concentrations of all analytes.

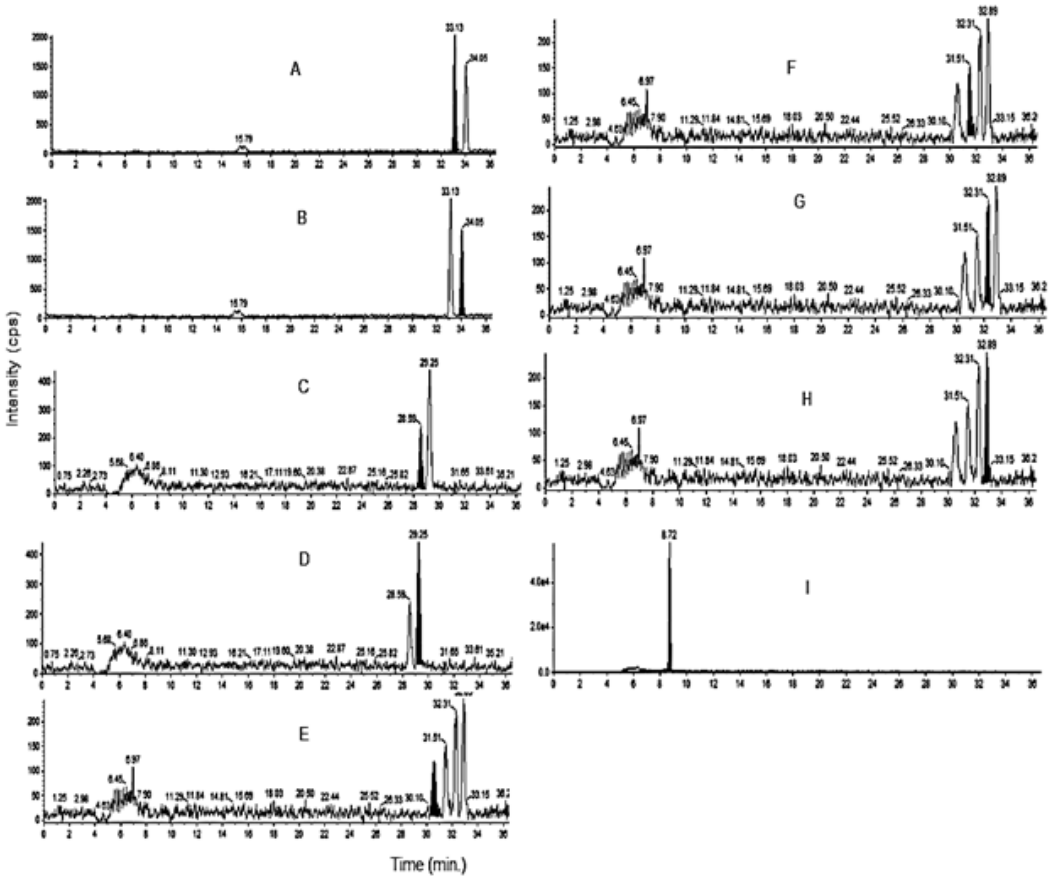

Figure 2. Chromatography of bupropion and metabolites at the lowest limit of quantification: 0.3ng/mL for R-bupropion (A), S-bupropion (B), (2R,3R)-4-hydroxybupropion (C), (2S,3S)-4-hydroxybupropion (D); and 0.15ng/mL for erythro-dihydrobupropion A (E), erythro-dihydrobupropion B (F), threo-dihydrobupropion A(G), threo-dihydrobupropion B (H), and the internal standard acetaminophen (I). The filled peak is the analyte of interest.

R- and S-BUP were incubated with S9 fraction to assess the possible metabolic pathways of the enantiomers of erythro- and threo-dihydrobupropion. The metabolism of R- and S-bupropion in human liver S9-fractions and NADPH is shown in Table 4. When R- and S-bupropion were incubated with human liver S9-fractions without NADPH, no R, R-OH BUP or S, S-OH BUP metabolite peak was detected and this finding (data not shown) was expected because CYP-mediated reactions do not occur in the absence of a co-factor. On the other hand, the reductive metabolites of bupropion were generated in similar patterns as described above for S9-fraction + NADPH. The

data show that R-BUP primarily forms R, R-OH BUP, Ery A and Thro A, while S-BUP primarily forms S, S-OH BUP, Ery B, and Threo B. Based on these preliminary in vitro data and the possible structural configuration, a tentative proposal is suggested that R-bupropion was reduced mainly to (1S, 2R)-dihydrobupropion (Ery A) and (1R, 2R)-dihydrobupropion (Threo A), while S-bupropion was reduced mainly to (1R, 2S)-dihydrobupropion (Ery B) and (1S, 2S)-dihydrobupropion (Threo B) (Figure 3).

	Peak Area (counts per second)					
	R, R-OH BUP	**S, S-OH BUP**	**Ery A**	**Ery B**	**Threo A**	**Threo B**
R-BUP + S9	26800	12900	6440	3090	43900	211000
S-BUP + S9	4370	41300	351	15000	3800	562000
Metabolic Ratio (R-BUP/S-BUP)	6.1	0.3	18.3	0.21	11.6	0.38

Table 2. The metabolism of R- and S-bupropion with human S9 fraction. R-bupropion and S-bupropion (10 μM) were incubated (30 min) with human S9-fractions (1mg) and NADPH. The incubation mixture was precipitated with methanol, mixed, centrifuged and the supernatant was transferred to a tube. The supernatant was evaporated to dryness overnight, reconstituted with mobile phase and an aliquot of the mobile phase was injected into the HPLC-MS/MS. Peak areas were estimated for all metabolites.

