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Development, validation and application of a comprehensive stereoselective LC/MS–MS assay for bupropion and oxidative, reductive, and glucuronide metabolites in human urine

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Abstract

A stereoselective assay was developed for the quantification of bupropion and oxidative, reductive, and glucuronide metabolites (16 analytes total) in human urine. Initially, authentic glucuronide standards obtained from commercial sources were found to be incorrectly labeled with regard to stereochemistry; the correct stereochemistry was unequivocally reassigned. A trifurcated urine sample preparation and analysis procedure was employed for the stereoselective analysis of bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion enantiomers, and hydroxybupropion, erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers in urine. Method 1 stereoselectively analyzed bupropion (*R* and *S*), and unconjugated free hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*), and threohydrobupropion (1*R*,2*R* and 1*S*,2*S*) using chiral chromatography with an α_1 -acid glycoprotein column. Because no hydroxybupropion β -D-glucuronide standards were commercially available, method 2 stereoselectively analyzed total hydroxybupropion aglycones (*R,R* and *S,S*-hydroxybupropion) after urine hydrolysis by β -glucuronidase. Hydroxybupropion β -D-glucuronide (*R,R* and *S,S*) urine concentrations were calculated as the difference between total and free hydroxybupropion (*R,R* and *S,S*) concentrations. Due to incomplete μ -glucuronidase hydrolysis of erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers, method 3 stereoselectively analyzed intact erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers using C18 column chromatography. All analytes were quantified by positive ion electrospray tandem mass spectrometry. The assay was fully validated over analyte-specific concentrations. Intra- and inter assay precision were within 15% for each analyte. The limits of quantification for bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), threohydrobupropion (1*S*,2*S* and 1*R*,2*R*), erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*) were 10, 50,

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Conflict of interest

No author has any conflict of interest.

100, and 100 ng/mL, respectively. The limits of quantification for (1*R*,2*R*)-threohydrobupropion β -D-glucuronide, (1*S*,2*S*)-threohydrobupropion β -D-glucuronide, and (1*R*,2*R*)-erythrohydrobupropion β -D-glucuronide were each 50 ng/mL. Due to the abundance of bupropion and metabolites in human urine, no efforts were made to optimize sensitivity. All analytes were stable following freeze thaw cycles at -80°C . This assay was applicable to clinical pharmacokinetic investigations of bupropion in patients and to *in vitro* metabolism of the primary bupropion metabolites to their glucuronides.

Keywords

Bupropion; Urine; LC–MS/MS; Analytical method; Stereoselectivity

1. Introduction

Bupropion is commonly prescribed to treat major depressive disorder and for smoking cessation. Clinically, bupropion is dosed as a racemic mixture, (*R,S*)-bupropion, and is extensively metabolized. Primary metabolites identified in humans are hydroxybupropion, erythrohydrobupropion, and threohydrobupropion (Fig. 1). Each of these metabolites exist as enantiomeric or diastereomeric pairs because metabolism creates a new chiral center on the parent molecule. (*R,S*)-bupropion undergoes stereoselective oxidation by cytochrome P450 2B6 (CYP2B6) to form (*R,R*)- and (*S,S*)-hydroxybupropion [1]. Additionally, bupropion is reduced by carbonyl reductase(s) to form pairs of erythrohydrobupropion and threohydrobupropion enantiomers [2]. The formation of at least one threohydrobupropion enantiomer is dependent on 11 β -hydroxysteroid dehydrogenase 1 reduction of (*R*)-bupropion *in vitro* [3], however, the enzyme that forms erythrohydrobupropion enantiomers has yet to be elucidated. Additionally, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion form corresponding β -D-glucuronides, catalyzed by uridine 5'-diphospho-glucuronosyl transferases (UGT) [4–6].

Numerous non-stereoselective assays have been developed to measure bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion simultaneously in urine, using LC-UV and LC–MS/MS [4,5,7]. Most recently, a method using an achiral HPLC column was developed to analyze β -D-glucuronide metabolites of hydroxybupropion, erythrohydrobupropion, and threohydrobupropion β -D-glucuronides from patient urine [6]. Only one stereoselective assay has been developed and validated which measures bupropion and hydroxybupropion enantiomers in human urine [8]. However, no method quantifies urine concentrations of erythrohydrobupropion enantiomers, threohydrobupropion enantiomers, and no comprehensive stereoselective assay for bupropion and all the major bupropion metabolites in urine is available. We recently developed and validated a stereoselective LC–MS/MS assay for bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion in human plasma [9].

The purpose of this investigation was to develop and validate a comprehensive LC–MS/MS assay for the stereoselective analysis of bupropion (*R* and *S*), the primary metabolites hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*) and

threohydrobupropion (1*R*,2*R* and 1*S*,2*S*), and the glucuronide metabolites erythrohydrobupropion β -D-glucuronides (1*R*,2*S* and 1*S*,2*R*), and threohydrobupropion β -D-glucuronides (1*R*,2*R* and 1*S*,2*S*) in human urine. The assay was applied to the quantification of bupropion and metabolites in urine from a patient receiving extended release bupropion, and from *in vitro* metabolism of the primary bupropion metabolites to their glucuronides.

2. Materials and methods

2.1. Materials

Standard methanolic solutions of *rac*-bupropion HCl, *rac*-hydroxybupropion, *rac*-bupropion- d_9 , *rac*-hydroxybupropion- d_6 were from Cerilliant Corporation (Round Rock, TX). The following materials were purchased from Toronto Research Chemicals (TRC, Toronto, ON, Canada): *rac*-threohydrobupropion and *rac*-erythrohydrobupropion maleate 1:1 mixture, *rac*-erythrohydrobupropion- d_9 , and *rac*-threohydrobupropion- d_9 . The TRC compound (Lot # 4-TKA-107-1) labeled *rac*-erythro-dihydro bupropion β -D-glucuronide,¹ purportedly *rac*-erythrohydrobupropion β -D-glucuronide, was later experimentally determined to be (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide single diastereomer (see Section 3.1). The TRC compound (Lot # 8-MWC-118-1) labeled (*S*,*S*)-dihydro bupropion β -D-glucuronide,² purportedly (1*S*,2*S*)-threohydrobupropion β -D-glucuronide, was later experimentally determined to be (1*R*,2*R*)-threohydrobupropion β -D-glucuronide (see Section 3.1). The TRC compound (Lot 8-MWC-117-1) labeled (*R*,*R*)-dihydro bupropion β -D-glucuronide,³ purportedly (1*R*,2*R*)-threohydrobupropion β -D-glucuronide, was later experimentally determined to be (1*S*,2*S*)-threohydrobupropion β -D-glucuronide (see Section 3.1). The TRC compound labeled *rac*-erythro-dihydro β -D-glucuronide bupropion- d_9 ,⁴ purportedly erythrohydrobupropion β -D-glucuronide- d_9 , was later experimentally determined to be (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide- d_9 single diastereomer (see Section 3.1).

Blank urine was obtained from healthy volunteers. HPLC-grade methanol and dichloromethane (CH₂Cl₂), ammonium formate, ϕ -glucuronidase from *Helix pomatia*, activated manganese dioxide (MnO₂), formic acid, trizma base, trizma hydrochloride, magnesium chloride (MgCl₂), and uridine 5'-diphosphoglucuronic acid (UDPGA) were from Sigma Aldrich (St. Louis, MO). HPLC-grade acetonitrile and ammonium acetate were from Fisher Scientific (Pittsburgh, PA). Ultra-pooled human liver microsomes (HLM, pool of 200, mixed sex), human kidney microsomes (HKM, pool of 13, mixed sex), and human intestinal microsomes (HIM, mixed sex, pool of 10) were purchased from Xenotech (Kansas

¹http://www.trc-canada.com/product-detail/?CatNum=D448655&CAS=&Chemical_Name=rac%20erythro-Dihydro%20Bupropion%20CE%20B2-D-Glucuronide%0B%0BDiscontinued&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%82%E2%82%88CINO%E2%82%87 Last accessed March 22, 2016.

²[http://www.trc-canada.com/product-detail/?CatNum=D448175&CAS=&Chemical_Name=\(S,S\)-Dihydro%20Bupropion%20CE%20B2-D-Glucuronide&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%82%E2%82%88CINO%E2%82%87](http://www.trc-canada.com/product-detail/?CatNum=D448175&CAS=&Chemical_Name=(S,S)-Dihydro%20Bupropion%20CE%20B2-D-Glucuronide&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%82%E2%82%88CINO%E2%82%87) Last accessed March 22, 2016.

³[http://www.trc-canada.com/product-detail/?CatNum=D448165&CAS=&Chemical_Name=\(R,R\)-Dihydro%20Bupropion%20CE%20B2-D-Glucuronide&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%82%E2%82%88CINO%E2%82%87](http://www.trc-canada.com/product-detail/?CatNum=D448165&CAS=&Chemical_Name=(R,R)-Dihydro%20Bupropion%20CE%20B2-D-Glucuronide&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%82%E2%82%88CINO%E2%82%87) Last accessed March 22, 2016.

⁴http://www.trc-canada.com/product-detail/?CatNum=D448657&CAS=&Chemical_Name=rac%20Erythro%20Dihydrobupropion%20CE%20B2-D-Glucuronide-d9&Mol_Formula=C%E2%82%81%E2%82%89H%E2%82%81%E2%82%89D%E2%82%89CINO%E2%82%87 Last accessed March 22, 2016.

City, KS). All solutions were prepared in ultrapure water 18.2 M Ω cm from a Milli-Q Gradient A10 Filtration System (EMD Millipore, Billerica, MA).

2.2. Calibrator, quality control (QC), and internal standard (IS) solutions

For reference or internal standards obtained in salt form, all reported concentrations reflect the concentration of free base and individual enantiomer. Individual working stock solutions of bupropion (*R* and *S*) and hydroxybupropion (*R,R* and *S,S*) were prepared by diluting certified authentic solutions in water. Three working stock concentrations of bupropion (*R* and *S*) were prepared at 500, 5000, and 50,000 ng/mL and two working stock concentrations of hydroxybupropion (*R,R* and *S,S*) were prepared at 5000 and 50,000 ng/mL and immediately stored at -80°C . The *rac*-erythrohydrobupropion and *rac*-threo hydrobupropion (1:1) powder was accurately weighed and prepared as a 1.0 mg/mL stock solution containing 500 $\mu\text{g/mL}$ *rac*-erythrohydrobupropion and 500 $\mu\text{g/mL}$ *rac*-threo hydrobupropion in DMSO. Two working stock solutions were prepared in water at 5000 ng/mL and 50,000 ng/mL erythrohydrobupropion (1*R*,2*S*) and (1*S*,2*R*) and threo hydrobupropion (1*S*,2*S*) and (1*R*,2*R*). Working stock concentrations of (1) purchased *rac*-erythro-dihydro bupropion β -D-glucuronide (purportedly *rac*-erythrohydrobupropion β -D-glucuronide but in actuality (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide), (2) Purchased (*R,R*)-dihydro bupropion β -D-glucuronide (purportedly (1*R*,2*R*)-threo hydrobupropion β -D-glucuronide but in actuality (1*S*,2*S*) threo hydrobupropion β -D-glucuronide), and 3) Purchased (*S,S*)-threo hydrobupropion β -D-glucuronide (purportedly (1*S*,2*S*) threo hydrobupropion β -D-glucuronide but in actuality (1*R*,2*R*) threo hydrobupropion β -D-glucuronide) were individually prepared at 5000 and 50,000 ng/mL. Deuterated internal standards erythrohydrobupropion- d_9 (1*R*,2*S*) and (1*S*,2*R*), and threo hydrobupropion- d_9 (1*S*,2*S*) and (1*R*,2*R*), and *rac*-erythrohydrobupropion β -D-glucuronide- d_9 (purchased as *rac*-erythro dihydro bupropion- d_9 β -D-glucuronide, but in actuality (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide- d_9) were individually prepared as 250 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, and 500 $\mu\text{g/mL}$ DMSO stock solutions, respectively, and stored at -80°C . A working stock of 535 ng/mL bupropion- d_9 (*R* and *S*), 3344 ng/mL hydroxybupropion- d_6 (*R,R* and *S,S*), 1338 ng/mL erythrohydrobupropion- d_9 (1*R*,2*S*) and (1*S*,2*R*), and 1338 ng/mL threo hydrobupropion- d_9 (1*S*,2*S*) and (1*R*,2*R*), 669 ng/mL, and *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion- d_9 β -D-glucuronide, but in actuality (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide- d_9) was divided into 1 mL aliquots which were stored at -80°C . On the day of an experiment, a fresh aliquot of the internal standards solution was removed from the freezer and utilized for 1 plate of samples.

All calibrators and QC samples at the enantiomeric or diastereomeric concentrations described in Table 1 were prepared in individual 1.5 mL tubes. Each calibration or QC sample was subsequently aliquoted (0.04 mL) into 0.65 mL tubes and stored at -80°C until analysis.

2.3. Sample preparation

Calibration standards, QCs, and patient samples were thawed at room temperature, vortexed, and transferred (25 μL) to a 1.0 mL DeepWell 96-well plate (Nunc, Thermofisher, Pittsburgh, PA). Internal standard aqueous solution (10 μL) containing 535 ng/mL

bupropion-d₉ (*R* and *S*), 3344 ng/mL hydroxybupropion-d₆ (*R,R* and *S,S*), 1338 ng/mL erythrohydrobupropion-d₉ (1*R*,2*S*) and (1*S*,2*R*), 1338 ng/mL threo hydrobupropion-d₉ (1*S*,2*S*) and (1*R*,2*R*), and 669 ng/mL of *rac*-erythrohydrobupropion β-D-glucuronide-d₉ (purchased as *rac*-erythro-dihydro bupropion-d₉ β-D-glucuronide, but in actuality (1*R*,2*S*)-erythrohydrobupropion β-D-glucuronide-d₉) was added to each well and the plate was shaken for 1 min. Next, 500 μL of 100 mM ammonium acetate (pH 5.0) either with or without 5000 units/mL β-glucuronidase was added and the plate shaken for 2 min. For samples that did not contain β-glucuronidase, the plate was centrifuged at 6100g at 4 °C for 15 min, and the supernatant (100 μL) was transferred to a MicroWell 96-well plate (Nunc, Thermofisher, Pittsburgh, PA). For samples that contained β-glucuronidase, the plate was incubated overnight at 37 °C. Hydrolyzed samples were then centrifuged at 6100g at 4 °C for 15 min, and the supernatant (100 μL) was transferred to a MicroWell 96-well plate.

2.4. Instrumentation

AB Sciex 3200: HPLC-ESI-MS/MS was conducted on a Shimadzu (Columbia, MD) HPLC system composed of two LC-20AD pumps with a CTO-20A column oven, DGU-20A3 degasser, FCV-11AL solvent selection valve, and CBM 20A controller connected to a MPS 3C temperature regulated autosampler equipped with an active wash station (Gerstel, Linthicum, MD) all interfaced with an API Sciex 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Analyst 1.4.2 software (AB Sciex, Foster City, CA) was utilized for instrument control, data acquisition, and analyte mass spectrometric parameter optimization.

AB Sciex 4000: LC/MS-MS analysis was performed on a Shimadzu HPLC system composed of two LC-20AD XR pumps, DGU20A3 degasser, CTO-20A column oven, FCV-11AL solvent selection valve, SIL-20AC XR temperature regulated autosampler, and an external Valco divert valve installed between the LC and mass spectrometer. The LC system was coupled to an API 4000 linear ion trap triple quadrupole (QTRAP) tandem mass spectrometer operated with Analyst 1.5.2.

Multiquant 3.0.1 (AB Sciex) was utilized for peak integration, generation of calibration curves, and data analysis.

2.5. Chromatographic conditions

Chiral chromatographic separation of bupropion, hydroxybupropion, erythrohydrobupropion, threo hydrobupropion, and respective internal standards was achieved utilizing a chiralpak α₁-acid glycoprotein (AGP) column as previously described [1,9]. Chromatographic separation of the β-D-glucuronide diastereomers was achieved with a Sunfire C18 (150 × 2.1 mm, 3.5 μM, Waters, Milford, MA) analytical column equipped with a C18 VanGuard cartridge (2.1 mm × 5 mm, 3.5 μM, Waters, Milford, MA). A 0.25 μM inline filter was additionally added prior to the sample entering the column. The flow rate was 0.22 mL/min with a mobile phase consisting of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B). The time program to achieve the separation was 18% B for 1.0 min, linear gradient to 40% B until 6 min, and equilibration at 10% B until 8 min. The column oven was at ambient temperature and the autosampler was at 4 °C. Flow was

directed into the mass spectrometer at 0.5 min and diverted to waste at 7.5 min. Under these conditions, the order of elution for the glucuronide standards was TRC-labeled (*R,R*)-dihydro bupropion β -D-glucuronide (purportedly (*1R,2R*) threohydrobupropion β -D-glucuronide), TRC-labeled (*S,S*)-dihydro bupropion β -D-glucuronide (purportedly (*1S,2S*) threohydrobupropion β -D-glucuronide), and TRC-labeled *rac*-erythro-dihydro bupropion β -D-glucuronide (purportedly *rac*-erythrohydrobupropion β -D-glucuronide). However, these were later identified as (*1S,2S*) threohydrobupropion β -D-glucuronide, (*1R,2R*) threohydrobupropion β -D-glucuronide, and (*1R,2S*)-erythrohydrobupropion β -D-glucuronide, eluting in the same order.