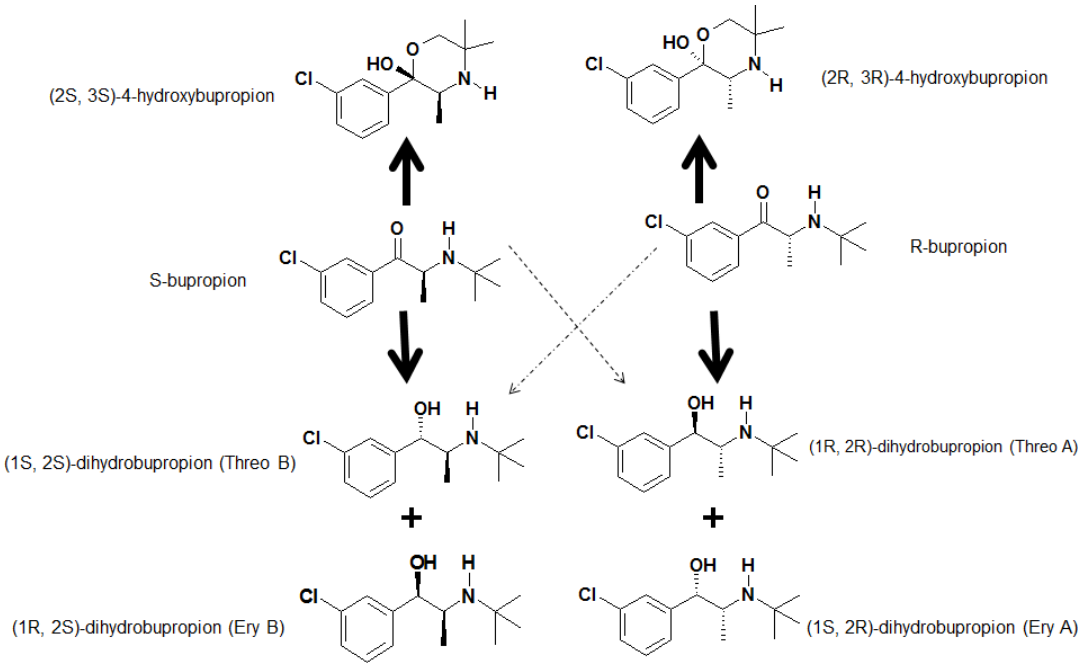

Figure 3. Structures and proposed metabolic pathway of R-bupropion, S-bupropion, (2R,3R)-4-hydroxybupropion, (2S,3S)-4-hydroxybupropion, erythro-dihydrobupropion (Ery A and Ery B), threo-dihydrobupropion (Threo A and Threo B). The main pathways are expressed with the bold arrows, while the minor pathways are shown with the dotted arrows.

Pharmacokinetic parameters of racemic and enantiomers of bupropion and metabolites in 15 healthy volunteers given a single 100 mg oral dose of bupropion are shown in Table 3. Geometric means of log-transformed data were utilized to compare pharmacokinetic parameters of all analytes due to the large inter-individual variability observed with bupropion metabolism and the small number of subjects (n=15) in the study, which preclude a normal distribution of the data. Arithmetic means were calculated but are not presented; and statistically significant differences are equivocal with the geometric mean data. There were significant differences in the stereoselective metabolism of all enantiomers. The plasma exposure of R, R-OHBUP was, predictably, the greatest of all the analytes. It had a statistically significant greater $AUC_{0-\infty}$, almost 65 fold higher, than S, S-OHBUP, and a C_{max} 35 fold higher. The $AUC_{0-\infty}$ of R, R-OHBUP was over 32 fold greater than R-BUP, and had a C_{max} about 4 fold greater than R-BUP.

The $t_{1/2}$ was longer (median 19.3 hours) for R,R-OH BUP in comparison to S,S-OH BUP (median 14.6 hours), and R-BUP (median 11.6) hours.

Analyte	Pharmacokinetic Outcomes					
	$t_{1/2}$ (hr.)	C_{max} (nM)	t_{max} (hr.)	$AUC_{0-\infty}$ (nM*hr.)	V_d/F per kg	CL/F per kg
Enantiomers:						
R-BUP	11.6 (49)	288 (74)	1 [1-2.5]	1,162 (58)	40.7 (78)	2.42 (56)
S-BUP	7.2 (103)	47.0 (74)	1.5 [1-3]	193 (72)	152 (83)	14.6 (67)
BUP (R/S)	1.6 [1.1-2.4]	6.1 [5.3-7.1]*	-	6.0 [5.2-7.0]*	0.26 [0.2-0.36]*	0.17 [0.14-0.19]*
R, R-OHBUP	19.3 (20)	1,240 (55)	2.5 [1-10]	37,421 (49)		
S, S-OHBUP	14.6 (36)	35.9 (74)	2.5 [1-6]	580 (55)		
OH-BUP (RR/SS)	1.3 [1.2-1.5]*	34.7 [28.6-42.1]*	-	64.5 [55.4-75.1]*		
Ery A	24.2 (39)	30.5 (37)	3 [1-10]	1182 (60)		
Ery B	14.6 (61)	10.6 (52)	2 [1-4]	185 (71)		
Ery (A/B)	1.7 [1.3-2.1]*	2.9 [2.4-3.5]*	-	6.4 [5.1-8.0]*		
Threo A	45.5 (40)	79.9 (34)	6 [1-12]	5,527 (61)*		
Threo B	8.2 (48)	168 (67)	2 [1-4]	1,433 (84)		
Threo (A/B)	5.5 [4.2-7.3]*	0.47 [0.38-0.60]*	-	3.9 [2.6-5.7]		
Racemic:						
BUP	10.8 (54)	335 (73)	1 [1-2.5]	1,334 (61)	65.7 (80)	4.21 (58)
OH-BUP	19.2 (21)	1,280 (56)	2.5 [1-10]	37,984 (49)		
Ery	21.6 (36)	40.0 (37)	2.5 [1-10]	1,330 (57)		
Threo	30.8 (41)	235 (53)	2 [1-4]	6,632 (45)		