2.6. Mass spectrometry conditions

Operating conditions for analysis on an AB Sciex 3200 mass spectrometer were as previously described [9]. All glucuronide analytes were optimized and analyzed on an AB Sciex 4000 mass spectrometer. Global parameters were optimized across all analytes: curtain gas 20 psig, ion spray voltage 5500 V, source temperature 450 °C, Gas 1 30 psig, and Gas 2 40 psig. Dwell times were 150 ms. Optimized analyte- and internal standard-specific MRM transitions, declustering potential, entrance potential, collision energy, and exit potential are described in Table 2 for glucuronide metabolites.

2.7. Hydrolysis of commercial glucuronide standards by β -glucuronidase

A 1 μ g/mL solution of 1) (*1R,2R*) threohydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide, 2) (*1S,2S*) threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide, and 3) *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide) were individually prepared in blank human urine. An aliquot (25 μ L) was added to 500 μ L of 100 mM ammonium acetate pH 5 and hydrolyzed overnight with 5000 units/mL β -glucuronidase at 37 °C. Subsequently, the samples were spun at 6100g and the supernatant was analyzed by chiral LC–MS/MS.

2.8. Qualitative synthesis of (*R*)- and (*S*)-bupropion from hydrolyzed glucuronide standards

Individual hydrolysates of: 1) (*1R,2R*) threohydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide, 2) (*1S,2S*) threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide), and 3) *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide) were prepared as described in Section 2.7. Following centrifugation at 6100g, the supernatants were transferred to 1.5 mL tubes and evaporated to dryness with a Savant DNA 120 speed vacuum (Thermo Scientific, Waltham, MA). The residues were reconstituted in dichloromethane (500 μ L) and 100 mg of activated manganese dioxide was added. The mixture was placed on a titer plate shaker (Thermo Scientific, Waltham, MA) for 1 h and subsequently spun at 15,000g for 5 min. The supernatant was evaporated to dryness and the residue was reconstituted in 20 mM ammonium acetate, pH 5 mobile phase buffer. The supernatants were analyzed by chiral LC–MS/MS.

2.9. Erythrohydrobupropion or threohydrobupropion glucuronidation in human microsomal preparations

Microsomal incubations contained human liver, kidney, or intestine microsomal protein (1.0 mg microsomal protein/mL), 100 mM Tris, pH 7.5 at 37 °C, 1 mM *rac*-erythrohydrobupropion or *rac*-threohydrobupropion, (1*S*,2*R*)-erythrohydrobupropion and (1*R*,2*R*)-threohydrobupropion 80:20 mixture or (1*R*, 2*S*)-erythrohydrobupropion and (1*S*, 2*S*)-threohydrobupropion 80:20 mixture, MgCl₂ (5 mM), alamethicin (10 µg/mL), and UDPGA (5 mM). The 80:20 mixture of (1*S*,2*R*)-erythrohydrobupropion and (1*R*,2*R*)-threohydrobupropion was produced from the sodium borohydride reduction of (*R*)-bupropion [9]. Similarly, the 80:20 mixture of (1*R*,2*S*)-erythrohydrobupropion and (1*S*,2*S*)-threohydrobupropion 80:20 mixture was produced from the sodium borohydride reduction of (*S*)-bupropion. All incubations contained 1% DMSO. Incubation samples were stored on ice for 15 min, pre-incubated in a 37 °C water bath for 2 min followed by the addition of UDPGA (5 mM final concentration). Samples were incubated for 60 min and 20 µL 15% ZnSO₄ (aq.) was added to quench the reactions. The samples were spun at 15,000g, and 20 µL of the supernatant was injected onto the LC–MS/MS for analysis.

2.10. Method validation

Validation experiments and run acceptance criteria were based on the United States Food and Drug Administration Bioanalytical Method Validation Guidance [10].

2.10.1. Accuracy and precision—The accuracy and precision for each analyte in pooled blank human urine were determined at the QC concentrations. Intra-assay and inter-assay accuracy and precision were calculated at five samples per concentration. Accuracy is expressed as a percentage of the nominal concentration and precision is expressed as percent coefficient of variation (%CV). Acceptance criteria was defined as variation and deviation 15% for all samples.

2.10.2. Selectivity—Six individual lots of human urine were prepared as described in Section 2.3 without internal standard and were confirmed to be negative for all analytes and their respective internal standards. Four of the individual lots of blank human urine were pooled to generate the matrix for preparing calibration and quality control samples. At the lower limit of quantification, there was no significant matrix interference for each analyte.

2.10.3. Ion suppression—To investigate any loss of signal due to urine matrix effect on ion suppression, a solution of all analytes and internal standards 100 ng/mL bupropion (*R* and *S*), 100 ng/mL bupropion-d₉ (*R* and *S*), 1500 ng/mL hydroxybupropion (*R,R* and *S,S*), 1500 ng/mL hydroxybupropion-d₆ (*R,R* and *S,S*), 500 ng/mL erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*), 500 ng/mL erythrohydrobupropion-d₉ (1*R*,2*S* and 1*S*,2*R*), 500 ng/mL threohydrobupropion (1*S*,2*S* and 1*R*,2*R*), and 500 ng/mL threohydrobupropion-d₉ (1*S*,2*S* and 1*R*,2*R*) were infused into the AB Sciex 3200 mass spectrometer while simultaneously injecting a processed blank human urine sample. Additionally, a solution of: 1) (1*R*,2*R*)-threohydrobupropion β-D-glucuronide (purchased as (*R,R*)-dihydro bupropion β-D-glucuronide, but in actuality (1*S*,2*S*)-threohydrobupropion β-D-glucuronide), 2) (1*S*,2*S*)-threohydrobupropion β-D-glucuronide (purchased as (*S,S*)-dihydro bupropion β-D-

glucuronide, but in actuality (1*R*,2*R*) threohydrobupropion β -D-glucuronide), 3) *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide, but in actuality (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide), and 4) *rac*-erythrohydrobupropion β -D-glucuronide-*d*₉ (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide-*d*₉, but in actuality (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide-*d*₉) (each 500 ng/mL) were infused into the AB Sciex 4000 mass spectrometer while simultaneously injecting a processed human urine sample.

2.10.4. Stability—Stability experiments utilizing low and high QC samples for each analyte were performed after 3 subsequent freeze/thaw cycles at -80°C , processed as described in Section 2.3, and analyzed by LC–MS/MS.

2.11. Clinical samples

The validated bioanalytical assay was applied to a clinical study approved by the Washington University in St Louis Institutional Review Board. Urine was obtained continuously 0–24 h after dosing 300 mg extended release bupropion at steady-state. Urine was stored at -80°C until analysis.

3. Results

3.1. Determination of erythrohydrobupropion and threohydrobupropion β -D-glucuronide stereochemistry

Initial experiments were performed to determine whether erythrohydrobupropion and threohydrobupropion β -D-glucuronide should be analyzed intact, or hydrolyzed and analyzed as their respective aglycones. Experiments therefore assessed the hydrolysis of these glucuronides. (1*R*,2*R*) threohydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide), (1*S*,2*S*) threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro β -D-glucuronide), and *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide) were incubated in the presence of β -glucuronidase for 24 or 48 h. The samples were processed according to the procedure outlined in Section 2.3 and analyzed by chiral LC–MS/MS. The resulting aglycone peaks were compared with the retention times of a calibration standard containing authentic *rac*-threohydrobupropion and *rac*-erythrohydrobupropion aglycone standards (Fig. 2). The elution order for authentic threohydrobupropion and erythrohydrobupropion aglycones was previously confirmed to be (1*S*,2*S*)-threohydrobupropion, (1*R*,2*R*)-threohydrobupropion, (1*R*,2*S*)-erythrohydrobupropion, and (1*S*,2*R*)-erythrohydrobupropion (Fig. 2A) [9].

Unexpectedly, the aglycone peak resulting from the hydrolysis of (1*R*,2*R*) threohydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion glucuronide) corresponded to the (1*S*,2*S*)-threohydrobupropion peak (Fig. 2B). The aglycone peak resulting from the hydrolysis of (1*S*,2*S*) threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion glucuronide) unexpectedly corresponded to the (1*R*,2*R*)-threohydrobupropion peak (Fig. 2C). The aglycone peak resulting from the hydrolysis of *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -

D-glucuronide) unexpectedly corresponded to the (1*R*,2*S*)-erythrohydrobupropion peak (Fig. 2D). These results suggested that the purchased standards were incorrectly labeled.