Table 3. Pharmacokinetic parameters of racemic and enantiomers of bupropion and metabolites in healthy volunteers (n=15) administered a single 100 mg oral dose of bupropion. Bupropion (BUP); hydroxybupropion (OH-BUP); erythro-hydrobupropion (Ery); and threo-hydrobupropion (Threo). Nomenclature of the enantiomers for Ery and Threo was based on the chromatography of the first eluting peak as "A" and the second eluting peak as "B". Thus enantiomers of Ery were denoted as Ery A and Ery B and those of Threo were denoted as Threo A and Threo B. Tmax: median [range]; Ratios: geometric mean ratio [90% confidence intervals]; all other outcomes reported as geometric mean (CV%). *P<0.05

The plasma exposure of Threo A was the second highest of all of the analytes and had the longest t_{max} (median 6 hours). It had an $AUC_{0-\infty}$ over 5 fold higher than Threo B and 7 fold greater than S-BUP. Although the AUC_{0-T} was greater for Threo A, Threo B had a significantly higher C_{max} at 168 nM compared to Threo B at 79.9 nM. The elimination for Threo A was the longest for all analytes (median $t_{1/2}$ of 45.5 hours), and was over 5 times longer than Threo B.

Ery A and R-BUP had similar $AUC_{0-\infty}$ as did Ery B in comparison to S-BUP. Ery A had over 6 fold greater $AUC_{0-\infty}$ than Ery B, and a significantly higher $t_{1/2}$, and almost 3 fold higher C_{max}. Ery A had the second longest elimination of all of the analytes at a median $t_{1/2}$ of 24.2 hours.

An abundance of plasma exposure of racemic OH-BUP and Threo was observed in comparison to racemic BUP and ERY (Table 3, Figure 4). The individual enantiomers; R, R-OH BUP and Threo A are the foremost contributors to this elevated exposure. The metabolite enantiomers plasma decay curves, excluding Threo B, all have significantly slower elimination rates than the parent, therefore are elimination rate limited. Threo B has a similar elimination rate to S-BUP, but a much higher AUC, characterizing it as elimination rate limited as well.

The plasma concentration vs. time profiles of BUP and metabolites, a combination of both racemic and enantiomers, shows stereoselective differences (Figure 4). Racemic BUP, OH-BUP, and ERY all have similar plasma concentrations and profiles in comparison to R-BUP, R, R-OHBUP, and ERY A, respectively, illustrating stereoselective metabolism towards these analytes. S-BUP, S, S-OHBUP, and ERY B all have lower plasma concentrations but similar profile to the racemic curves. Threo A and B show the most significant difference between the racemic and enantiomer profiles.

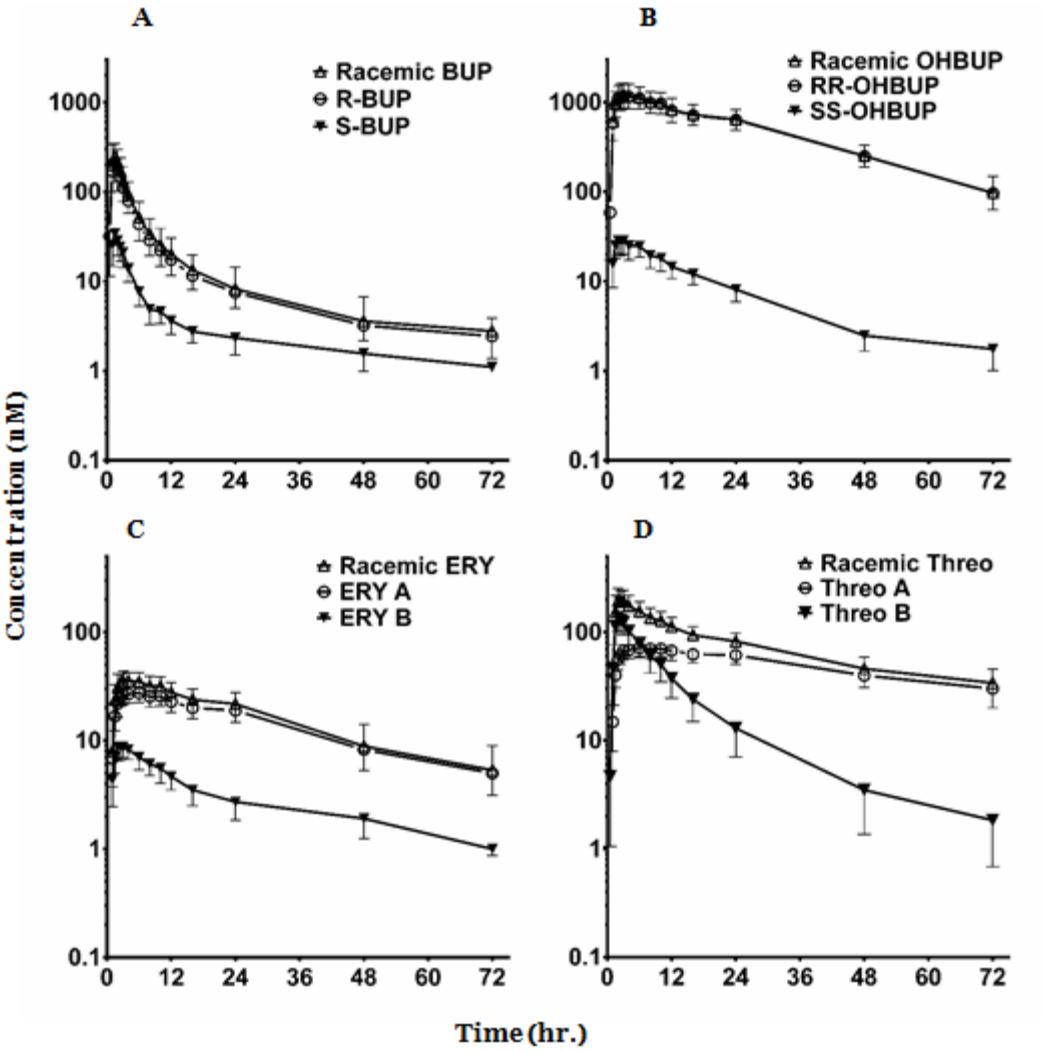

Figure 4. Geometric mean concentration-time profiles of racemic bupropion, R-bupropion, and S-bupropion (A); racemic hydroxybupropion, (2R,3R)-4-hydroxybupropion, and (2S,3S)-4-hydroxybupropion (B); racemic erythro-dihydrobupropion (Ery), Ery A and Ery B (C); racemic threo-dihydrobupropion (Threo), Threo A, and Threo B (D) in 15 healthy volunteers following a single 100 mg oral dose of racemic bupropion. Symbols and error bars denote observed geometric means and limits of the 95% confidence interval, respectively.

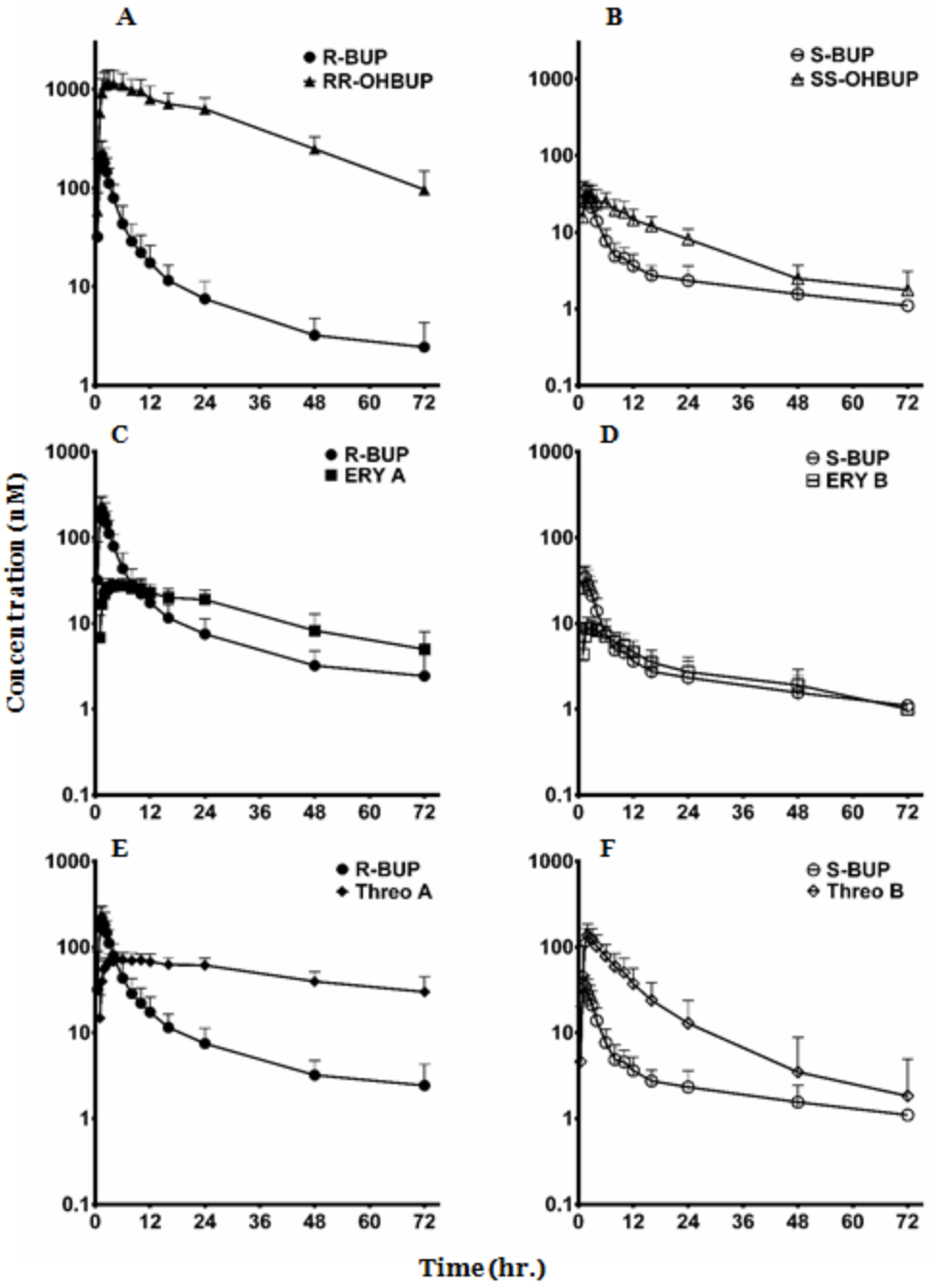

Figure 5. Geometric mean concentration-time profiles of bupropion metabolites and predicted substrates (denoted as predicted substrate → predicted metabolite) based upon proposed metabolic pathways; R-bupropion → (2R,3R)-4-hydroxybupropion (A), S-bupropion → (2S,3S)-4-hydroxybupropion (B), R-bupropion → Ery A (C), S-bupropion → Ery B (D), R-bupropion → Threo A (E), and S-bupropion → Threo B (F). Symbols and error bars denote observed geometric means and limits of the 95% confidence interval, respectively.