Further experiments were conducted to confirm the structural assignments of these standards. Activated manganese dioxide was added to the hydrolysates from the overnight incubations of *erythro*- and threo hydrobupropion glucuronides with β -glucuronidase to oxidize the resulting aglycones back to either (*R*)- or (*S*)-bupropion. A LC-MS/MS chromatogram illustrating the separation of (*R*)- and (*S*)-bupropion is shown in Fig. 2E. Oxidation of the aglycone produced from (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide) hydrolysis generated (*S*)-bupropion (Fig. 2F), suggesting that the correct stereochemistry of the original glucuronide was (1*S*,2*S*). Oxidation of the aglycone produced by (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide) hydrolysis generated (*R*)-bupropion (Fig. 2G), suggesting that the correct stereochemistry of the original glucuronide was (1*R*,2*R*). Oxidation of the aglycone produced from the *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro bupropion β -D-glucuronide) hydrolysis generated (*S*)-bupropion (Fig. 2H), suggesting that the correct stereochemistry of the original glucuronide was (1*R*,2*S*). Each of the bupropion enantiomers generated had the opposite stereochemistry at C2 than the originally labeled glucuronide standards, which provides further evidence that the purchased standards were incorrectly labeled.

Due to the above surprising results of the glucuronide hydrolysis experiments, additional experiments were performed using glucuronide biosynthesis, to further elucidate the stereochemistry of the glucuronide standards (Fig. 3). The commercial standards eluted in the following order: (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide), (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide), and *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro β -D-glucuronide) (Fig. 3A). The *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro β -D-glucuronide), however, only showed one peak, suggesting the product as only one diastereomer. This was subsequently confirmed in a communication with the manufacturer (Dr. Phillip Chan, Personal Communication, December 21, 2015). Sodium borohydride reduction of (*S*)-bupropion yields a 80:20 mixture of (1*R*,2*S*)-erythrohydrobupropion and (1*S*,2*S*)-threo hydrobupropion, respectively [9,11]. A human liver microsomal incubation with the 80:20 mixture of (1*R*,2*S*)-erythrohydrobupropion and (1*S*,2*S*)-threo hydrobupropion supplemented with UDPGA produced both (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide and (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide (Fig. 3B). However, the (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide purchased as (*S,S*)-dihydro bupropion β -D-glucuronide standard (Fig. 3A) did not align with the biosynthesized (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide diastereomer (Fig. 3B). Rather, the (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide) standard (Fig. 3A) in fact aligned with the biosynthesized (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide diastereomer (Fig. 3B). Additionally, the *rac*-erythrohydrobupropion β -D-glucuronide purchased as *rac*-erythro-dihydro β -D-glucuronide (Fig. 3A) aligned with biosynthesized (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide (Fig. 3B). Sodium borohydride reduction of (*R*)-bupropion yields a 80:20 mixture of (1*S*,2*R*)-

erythrohydrobupropion and (1*R*,2*R*)-threo hydrobupropion, respectively [9,11]. A human liver microsomal incubation with a 80:20 mixture of (1*S*,2*R*)-erythrohydrobupropion and (1*R*,2*R*)-threo hydrobupropion supplemented with UDPGA only produces (1*S*,2*R*)-erythrohydrobupropion β -D-glucuronide (Fig. 3C).

Thus, based on the β -D-glucuronide hydrolysis and chemical oxidation of the hydrolysates (Fig. 2), and the inaccurate alignment of biosynthesized (1*S*,2*S*) threo hydrobupropion β -D-glucuronide with the authentic standard (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (purchased as (*S*,*S*)-dihydro bupropion β -D-glucuronide) (Fig. 3), the stereochemistry of the (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (purchased as (*R*,*R*)-dihydro bupropion β -D-glucuronide) and (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (purchased as (*S*,*S*)-dihydro bupropion β -D-glucuronide) standards was reassigned. Additionally, (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide corresponds to *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro β -D-glucuronide). Hence, (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (purchased as (*R*,*R*)-dihydro bupropion β -D-glucuronide) is really (1*S*,2*S*) threo hydrobupropion β -D-glucuronide. (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (purchased as (*S*,*S*)-dihydro bupropion β -D-glucuronide) is really (1*R*,2*R*) threo hydrobupropion β -D-glucuronide. *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro β -D-glucuronide) is really (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide. Throughout the remainder of this manuscript, correct structural nomenclature is used.

An LC-MS/MS chromatogram illustrating β -D-glucuronides in patient urine is shown in Fig. 3D. The elution order and correct structural reassignment for the β -D-glucuronides are: (1*S*,2*S*) threo hydrobupropion β -D-glucuronide, (1*S*,2*R*)-erythrohydrobupropion β -D-glucuronide, (1*R*,2*R*) threo hydrobupropion β -D-glucuronide, and (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide.

3.2. Evaluation of erythrohydrobupropion and threo hydrobupropion β -D-glucuronide hydrolysis

Following the reassignment of standards' stereochemistry, time dependent hydrolysis of (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide, (1*S*,2*R*)-erythrohydrobupropion (1*R*,2*R*)-threo hydrobupropion β -D-glucuronide, (1*R*,2*S*)-erythrohydrobupropion was performed to determine whether erythrohydrobupropion and threo hydrobupropion glucuronides should be analyzed as intact or hydrolyzed and analyzed as their respective aglycones. Patient urine was hydrolyzed by β -glucuronidase for 4, 8, and 24 h at 37 °C. After 24 h, the percent glucuronide remaining for (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide, (1*S*,2*R*)-erythrohydrobupropion β -D-glucuronide, (1*R*,2*R*)-threo hydrobupropion β -D-glucuronide, and (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide were 0, 1, 73, and 75%, respectively (Table 3). Thus, erythrohydrobupropion and threo hydrobupropion glucuronides undergo diastereomer-specific β -glucuronidase hydrolysis. Due to the differences in hydrolysis between the glucuronide diastereomers, and the inability to completely hydrolyze the glucuronides, erythrohydrobupropion and threo hydrobupropion glucuronide metabolites in urine were analyzed as their intact conjugates. Because conjugated and unconjugated erythrohydrobupropion and threo hydrobupropion metabolites could not all be analyzed as

free aglycones, it then became necessary to measure erythrohydrobupropion and threo hydrobupropion glucuronides, as well as their free aglycones.

3.3. Assay development

A trifurcated urine sample preparation and analysis procedure was employed for the chiral analysis of bupropion, hydroxybupropion, erythrohydrobupropion, and threo hydrobupropion and the achiral analysis of erythrohydrobupropion and threo hydrobupropion β -D-glucuronides from a urine sample (Fig. 4). The trifurcated sample preparation procedure was utilized for the following reasons: (1) there are no commercially available hydroxybupropion β -D-glucuronide standards, (2) hydroxybupropion β -D-glucuronides in urine require hydrolysis prior to generate hydroxybupropion aglycones for accurate quantification of hydroxybupropion enantiomeric aglycone concentrations, and (3) initially attempted separation of erythrohydrobupropion and threo hydrobupropion β -D-glucuronide diastereomers with the α_1 acid glycoprotein chiral column was not achievable, and therefore these needed to be separated and analyzed by a different method

Method 1 (Fig. 4) stereoselectively analyzed free bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (*1R,2S* and *1S,2R*), and threo hydrobupropion (*1R,2R* and *1S,2S*) using chiral chromatography, as based on a previous method [8,9]. A 25 μ L urine aliquot was diluted with 100 mM ammonium acetate pH 5, centrifuged at 6100g, injected onto a α_1 -acid glycoprotein (AGP) column, and analyzed by MS/MS. Stereoselective chromatographic separation of bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (*1R,2S*) and (*1S,2R*) and threo hydrobupropion (*1R,2R*) and (*1S,2S*) was achieved (Fig. 5A, D, G).

Method 2 (Fig. 4) stereoselectively analyzed total hydroxybupropion aglycones (*R,R* and *S,S*-hydroxybupropion) – both free aglycones and those generated from urine hydrolysis by β glucuronidase. A 25 μ L urine aliquot was diluted with 100 mM ammonium acetate pH 5 containing and incubated overnight with 5000 units/mL of β -glucuronidase. The sample was vortexed, centrifuged at 6100g, and the supernatant was injected onto a α_1 -acid glycoprotein (AGP) column for MS/MS analysis of hydroxybupropion enantiomer aglycones (*R,R* and *S,S*)-hydroxybupropion. Hydroxybupropion β -D-glucuronide (*R,R* and *S,S*) urine concentrations were calculated as the difference between total and free hydroxybupropion (*R,R* and *S,S*) concentrations.