The geometric mean concentration-time profiles of bupropion and predicted metabolites are shown in Figure 5. Based on the in vitro data; R-BUP is predicted to metabolize to R, R-OH BUP, Ery A, and Threo A. S-BUP is predicted to metabolize to S, S-OH BUP, Ery B, and Threo B. All of the metabolic profiles differ from that of the parent illustrating that all of the metabolites are elimination rate limited.

Another way of observing the stereoselectivity of the enantiomers is by illustrating the mean metabolic ratios of the enantiomers over time (Figure 6). If the ratio equals one then it will fall on the line of unity (dotted line), which signifies that there is no stereoselectivity occurring for that pair of enantiomers. An increasing ratio shows that over time stereoselectivity is increasing, while a decreasing ratio shows a loss of stereoselectivity over time. The data show that for the metabolites of BUP; R, R-OHBUP/S, S-OHBUP, Ery A/Ery B, and Threo A/Threo B, stereoselectivity increases over time. Alternatively, for R-BUP/S-BUP, stereoselectivity decreases over time, with only a small increase in stereoselectivity from 6 to 12 hours, suggesting that a secondary process is happening, possibly with the clearance, protein binding, and/or volume of distribution. Interestingly, Threo is the only metabolite that has a portion of the profile below the line of unity less than 4 hours, then above the line of unity from 6 hours on.

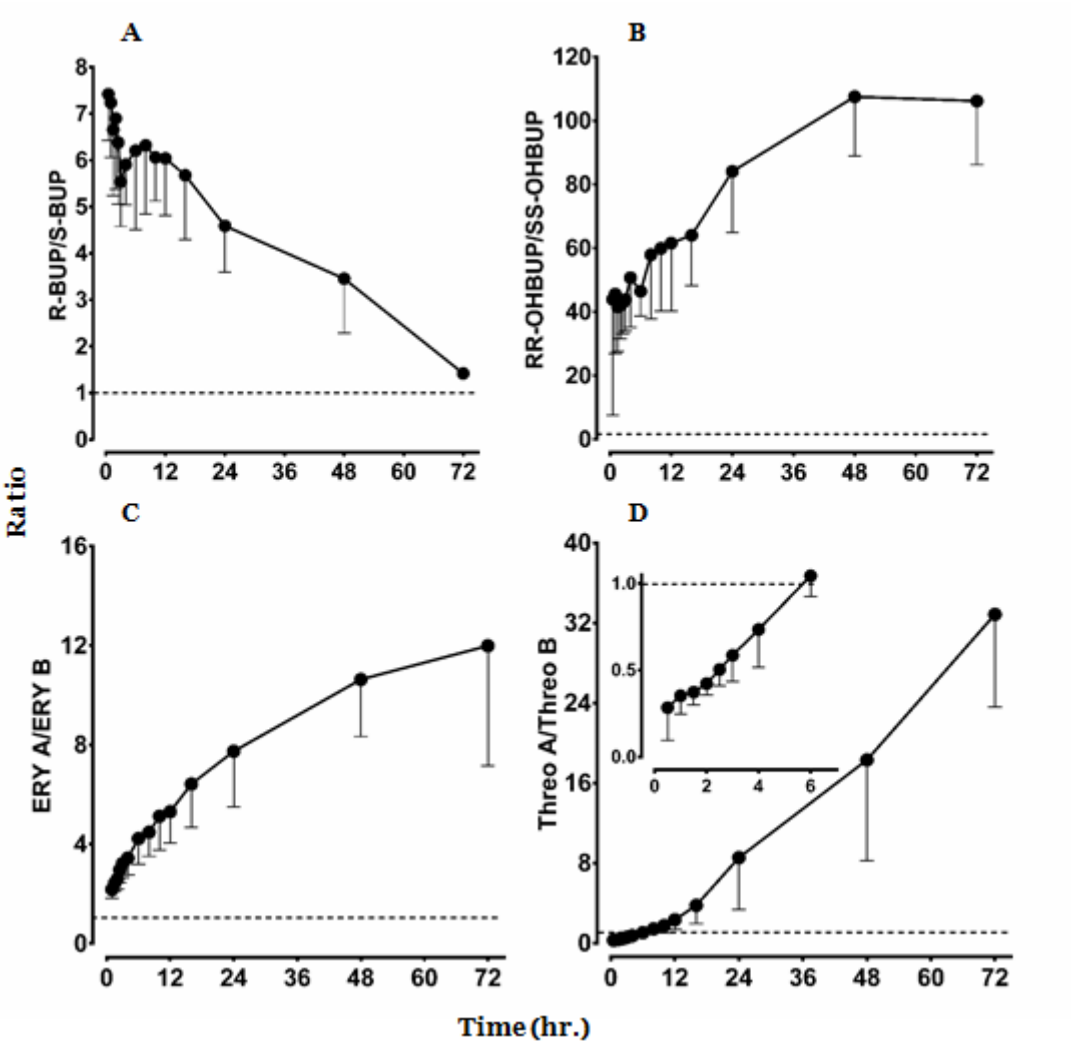

Figure 6. Mean metabolic ratios of R-bupropion and S-bupropion (A); (2R,3R)-4-hydroxybupropion and (2S,3S)-4-hydroxybupropion (B); Ery A and Ery B (C); Threo A, and Threo B (D) over time in 15 healthy volunteers following a single 100 mg oral dose of racemic bupropion. Inset in (D) to illustrate the switch in ratio directionality after 6 hours. Symbols and error bars denote observed mean ratios and lower limits of the 95% confidence interval, respectively. Dotted line represents the line of unity.