Method 3 (Fig. 4) stereoselectively analyzed erythrohydrobupropion and threo hydrobupropion β -D-glucuronide glucuronide diastereomers using C18 column chromatography. A 25 μ L urine aliquot was diluted with 100 mM ammonium acetate pH 5, centrifuged at 6100g, and injected onto a Sunfire C18 column for MS/MS analysis of: (*1S,2S*)-threo hydrobupropion β -D-glucuronide, (*1S,2R*)-erythrohydrobupropion β -D-glucuronide, (*1R,2R*)-threo hydrobupropion β -D-glucuronide, (*1R,2S*)-erythrohydrobupropion β -D-glucuronide. Erythrohydrobupropion and threo hydrobupropion β -D-glucuronide diastereomers were well resolved (Fig. 5J). There was no in source hydrolysis of the glucuronides, as determined by monitoring the aglycone transitions during the analytical run. Daughter ion spectra for (*1S,2S*)-threo hydrobupropion β -D-glucuronide, (*1S,2R*)-erythrohydrobupropion β -D-glucuronide, (*1R,2R*)-threo hydrobupropion β -D-glucuronide,

(1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide from a patient sample are shown in Fig. 6. With the collision energy was set to 25 electron volts, each glucuronide diastereomer had similar MS/MS spectra and the daughter ions conform to previously published values [4].

3.4. Assay validation

Analytical method validations for methods 1 and 2 were conducted utilizing an AB Sciex 3200 mass spectrometer. Free (method 1) and total (method 2) hydroxybupropion determinations had two different sample preparation procedures and are measured separately, and thus required two separate validations. Method 3 was validated utilizing an AB Sciex 4000 mass spectrometer. Calibration curves were analyzed by least squares regression analysis, with choice of model (linear or quadratic) and weighting ($1/x$ or $1/x^2$) optimized for each analyte and instrument. Analyte specific calibrator and QC concentrations were based on a preliminary analysis of urine samples from a clinical investigation in which patients received 300 mg extended release bupropion once daily, and from previously published clinical studies [5,8].

The final assay met all validation acceptance criteria according to the FDA Guidance for Bioanalytical Method Validation [10]. Accuracy and precision data for each analyte and the QC concentrations are shown in Tables 4–6. For each analyte at each respective concentration, the accuracy and precision was within 15%, including the limit of quantification. Fig. 5 shows the extracted ion chromatograms from mid-level urine calibration sample, a calibration sample at the limit of quantification, and blank urine devoid of analytes or internal standards for each analyte, respectively. Extracted ion chromatograms from ion suppression experiments are shown in Fig. 7. In each of the chromatograms, ion suppression from urine matrix components only occurred from 1 to 3 min and did not interfere with the respective analyte signals. Analyte stability following three subsequent freeze thaw cycles at $-80\text{ }^{\circ}\text{C}$ was evaluated. Freeze thaw stability experiments resulted in no analyte degradation over the course of three subsequent freeze thaw cycles (Table 7).

3.5. Assay application

Glucuronide metabolites from human liver, kidney, and intestinal microsomal incubations with either *rac*-erythrohydrobupropion or *rac*-threohydrobupropion supplemented with UDPGA were analyzed (Fig. 8). Incubation with *rac*-erythrohydrobupropion in human liver, kidney, and intestinal microsomes (Fig. 8A–C), show the formation of both (1*R*,2*S*) and (1*S*,2*R*) erythrohydrobupropion β -D-glucuronides. The formation of erythrohydrobupropion β -D-glucuronide in intestinal microsomes is less than in human liver or kidney microsomes. Incubations of *rac*-threohydrobupropion with liver, kidney, and intestinal microsomal produced (1*S*,2*S*)-threohydrobupropion β -D-glucuronide, with only minor formation of (1*R*,2*R*)-threohydrobupropion β -D-glucuronide (Fig. 8D–F). Threohydrobupropion β -D-glucuronides amounts formed by intestinal microsomes were lower than by liver and kidney microsomes.

The validated assay was successfully utilized to quantify urine concentrations of bupropion (*R* and *S*), total and free hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*), threohydrobupropion (1*R*,2*R* and 1*S*,2*S*), hydroxybupropion β -D-glucuronide

(*R,R* and *S,S*) erythrohydrobupropion β -D-glucuronide (1*R,2S* and 1*S,2R*), and threohydrobupropion β -D-glucuronide (1*R,2R* and 1*S,2S*) from a patient who received 300 mg oral extended release bupropion once daily (Fig. 9). Concentrations were within the calibration ranges. Hydroxybupropion, erythrohydrobupropion, and threohydrobupropion were excreted both as glucuronide conjugates and unconjugated aglycones. There were 4- to 6-fold differences in concentration for both erythrohydrobupropion enantiomers and threohydrobupropion enantiomers, respectively.

4. Discussion

4.1. Assay development and validation

This is the first stereoselective, comprehensive bioanalytical assay to quantify enantiomers of bupropion, the primary metabolites hydroxybupropion, erythrohydrobupropion, and threohydrobupropion, and the metabolite diastereomers hydroxybupropion β -D-glucuronides, erythrohydrobupropion β -D-glucuronides, and threohydrobupropion β -D-glucuronides. To accurately quantify bupropion and oxidative, reductive, and glucuronide metabolites from human urine, a trifurcated sample preparation procedure and analysis was developed. In method 1, urine was analyzed for bupropion (*R* and *S*), free hydroxybupropion (*R,R* and *S,S*), threohydrobupropion (1*R,2R* and 1*S,2S*), and erythrohydrobupropion (1*R,2S* and 1*S,2S*) by simply diluting the urine, centrifuging, and analyzing the supernatant. Since there are no commercially available hydroxybupropion glucuronide standards, total hydroxybupropion (*R,R* and *S,S*) aglycones were analyzed by method 2 following an overnight incubation with β -glucuronidase. Hydroxybupropion β -D-glucuronide (*R,R* and *S,S*) urine concentrations were calculated as the difference between free hydroxybupropion (*R,R* and *S,S*) concentrations measured by method 1 and total hydroxybupropion (*R,R* and *S,S*) concentrations measured by method 2. Erythrohydrobupropion and threohydrobupropion glucuronides undergo only partial, and diastereomer specific, β -glucuronidase hydrolysis. (1*S,2S*)-threohydrobupropion β -D-glucuronide and (1*S,2R*)-erythrohydrobupropion are susceptible to hydrolysis while (1*R,2R*)-threohydrobupropion and (1*R,2S*)-erythrohydrobupropion are comparatively resistant. It is interesting to note that glucuronides with the *S* configuration at C1 are more prone to hydrolysis than glucuronides with *R* configuration at C1. Initially, erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers were analyzed by method 1; however, method 1 was unsuitable for complete resolution of each glucuronide diastereomer. As a result, method 3 was developed for analysis of erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers. Each method was validated in compliance with the FDA bioanalytical guidelines.

To date there is one published report for the stereoselective analysis of bupropion and hydroxybupropion in human urine [8], and one published report for the direct quantitative analysis of glucuronide metabolites in human urine [6]. Concentrations of hydroxybupropion, erythrohydrobupropion, and threohydrobupropion β -D-glucuronides in human urine have also been quantified [5]; albeit indirectly by subtracting the aglycones measured in non-treated urine from the total aglycones measured in β -glucuronidase treated urine. Since erythrohydrobupropion β -D-glucuronide and threohydrobupropion β -D-

glucuronide undergo variable diastereomer-selective hydrolysis by β -glucuronidase, amounts of free erythrohydrobupropion and threohydrobupropion quantified after hydrolysis may warrant re-evaluation. The previous stereoselective assay for bupropion and hydroxybupropion had a total run time of 20 min and was utilized sample clean up by solid phase extraction (mixed-mode cation exchange) [8]. The current stereoselective assay analyzes bupropion (*R* and *S*) and hydroxybupropion (*R,R* and *S,S*), as well as threohydrobupropion (*1S,2S* and *1R,2R*) and erythrohydrobupropion (*1R,2S* and *1S,2R*), all within 12 min, and needs sample preparation of only dilution and centrifugation, and is therefore shorter, easier, and more comprehensive compared with the previous stereoselective assay. A previous chromatographic method analyzed glucuronide metabolites stereoselectively, and bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion non-stereoselectively in 20 min [6]. The current assay analyzes erythrohydrobupropion and threohydrobupropion β -D-glucuronide diastereomers stereoselectively within 8 min and additionally analyzes bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion stereoselectively. Due to the abundance of bupropion and metabolites detected in human urine, no efforts were made to optimize sensitivity in the present assay.

4.2. Clinical bupropion disposition

At steady-state bupropion (300 mg extended release), the most abundant urinary unconjugated metabolites were (*1R,2R*)-threohydrobupropion and (*1S,2S*)-threohydrobupropion, and the most abundant conjugated metabolites were the threohydrobupropion β -D-glucuronide (*1R,2R* and *1S,2S*) diastereomers, (*1S,2R*)-erythrohydrobupropion, and (*R,R*)-hydroxybupropion β -D-glucuronide. Overall, most abundant were total (*R,R*)- and (*S,S*)-threohydrobupropion, (*1S,2R*)-erythrohydrobupropion, and (*R,R*)-hydroxybupropion. In other published clinical studies, which non-stereoselectively measured hydroxybupropion, erythrohydrobupropion, and threohydrobupropion metabolites, the amounts of threohydrobupropion were greater than hydroxybupropion and erythrohydrobupropion [5,6].

4.3. Metabolite standards

Initial glucuronide hydrolysis experiments demonstrated the need to establish the correct stereochemistry of the glucuronide standards obtained from Toronto Research Chemicals. The hydrolysis experiments, chemical synthesis, and biosynthesis experiments (Figs. 2 and 3) unequivocally confirmed that: 1) (*1R,2R*) threohydrobupropion β -D-glucuronide (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide) is actually (*1S,2S*)-threohydrobupropion β -D-glucuronide. 2) (*1S,2S*) threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide) is actually (*1R,2R*)-threohydrobupropion β -D-glucuronide, and 3) *racerythro*hydrobupropion β -D-glucuronide (purchased as *rac-erythro*-dihydro β -D-glucuronide) is actually (*1R,2S*)-erythrohydrobupropion β -D-glucuronide. The glucuronide stereochemistry was reassigned for all subsequent quantitative analyzes.

Recently, the quantification of erythrohydrobupropion and threohydrobupropion β -D-glucuronides from human urine was reported utilizing TRC standards [6]. As a result of the

incorrect labeling of the purchased standards, the reported data regarding erythrohydrobupropion and threohydrobupropion β -D-glucuronide concentrations warrant re-evaluation and may need revision.

5. Conclusion

A comprehensive LC–MS/MS assay for the stereoselective analysis of bupropion (*R* and *S*), the primary metabolites hydroxybupropion (*R,R* and *S,S*), erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*) and threohydrobupropion (1*R*,2*R* and 1*S*,2*S*), and the secondary metabolites hydroxybupropion- β -D-glucuronides (*R,R* and *S,S*), erythrohydrobupropion β -D-glucuronides (1*R*,2*S* and 1*S*,2*R*), and threohydrobupropion β -D-glucuronide (1*R*,2*R* and 1*S*,2*S*) in human urine was developed and validated. The validated stereoselective assay enabled quantification of bupropion and oxidative, reductive, and glucuronide metabolites (16 analytes total) from human urine. Differences in concentration for both erythrohydrobupropion enantiomers and threohydrobupropion enantiomers substantiate the requirement of a stereoselective assay for these metabolites. This assay was applicable to clinical pharmacokinetic investigations of bupropion in patients and to *in vitro* metabolism of the primary bupropion metabolites to their corresponding glucuronides.

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Fig. 1.
Oxidative, reductive, and glucuronide metabolites of (*R*)-bupropion and (*xi*)-bupropion.

Fig. 2.

Correct structural identification of commercial bupropion metabolite glucuronides. $(1R,2R)$ -threohydrobupropion β -D-glucuronide (purchased as (R,R) -dihydro bupropion β -D-glucuronide), $(1S,2S)$ -threohydrobupropion β -D-glucuronide (purchased as (S,S) -dihydro bupropion β -D-glucuronide), and rac -erythrohydrobupropion β -D-glucuronide (purchased as rac -erythro-dihydro β -D-glucuronide) were hydrolyzed to aglycones by β -glucuronidase and were subsequently oxidized back to (R) - or (S) -bupropion. A 1 μ g/mL solution of $(1R,2R)$ -threohydrobupropion β -D-glucuronide (purchased as (R,R) -dihydro bupropion β -D-glucuronide), $(1S,2S)$ -threohydrobupropion β -D-glucuronide (purchased as (S,S) -dihydro bupropion β -D-glucuronide), and rac -erythrohydrobupropion β -D-glucuronide (purchased as rac -erythro-dihydro β -D-glucuronide) were individually prepared in blank human urine, hydrolyzed overnight with β -glucuronidase, and subsequently analyzed by chiral LC-MS/MS (panels B–D). The remaining supernatant was evaporated to dryness and the residue was reconstituted in dichloromethane followed by the addition of manganese dioxide (MnO_2). The mixture was shaken for 1 h, evaporated and the residue was dissolved in 20 mM ammonium formate, pH 5 and injected onto the LC-MS/MS (panels F–H). (A) Extracted ion chromatogram (numbers in parenthesis refer to retention times in minutes and lowercase letters refer to corresponding peaks) of authentic standards $(1S,2S)$ -threohydrobupropion (6.68 a), $(1R,2R)$ -threohydrobupropion (7.59 b), $(1R,2S)$ -erythrohydrobupropion (8.24 c), and $(1S,2R)$ -erythrohydrobupropion (9.00 d). Peak assignment of erythrohydrobupropion enantiomers and threohydrobupropion enantiomers

was previously described [9]. (B) (1*S*,2*S*)-threohydrobupropion aglycone formed following hydrolysis of (1*R*,2*R*)-threohydrobupropion β -D-glucuronide standard (purchased as (*R,R*)-dihydro bupropion β -D-glucuronide). (C) (1*R*,2*R*)-threohydrobupropion aglycone formed following hydrolysis of (1*S*,2*S*)-threohydrobupropion β -D-glucuronide (purchased as (*S,S*)-dihydro bupropion β -D-glucuronide) standard. (D) (1*R*,2*S*)-erythrohydrobupropion aglycone formed following the hydrolysis of *rac*-erythrohydrobupropion β -D-glucuronide (purchased as *rac*-erythro-dihydro β -D-glucuronide) standard. Results show that the aglycones produced from each respective hydrolysis experiment have different stereochemistry from their corresponding glucuronides standards. (E) Representative MRM chromatogram of 1 μ g/mL (*R*)-bupropion and (*S*)-bupropion diluted in 20 mM ammonium formate pH 5. (F–H) Extracted ion chromatograms from the manganese dioxide oxidation of (F) (1*S*,2*S*)-threohydrobupropion, (G) (1*R*,2*R*)-threohydrobupropion, and (H) (1*R*,2*S*)-erythrohydrobupropion to bupropion. Results show that each of the bupropion enantiomers generated had the opposite stereochemistry at C2 than the original glucuronide standards, as labeled.

Fig. 3.

Correct structural identification of commercial bupropion metabolite glucuronides. Accurate identification of erythrohydrobupropion and threohydrobupropion glucuronide diastereomer peaks. Previously, sodium borohydride reduction of (*S*)-bupropion produced an 80:20 mixture of (1*R*,2*S*)-erythrohydrobupropion and (1*S*,2*S*)-threohydrobupropion, respectively. Additionally, sodium borohydride reduction of (*R*)-bupropion produced an 80:20 mixture of (1*S*,2*R*)-erythrohydrobupropion and (1*R*,2*R*)-threohydrobupropion, respectively [9]. Each respective mixture was incubated with human liver microsomes supplemented with uridine 5'-diphosphoglucuronic acid (UDPGA) to generate corresponding glucuronides (A): MRM chromatogram of TRC standards diluted in Tris buffer, pH 7.8. (numbers in parenthesis refer to retention times in minutes and lowercase letters refer to corresponding peaks). (1*R*,2*R*)-threohydrobupropion β-D-glucuronide standard (purchased as (*R,R*)-dihydro bupropion β-D-glucuronide) (5.07 a), (1*S*,2*S*)-threohydrobupropion β-D-glucuronide standard (purchased as (*S,S*)-dihydro bupropion β-D-glucuronide) (5.43 b), and *rac*-erythrohydrobupropion β-D-glucuronide standard (purchased as *rac*-erythro-dihydro β-D-glucuronide) (5.84 c). Note: *the rac*-erythrohydrobupropion β-D-glucuronide (purchased as *rac*-erythro-dihydro β-D-glucuronide) standard only produces one peak at 5.84 min (c), suggesting the product is one diastereomer. This was subsequently confirmed in a communication with the manufacturer (Dr. Phillip Chan, Personal Communication, December 21, 2015). (B) MRM chromatogram