Approximately 12% of the administered bupropion dose was recovered in the urine as parent and metabolites by 48 hours. Urinary excretion analysis revealed enantioselective elimination of bupropion and metabolites (Figure 7). Less than 1% of the administered bupropion dose was excreted unchanged in the urine as R- and S-bupropion with nearly 6-fold greater mean urinary recovery of R- versus S-bupropion (Table 4). Threo A, the predominant bupropion metabolite detected in urine, and Threo

B accounted for nearly 30% of all bupropion species recovered in the urine. 4-hydroxybupropion and erythro-dihydrobupropion urinary excretion were markedly enantioselective with 6- and 5- fold greater mean recovery of R, R-OHBUP and Ery A, respectively. The glucuronide conjugate of R, R-OHBUP was the single most prevalent bupropion conjugate recovered in the urine, accounting for a mean recovery of ~30% of all bupropion species detected.

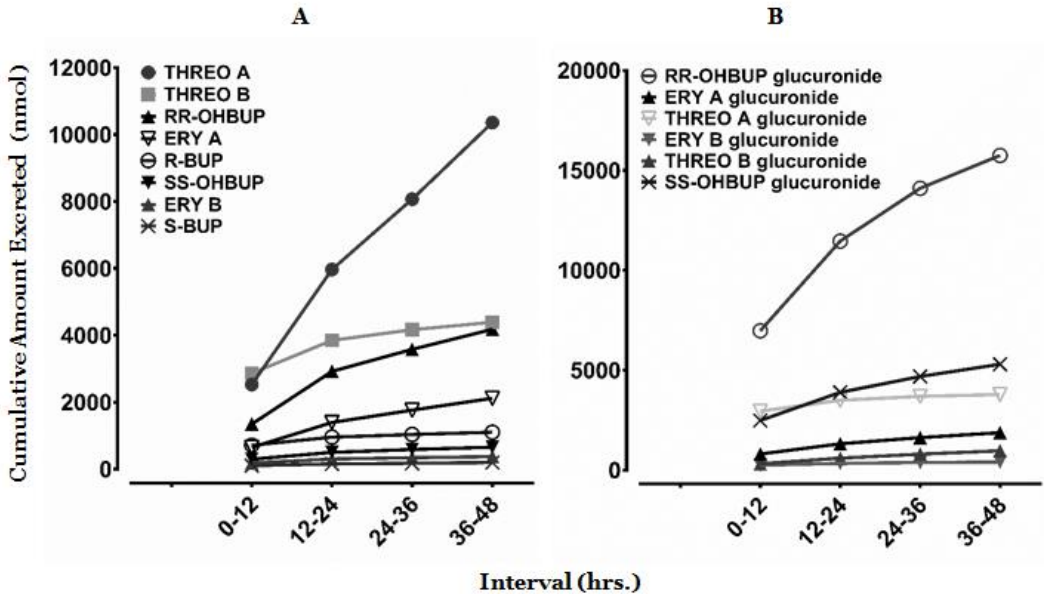

Figure 7. Urinary excretion kinetics of (A) bupropion metabolite enantiomers and (B) glucuronide conjugates. Urine was collected (0-48 hr.) from healthy volunteers (n=15) following oral administration of a single 100 mg dose of racemic bupropion. Symbols denote the geometric mean of the observed cumulative urinary excretion (nmol) at the end of each collection interval. CAE, cumulative amount (nmol) excreted. Variability is not shown for graphical clarity.

Analyte	48 hr. Recovery (nmol)		% of bupropion dose	
R-bupropion	1,106	(873-1,401)	0.27	(0.21-0.34)
S-bupropion	197	(164-238)	0.05	(0.04-0.06)
R, R-OH BUP	4,180	(3,167-5,517)	1.00	(0.76-1.32)
S, S-OH BUP	655	(508-845)	0.16	(0.12-0.20)
Threo A	10,361	(8,433-12,729)	2.48	(2.02-3.05)
Threo B	4,391	(2,887-6,679)	1.05	(0.69-1.60)
Ery A	2,120	(1,710-2,628)	0.51	(0.41-0.63)
Ery B	374	(282-497)	0.09	(0.07-0.12)
R, R-OH BUP Glucuronide	15,751	(12,598-19,693)	3.78	(3.02-4.72)
S, S-OH BUP Glucuronide	5,301	(4,228-6,646)	1.27	(1.01-1.59)
R, R-Threo Glucuronide	3,782	(2,879-4,969)	0.91	(0.69-1.19)
S, S-Threo Glucuronide	969	(827-1,135)	0.23	(0.20-0.27)
Ery A Glucuronide	1,878	(1,569-2,247)	0.45	(0.38-0.54)
Ery B Glucuronide	382	(302-482)	0.09	(0.07-0.12)

Table 4. Bupropion and metabolite urinary excretion data over 48 hours. Values denote geometric means for n= 15 subjects and the numbers in parentheses are 90% confidence intervals.

CHAPTER 4-DISCUSSION

Bupropion is a drug that is widely used in the management of depression and as a smoking cessation aid. BUP, administered orally as a racemate, undergoes extensive hepatic metabolism, with <1% of the administered dose excreted as unchanged in urine over 48 hours after bupropion dosing. Thus, BUP is associated with large inter-patient variability in clinical response [5-8], while some patients are at increased risk for adverse effects such as dose-dependent seizure [9] and often fatal outcomes [10] among drug users abusing bupropion.

Previous clinical studies with BUP have identified three primary metabolites: 4-hydroxybupropion, erythro-dihydrobupropion, and threo-dihydrobupropion. Each metabolite exhibits pharmacological and toxicological activity. It has been suggested that bupropion metabolites contribute significantly to the beneficial and/or adverse effects of bupropion [17-25]. Although the primary racemic metabolites have been identified for over three decades, only R-bupropion (R-BUP), S-bupropion (S-BUP), (2R,3R)-4-hydroxybupropion (R, R-OH BUP), and (2S,3S)-4-hydroxybupropion (S, S-OH BUP) have been stereoselectivity profiled, due to the lack of a chiral assay for erythro- and threo-dihydrobupropion. Therefore, the mechanisms underlying large interpatient variability in the exposure of these active metabolites are not fully understood.