of glucuronide products from a human liver microsomal incubation with (1*R*,2*S*)-erythrohydrobupropion and (1*S*,2*S*)-threo hydrobupropion (present in a 80:20 mixture). The 80:20 mixture was synthesized by sodium borohydride reduction of (*S*)-bupropion as previously described [9]. *Note the* (1*S*,2*S*)-threo hydrobupropion β -D-glucuronide standard (*purchased as* (*S,S*)-dihydro bupropion β -D-glucuronide) *at* 5.43 min (b) (Panel A) does not align with the biosynthesized (1*S*,2*S*) threo hydrobupropion β -D-glucuronide diastereomer at 5.06 min (a) (Panel B). The (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide peak at 5.82 min (c) (Panel B) corresponds with *rac*-erythrohydrobupropion β -D-glucuronide (*purchased as rac-erythro*-dihydro β -D-glucuronide) at 5.84 min (c) (Panel A). (C) MRM chromatogram of the glucuronide products from a human liver microsomal incubation with (1*S*,2*R*)-erythrohydrobupropion and (1*R*,2*R*)-threo hydrobupropion (present in a 80:20 mixture). Note the production of only (1*S*,2*R*)-erythrohydrobupropion β -D-glucuronide which elutes at 5.26 min (d). Thus, based on the β -glucuronidase hydrolysis and chemical synthesis experiments described in Fig. 2, and inaccurate alignment of biosynthesized (1*S*,2*S*) threo hydrobupropion β -D-glucuronide with (1*S*,2*S*) threo hydrobupropion β -D-glucuronide standard (*purchased as* (*S,S*)-dihydro bupropion β -D-glucuronide), the stereo-chemistry of the (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (*purchased as* (*R,R*)-dihydro bupropion β -D-glucuronide) and (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (*purchased as* (*S,S*)-dihydro bupropion β -D-glucuronide) standards were reassigned. Additionally, (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide corresponds to *rac*erythrohydrobupropion β -D-glucuronide (*purchased as rac-erythro*-dihydro β -D-glucuronide). (D) MRM chromatogram of glucuronides from patient urine with reassigned stereochemistry. (1*S*,2*S*) threo hydrobupropion β -D-glucuronide (5.02 min a), (1*S*,2*R*)-erythrohydrobupropion β -D-glucuronide (5.23 min d), (1*R*,2*R*) threo hydrobupropion β -D-glucuronide (5.47 min b), and (1*R*,2*S*)-erythrohydrobupropion β -D-glucuronide (5.88 min c).

Fig. 4.

Flow diagram for urine sample preparation and analysis for the stereoselective analyses of bupropion, hydroxybupropion, erythrohydrobupropion, threohydrobupropion, erythrohydrobupropion β-D-glucuronide and threohydrobupropion β-D-glucuronide.

Fig. 5.

Representative MRM chromatograms of a midrange urine calibration standard, urine calibration standard at the limit of quantification, and blank urine. Bupropion (*R* and *S*): (A) 100 ng/mL (B) 10 ng/mL, (C) blank urine. Hydroxybupropion (*R,R* and *S,S*): (D) 1000 ng/mL (E) 50 ng/mL (F) blank urine. Threohydrobupropion (1*S*,2*S* and 1*R*,2*R*) and erythrohydrobupropion (1*R*,2*S* and 1*S*,2*R*): (G) 2500 ng/mL (H) 100 ng/mL (I) blank urine. Threohydrobupropion β-D-glucuronide (1*S*,2*S* and 1*R*,2*R*) and (1*R*,2*S*)-erythrohydrobupropion β-D-glucuronide: (J) 250 ng/mL (K) 50 ng/mL (L) blank urine.

Fig. 6.

Product ion spectra of erythrohydrobupropion and threohydrobupropion β-D-glucuronide metabolites from patient urine. (A) (1*S*,2*S*)-threohydrobupropion β-D-glucuronide, (B) (1*S*,2*R*)-erythrohydrobupropion β-D-glucuronide, (C) (1*R*,2*R*)-threohydrobupropion β-D-glucuronide, and (D) (1*R*,2*S*)-erythrohydrobupropion β-D-glucuronide. Right: MS/MS fragments of *erythro*- or threohydrobupropion β-D-glucuronide.

Fig. 7.

Evaluation of ionization suppression. Extracted ion chromatograms were acquired after injecting analyte- and internal standard free human urine while simultaneously infusing a solution of analytes and internal standards into the AB Sciex 3200 or 4000 mass spectrometer: (A) bupropion (B) hydroxybupropion, (C) erythrohydrobupropion and threohydrobupropion, (D) erythrohydrobupropion glucuronide and threohydrobupropion glucuronide. Peak overlays are from a calibration standard to show the respective retention time of each analyte.

Fig. 8.

Microsomal incubations with *rac*-erythrohydrobupropion or *rac*-threo hydrobupropion. *rac*-erythrohydrobupropion incubation with (A) human liver microsomes (HLM), (B) human kidney microsomes (HKM), and (C) human intestinal microsomes (HIM). Peaks at 5.24 and 5.82 min represent (1*S*,2*R*)-erythrohydrobupropion β-D-glucuronide and (1*R*,2*S*)-erythrohydrobupropion β-D-glucuronide, respectively. *rac*-threo hydrobupropion incubation with (D) human liver microsomes (HLM), (E) human kidney microsomes (HKM), and (F) human intestinal microsomes (HIM). Peaks at 5.06 and 5.42 min represent (1*S*,2*S*)-threo hydrobupropion β-D-glucuronide and (1*R*,2*R*)-threo hydrobupropion β-D-glucuronide, respectively.

Fig. 9.

Urine concentrations of (*R*)-bupropion, (*S*)-bupropion, (*R,R*)-hydroxybupropion (free and total), (*S,S*)-hydroxybupropion (free and total), (*1S,2S*)-threohydrobupropion, (*1R,2R*)-threohydrobupropion, (*1R,2S*)-erythrohydrobupropion, (*1S,2R*)-erythrohydrobupropion, (*1R,2R*)-threohydrobupropion β-D-glucuronide, (*1S,2S*)-threohydrobupropion β-D-glucuronide, (*1S,2R*)-erythrohydrobupropion β-D-glucuronide, and (*1R,2S*)-erythrohydrobupropion β-D-glucuronide from a research subject who received 300 mg oral extended release bupropion each morning.

Table 1

Assay calibration standard and quality control concentrations.

Analyte	Enantiomeric calibrator concentrations (ng/mL)	Quality control concentrations (ng/mL) ^a					
		QC 1	QC 2	QC 3	QC 4	QC 5	QC 6
bupropion (<i>R</i> and <i>S</i>)	10, 25, 50, 75, 100, 150, 250, 500, 750, 1000, 2000, 3000	25	100	500	1000		
hydroxybupropion (<i>R,R</i> and <i>S,S</i>)	50, 100, 250, 500, 1000, 1500, 2500, 5000, 10,000, 15,000, 20,000, 30,000	100	500	2000	5000	10,000	15,000
threohydrobupropion (1 <i>S</i> ,2 <i>S</i> and 1 <i>R</i> ,2 <i>R</i>)	100, 250, 500, 1000, 2500	250	750	2500			
erythrohydrobupropion (1 <i>R</i> ,2 <i>S</i> and 1 <i>S</i> ,2 <i>R</i>)	100, 250, 500, 1000, 2500	250	750	2500			
erythrohydrobupropion β-D-glucuronide (1 <i>R</i> ,2 <i>S</i>) ^b	50, 100, 150, 200, 250, 500, 750, 1000, 1500, 2500, 5000	100	250	500	1000	2500	
threohydrobupropion β-D-glucuronide (1 <i>S</i> ,2 <i>S</i>)	50, 100, 150, 200, 250, 500, 750, 1000, 1500, 2500, 5000	100	250	500	1000	2500	
threohydrobupropion β-D-glucuronide (1 <i>R</i> ,2 <i>R</i>)	50, 100, 150, 200, 250, 500, 750, 1000, 1500, 2500, 5000	100	250	500	1000	2500	

^a Analyte-specific calibrator and QC concentrations chosen based on a preliminary analysis of patient urine from a clinical study in patients receiving 300 mg oral bupropion.

^b erythrohydrobupropion-glucuronide (1*S*,2*R*) was not commercially available.

Table 2

Glucuronide analyte and internal standard MS/MS optimized parameters on AB Sciex 4000 QTRAP mass spectrometer.

Analyte	MRM transition	Declustering Potential	Entrance Potential	Collision energy	Exit potential
erythrohydrobupropion β -D-glucuronide	418.0> 168.2	81	10	41	26
erythrohydrobupropion β -D-glucuronide-d ₉	427.1> 169.1	106	10	43	28
threohydrobupropion β -D-glucuronide	418.0> 168.2	81	10	41	26

Table 3

Time course of threohydrobupropion β -D-glucuronide and erythrohydrobupropion β -D-glucuronide hydrolysis in patient urine by β -glucuronidase.

Analyte	(%) Glucuronide Remaining^a (n = 3)		
	4h	8h	24h
(1 <i>S</i> ,2 <i>S</i>)-threohydrobupropion β -D-glucuronide	10 $\pm$ 1	1 $\pm$ 0	0 $\pm$ 0
(1 <i>S</i> ,2 <i>R</i>)-erythrohydrobupropion β -D-glucuronide	41 $\pm$ 1	19 $\pm$ 2	1 $\pm$ 0
(1 <i>R</i> ,2 <i>R</i>)-threohydrobupropion β -D-glucuronide	83 $\pm$ 5	80 $\pm$ 3	73 $\pm$ 3
(1 <i>R</i> ,2 <i>S</i>)-erythrohydrobupropion β -D-glucuronide	106 $\pm$ 3	88 $\pm$ 2	75 $\pm$ 5

^aReported values are the percentage of the initial observed peak area ratio of glucuronides in patient urine (mean $\pm$ standard deviation, n = 3).