The steady-state plasma exposure of racemic threo-dihydrobupropion is much higher than racemic erythro-dihydrobupropion [27]. There have been documented cases, in humans, which show the amount of threo-dihydrobupropion plasma exposure to be higher than that of 4-hydroxybupropion; in cerebrospinal fluid, after over-dose, or abuse of bupropion [34]. The activity and high plasma exposure of threo- and erythro-dihydrobupropion indicate these metabolites may have important stereoselective

pharmacological activities and disposition, but have only been studied as racemic mixtures.

To further understand the stereoselective metabolism and disposition of BUP, an accurate, precise, and sensitive LC-MS/MS method was developed to quantify the enantiomers of bupropion, 4-hydroxybupropion, threo-dihydrobupropion, and erythro-dihydrobupropion. Chromatographic separation of the enantiomers was optimized for injection volume, column temperature, mobile phase, and buffers, as slight deviations from these conditions resulted in co-elution of the enantiomers. In addition, the HPLC column was washed for 1 h with methanol: water; (90:10 v/v), then equilibrated with mobile phase A, overnight, at a flow rate of 400 µL/min. The advantages of the current method include good chromatographic separation of enantiomers of BUP, OH-BUP, threo-dihydrobupropion, and erythro-dihydrobupropion, a simple liquid–liquid extraction, and small sample volumes. The main disadvantages of this method are the long run time and a short column life span. The HPLC column was changed after approximately 500 sample injections because of loss of baseline resolution of analytes. This is less than other chiral columns life span of around 1000 injections. The method described above was successfully implemented to determine the stereoselective pharmacokinetics of BUP and its metabolites in subjects administered a single 100 mg oral dose of racemic bupropion.

The pharmacokinetics of BUP and metabolites were assessed in 15 normal healthy subjects. All subjects had detectable/quantifiable concentrations of BUP, OH-BUP, Ery A, Ery B, Threo A and Threo B. The pharmacokinetic data show highly stereoselective disposition of bupropion and its metabolites (Table 2). A previous report, Kharasch *et al.*, observed stereoselective differences in plasma bupropion and hydroxybupropion. Kharasch *et al.*, found R-BUP C_{max} and $AUC_{0-\infty}$ were 3-fold greater than those of S-BUP [28]. Kharasch *et al.*, data differ from the data in this study which

had a 6-fold difference between R- and S-BUP C_{max} and $AUC_{0-\infty}$. The $t_{1/2}$ data differ in that Kharasch *et al.*, found the $t_{1/2}$ to be 19 hours for R-BUP, and 17.6 hours for S-BUP, while this study observed 13 hours for R-BUP and 10 hours for S-BUP. There were additional differences in clearance. Also, Kharasch *et al.*, reported that R, R-OH BUP and S, S-OH BUP had a significant difference in T_{max} (4.6 vs. 2.2 hours, respectively), a C_{max} 20-fold greater for R, R-OH BUP, and an $AUC_{0-\infty}$ 44-fold greater for R, R-OH BUP [28]. Table 2 data show that R, R-OH BUP and S, S-OH BUP have the same T_{max}, a C_{max} 34.7-fold greater for R, R-OH BUP, and an $AUC_{0-\infty}$ over 64-fold greater for R, R-OH BUP. Kharasch *et al.*, also determined R, R-OH BUP to be elimination rate limited, while S, S-OH BUP to be formation rate limited [28]. This current study ascertained that R, R-OH BUP and S, S-OH BUP to be elimination rate limited. The disparities between the two studies could be due to the difference in the oral bupropion given. Kharasch *et al.*, study used immediate release, while this study used slow release [28].

Although there were discrepancies in the pharmacokinetic parameters between the Kharasch *et al.*, study and this one, the disposition of the enantiomers was similar [28]. Both studies found R, R-OH BUP had the highest $AUC_{0-\infty}$ and longest $t_{1/2}$ in comparison to R- and S-BUP and S, S-OH BUP. Both studies also found that although the clearance of S-BUP was faster than R-BUP, and the plasma concentration of S-BUP was less than that of R-BUP [28].

The enantioselective elimination rate-limited kinetics of bupropion metabolites compromise the utility of bupropion as a suitable probe of CYP2B6 activity in vivo, an issue reported previously [28, 36-39]. For an ideal CYP probe the parent drug clearance could possibly be used in determining the enzyme activity. However, in concurrence with a previous report [27], only 0.3% of the administered bupropion dose was recovered in the urine as unchanged drug. In addition to BUP being heavily metabolized, it is metabolized through multiple pathways. The data substantiate that CYP2B6

hydroxylation is a minor pathway since the most prevalent urinary bupropion metabolites, Threo A and the glucuronide conjugate of R, R-OH BUP, are excreted in similar amounts. This suggests that metabolism via carbonyl reductases and glucuronidation are among the most important contributors to overall bupropion disposition. The most abundant bupropion species detected in plasma, R, R-OH BUP, coincides with the most prevalent glucuronide conjugate detected in urine, R, R-OH BUP glucuronide. Urinary excretion reveals approximately 1.8 fold greater recovery of Threo A versus Threo B at 48 hours in concurrence with the observed 2.3 fold greater plasma exposure ($AUC_{0\text{-last}}$) of Threo A. Plasma exposure and urinary excretion of S, S-OH BUP have been previously proposed as improved in vivo markers of CYP2B6 activity [28] but limitations still exist. The potentially important contribution of stereoselective gut metabolism, observed enantioselective renal elimination, and elimination rate limited kinetics further confound the use of bupropion as a in vivo probe of CYP2B6 activity. Taken together, further elucidation of the enantioselective metabolism and urinary elimination of bupropion provides additional data to suggest that bupropion may not be an ideal marker of CYP2B6 activity.