Intra-day and inter-day accuracy and precision for determination of bupropion, oxidative, and reductive metabolites in human urine with analyte specific calibration ranges^a on an AB Sciex 3200 mass spectrometer.

Table 4

Analyte	Nominal concentration (ng/mL)	Intra-assay (n = 5)			Inter-assay (n = 5)		
		Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)	Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)
(R)-bupropion	100	103 ± 5	103	5	103 ± 8	102	8
	500	554 ± 35	111	6	568 ± 61	114	11
	1000	1013 ± 59	101	6	1032 ± 32	103	3
(S)-bupropion	25	25.9 ± 1.5	103	6	24.3 ± 3.0	97	12
	100	103 ± 12	103	12	101 ± 14	101	14
	500	557 ± 76	111	14	552 ± 34	110	6
(R,R)-hydroxybupropion ^c (free)	100	85 ± 8	85	9	94 ± 10	94	11
	500	494 ± 32	99	7	490 ± 41	96	8
	2000	2024 ± 213	101	11	1997 ± 121	98	6
(S,S)-hydroxybupropion ^c (free)	5000	5094 ± 434	102	9	5159 ± 323	102	6
	100	95 ± 6	95	7	93 ± 4	93	4
	500	524 ± 30	105	6	492 ± 50	98	10
(1S,2S)-threohydrobupropion	2000	2083 ± 221	104	11	2039 ± 171	102	8
	5000	5192 ± 386	104	7	5185 ± 363	104	7
	250	253 ± 15	101	6	253 ± 13	101	5
(1S,2S)-threohydrobupropion	750	686 ± 39	91	6	704 ± 40	94	6
	2500	2255 ± 231	90	10	2341 ± 62	94	3
(1R,2R)-threohydrobupropion	250	242 ± 17	97	7	252 ± 6	101	2
	750	692 ± 37	92	5	703 ± 40	94	6
	2500	2274 ± 168	91	7	2318 ± 39	93	2
(1R,2S)-erythrohydrobupropion	250	257 ± 21	103	8	255 ± 22	102	9
	750	694 ± 33	93	5	699 ± 34	93	5
	2500	2214 ± 175	89	8	2359 ± 24	94	1
(1S,2R)-erythrohydrobupropion	250	255 ± 13	102	5	255 ± 11	102	4
	750	705 ± 36	94	5	704 ± 41	94	6

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Analyte	Nominal concentration (ng/mL)	Intra-assay (n = 5)			Inter-assay (n = 5)		
		Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)	Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)
	2500	2264 ± 188	91	8	2340 ± 72	94	3

^aCalibration ranges are provided in Table 1.

^bMean ± standard deviation.

^cFree hydroxybupropion concentrations determined following sample preparation method 1 (Fig. 4).

Table 5

Intra-day and inter-day accuracy and precision for determination of total^a hydroxybupropion (*R,R* and *S,S*) in human urine with analyte specific calibration ranges^b on an AB Sciex 3200 mass spectrometer.

Analyte	Nominal concentration (ng/mL)	Intra-assay (n = 5)			Inter-assay (n = 5)		
		Calculated Concentration (ng/mL) ^c	Accuracy (%)	Precision (%)	Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)
<i>(R,R)</i> -hydroxybupropion	100	91.3 ± 8.2	91	9	103 ± 6	103	6
	500	517 ± 46	103	9	487 ± 43	97	9
	2000	1983 ± 147	99	7	1870 ± 73	94	4
	5000	5086 ± 350	102	7	4848 ± 129	97	3
	10,000	9907 ± 536	99	5	9056 ± 337	91	4
<i>(S,S)</i> -hydroxybupropion	15,000	15338 ± 381	102	2	14802 ± 774	99	5
	100	95.8 ± 13.7	96	14	93.9 ± 8.1	94	9
	500	523 ± 36	105	7	472 ± 54	94	12
	2000	2000 ± 96	100	5	1927 ± 74	96	4
	5000	5346 ± 133	107	2	4976 ± 252	100	5
	10,000	10004 ± 413	100	4	9343 ± 389	93	4
	15,000	15082 ± 818	101	5	14592 ± 570	97	4

^aTotal hydroxybupropion concentrations determined following sample preparation method 2 (Fig. 4).

^bCalibration ranges are provided in Table 1.

^cMean ± standard deviation.

Table 6

Intra-day and inter-day accuracy and precision for determination of threo hydrobupropion and erythro hydrobupropion β -D-glucuronide diastereomers in human urine with analyte specific calibration ranges^a on an AB Sciex 4000 mass spectrometer.

	Nominal concentration (ng/mL)	Intra-assay (n = 5)			Inter-assay (n = 5)		
		Calculated Concentration (ng/mL) ^b	Accuracy (%)	Precision (%)	Calculated concentration (ng/mL) ^b	Accuracy (%)	Precision (%)
(1 <i>S</i> ,2 <i>S</i>) threo hydrobupropion β -D-glucuronide	100	98.0 $\pm$ 6.8	98	7	98.4 $\pm$ 4.0	98	4
	250	254 $\pm$ 11	102	4	256 $\pm$ 18	102	7
	500	519 $\pm$ 48	104	9	547 $\pm$ 27	109	5
	1000	959 $\pm$ 67	96	7	973 $\pm$ 40	97	4
	2500	2604 $\pm$ 131	104	5	2563 $\pm$ 113	103	4
(1 <i>R</i> ,2 <i>R</i>) threo hydrobupropion β -D-glucuronide	100	98.1 $\pm$ 7.8	98	8	100.4 $\pm$ 5.5	100	6
	250	248 $\pm$ 16	99	6	252 $\pm$ 12	101	5
	500	495 $\pm$ 51	99	10	525 $\pm$ 30	105	6
	1000	986 $\pm$ 63	99	6	993 $\pm$ 60	99	6
	2500	2639 $\pm$ 132	106	5	2614 $\pm$ 78	105	3
(1 <i>R</i> ,2 <i>S</i>) erythro hydrobupropion β -D-glucuronide	100	99.3 $\pm$ 5.3	99	5	98.0 $\pm$ 4.4	98	4
	250	245 $\pm$ 16	98	6	248 $\pm$ 15	99	6
	500	490 $\pm$ 42	98	9	508 $\pm$ 19	102	4
	1000	1040 $\pm$ 74	104	7	1054 $\pm$ 56	105	5
	2500	2570 $\pm$ 126	103	5	2537 $\pm$ 62	101	2

^a Calibration ranges are provided in Table 1.

^b Mean $\pm$ standard deviation.

Table 7

Stability of bupropion and metabolites in human urine.

Analyte	Stability in urine (%) ^a (n = 3)			
	Nominal Concentration (ng/mL)	1stfreeze-thaw	2ndfreeze-thaw	3rdfreeze-thaw
(R)-bupropion	25	101 ± 8	98 ± 5	88 ± 4
	1000	103 ± 2	98 ± 11	91 ± 3
(S)-bupropion	25	112 ± 6	99 ± 5	93 ± 11
	1000	108 ± 7	95 ± 12	90 ± 6
(R,R)-hydroxybupropion	100	96 ± 7	86 ± 10	88 ± 5
	15,000	105 ± 5	99 ± 5	97 ± 6
(S,S)-hydroxybupropion	100	92 ± 2	88 ± 9	88 ± 5
	15,000	105 ± 6	100 ± 7	97 ± 6
(1S,2S)-threohydrobupropion	250	97 ± 8	96 ± 2	88 ± 6
	2500	104 ± 4	98 ± 7	95 ± 6
(1R,2R)-threohydrobupropion	250	93 ± 7	99 ± 3	88 ± 6
	2500	103 ± 6	97 ± 7	94 ± 7
(1R,2S)-erythrohydrobupropion	250	96 ± 11	96 ± 4	87 ± 7
	2500	105 ± 6	98 ± 6	95 ± 7
(1S,2R)-erythrohydrobupropion	250	97 ± 9	99 ± 5	89 ± 5
	2500	104 ± 4	97 ± 6	94 ± 6
(1S,2S)-threohydrobupropion β-D-glucuronide	100	99 ± 10	97 ± 2	91 ± 4
	2500	105 ± 5	100 ± 9	97 ± 7
(1 R,2R)-threohydrobupropion β-D-glucuronide	100	100 ± 11	103 ± 1	97 ± 9
	2500	105 ± 7	102 ± 8	97 ± 7
(1R,2S)-erythrohydrobupropion β-D-glucuronide	100	102 ± 5	104 ± 3	97 ± 5
	2500	105 ± 6	100 ± 7	98 ± 7

^aReported values are the percentage of the initial observed concentration (prepared freshly with no freeze thaw cycles), mean ± standard deviation, n = 3.