The urinary data in this study corroborate similar results to a previous study performed by Gufford et al. They found that 10% of all analytes were recovered in the urine [26], while this study found 12% recovery of all analytes. Threo A and R, R-OH BUP glucuronide accounted for over 50% excreted of that 12% recovered. Urinary excretion reveals approximately 1.8 fold greater recovery of Threo A versus Threo B at 48 hours in concurrence with the observed 2.3 fold greater plasma exposure ($AUC_{0\text{-}72}$) of Threo A. These data are consistent with a previous publication [26].

These data are the first to demonstrate unique stereoselective disposition of erythro- and threo-dihydrobupropion in humans. All previous studies quantified Threo and Ery as racemic mixtures and the pharmacokinetic data were analyzed accordingly.

The marked differences in the elimination half-life of threo-dihydrobupropion and to some extent erythro-dihydrobupropion suggest differential accumulation of these metabolites during chronic dosing with racemic bupropion. For example, Ery A and Threo A, which show relatively longer half-lives than the parent, would be expected to accumulate during multiple dosing compared to Ery B and Threo B.

Another unique observation of Threo metabolism is the ratio of Threo A/Threo B over time (Figure 3). Interestingly the ratio of Threo A/Threo B falls below the line of unity <4 hours, then above the line of unity from 6 hours up to 72 hours; meaning that the initial rate of formation for Threo B is faster than the rate of formation of Threo A. This suggests that S-BUP is forming Threo B at a much faster rate than Threo A is being formed by R-BUP. The explanation may be that R-BUP is a better substrate to CYP2B6 or S-BUP is a better substrate to 11β-hydroxysteroid dehydrogenase, or a combination of the two. It is possible that differential absorption or first-pass metabolism of R- and S-BUP may also contribute to the unique pharmacokinetics of Threo A and Threo B. Pre-systemic metabolism of bupropion by intestinal CYP2B6 and carbonyl reductases (primarily AKR7) is a plausible mechanism underlying these observations [40]. The oral dose of BUP was a slow release formulation, which may also give rise to the unique pharmacokinetics. Interestingly, an observation of the profile of the ratio of R-BUP/S-BUP corresponds to the Threo A/B ratio data shown in Figure 6. There is a rapid stereoselective reduction happening from time 0.5 hours to 6 hours, then an increase in stereoselectivity from 6 hours to 12 hours, then a gradual loss of stereoselectivity to 72 hours. The time-dependent decrease in R-BUP/S-BUP ratio and corresponding time-dependent increase in R, R-OHBUP/S, and S-OHBUP agree well with a previous report [28].

The in vitro data allow, for the first time, a proposal for the possible structural configuration of Threo A and B and Ery A and B. As expected, R- and S-bupropion were

metabolized predominantly to R, R-OH BUP and S, S-OH BUP, respectively, and served as a positive control. In addition, the data show that Ery A and Threo A are mainly formed from R-bupropion, while Ery B and Threo B are mainly formed from S-bupropion. However, data also suggest potential chiral inversion of the substrates or the metabolites during the incubation as there was no absolute selectivity, even though pure enantiomers were used during the incubation (ratios in Table 2).

In summary, a novel reversed phase chiral-HPLC-MS/MS method involving a simple liquid-liquid extraction procedure and a small plasma sample volume (50µL) was developed that allowed simultaneous separation and quantification of enantiomers of bupropion, 4-hydroxybupropion, and those of threo- and erythro-dihydrobupropion in human plasma. This method was successfully implemented to determine the unique stereoselective disposition of bupropion and its metabolites in 15 research human volunteers administered a single 100 mg oral dose of racemic bupropion. This is the first evidence showing stereoselective reduction of bupropion in vitro and in vivo. Although needs further confirmation, also provide plausible structural features of threo- and erythro-dihydrobupropion (Figure 3). In addition, clarification of the enantioselective metabolism and urinary elimination of bupropion provides additional data to suggest that bupropion may not be an ideal marker of CYP2B6 activity. The importance of this study was, for the first time, to be able to characterize the stereoselective metabolism of BUP and its three major chiral metabolites. This new quantification method and subsequent pharmacokinetic data should now enhance further research into bupropion stereoselective metabolism, drug interactions, and effect.

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CURRICULUM VITAE

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Education

1999-2004	Ball State University B.S. Chemistry
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Research and Training Experience

2005	Applied Biosystems Analyst training class for the API2000/3000/4000 Q (Foster City, CA)

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2004-2007	Research Scientist, Indiana University Simon Cancer Center
2003-2004	Technician, Sherry Laboratories, Muncie, IN
2001-2003	Certified Pharmacy Technician, CVS, Muncie, IN

Technical Experience

- Expertise in the identification, validation, implementation, and troubleshooting of the following analytical instruments:
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- Proficient with the following software: Analyst, Chemstation, XCalibur, Word, Power Point, Excel, and statistical software SPSS

- Responsible for bioanalytical method development, method validation and sample processing
- Maintenance, calibration and troubleshooting of all analytical instruments and other equipment necessary for drug quantification
- Develop and implement standard operating procedures (SOPs)
- Documentation of bioanalytical procedures
- Train and direct laboratory staff to perform work according to SOPs
- Research new technologies and approaches
- Preparation of bioanalytical protocols, validation reports, study sample analysis reports and relevant SOPs
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Additional capabilities: Oversee all functions of the core laboratory including: supervision of research personnel, preparation of research reports, maintenance of laboratory equipment and records, development of research projects, ordering of supplies, and disposal of biological wastes. Serve as a resource to other laboratories.

Publications

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