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Development and validation of a high-throughput stereoselective LC-MS/MS assay for bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion in human plasma

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Abstract

A stereoselective analytical method was developed and validated for the quantification of bupropion, and principle metabolites hydroxybupropion, erythrohydrobupropion and threohydrobupropion in human plasma. Separation of individual enantiomers (*R*)-bupropion, (*S*)-bupropion, (*R,R*)-hydroxybupropion, (*S,S*-hydroxybupropion), (*1S,2S*)-threohydrobupropion, (*1R, 2R*)-threohydrobupropion, (*1R,2S*)-erythrohydrobupropion, and (*1S,2R*)-erythrohydrobupropion was achieved utilizing an α_1-acid glycoprotein column within a 12-minute run time. Chromatograph separation was significantly influenced by mobile phase pH and variability between columns. Analytes were quantified by positive ion electrospray tandem mass spectrometry following plasma protein precipitation with 20% trichloroacetic acid. Identification of erythrohydrobupropion enantiomer peaks and threohydrobupropion enantiomer peaks was achieved by sodium borohydride reduction of enantiopure (*R*)- and (*S*)-bupropion. Initial assay validation and sensitivity determination was on AB Sciex 3200, 4000 QTRAP, and 6500 mass spectrometers. Accuracy and precision were within 15% for each analyte. The assay was fully validated over analyte-specific concentrations using an AB Sciex 3200 mass spectrometer. Intra- and inter-assay precision and accuracy were within 12% for each analyte. The limits of quantification for bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), threohydrobupropion (*1S,2S* and *1R,2R*), and erythrohydrobupropion (*1R,2S* and *1S,2R*) were 0.5, 2, 1, and 1 ng/mL, respectively. All analytes were stable following freeze thaw cycles at −80°C and while stored at

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4°C in the instrument autosampler. This method was applicable to clinical pharmacokinetic investigations of bupropion in patients. This is the first chromatographic method to resolve erythrohydrobupropion and threohydrobupropion enantiomers, and the first stereoselective LC-MS/MS assay to quantify bupropion, and principle metabolites hydroxybupropion, erythrohydrobupropion, and threohydrobupropion in human plasma.

Keywords

bupropion; hydroxybupropion; erythrohydrobupropion; threohydrobupropion; LC-MS/MS; stereoselectivity

1. Introduction

Bupropion is an antidepressant that is efficacious in the treatment of major depressive disorder, relapse prevention, and smoking cessation. It is available in immediate release, twice daily sustained release (SL), and once-daily extended release (XL) formulations. Clinically, bupropion is dosed as a racemic mixture, (*R,S*)-bupropion, and is metabolized to three main metabolites: hydroxybupropion, erythrohydrobupropion, and threohydrobupropion. Clinical pharmacokinetics of (*R,S*)-bupropion have been well-described in achiral assays showing that metabolite concentrations exceed those of the parent drug [1].

Each of these metabolites, however, actually exist as enantiomeric or diastereomeric pairs because biochemical metabolism creates a new chiral center on the parent molecule (Figure 1). (*R,S*)-bupropion undergoes stereoselective oxidative metabolism by cytochrome P450 2B6 (CYP2B6) to form (*R,R*)- and (*S,S*)-hydroxybupropion [2]. Additionally, bupropion is stereoselectively reduced by carbonyl reductase(s) to form erythrohydrobupropion and threohydrobupropion [3]. The formation of threohydrobupropion is dependent on 11β-hydroxysteroid dehydrogenase 1 reduction of (*R*)-bupropion in vitro [4]; however, the enzyme that forms erythrohydrobupropion has yet to be elucidated.

Clinical bupropion disposition is remarkably stereoselective [5–9]. Peak plasma concentrations and area under the curve values were 3-fold greater for (*R*)- than for (*S*)-bupropion, and bupropion apparent oral clearance was 3- and 2-fold greater for (*S*)- than for (*R*)- and (*R,S*)-bupropion, respectively. Plasma area under the curve and elimination half-life were significantly less for (*S,S*)- than (*R,R*)-hydroxybupropion. (*S,S*)-hydroxybupropion was formation rate limited, whereas (*R,R*)-hydroxybupropion was elimination rate limited. Moreover, while (*R*)- and (*S*)-bupropion have similar antidepressant activity [10], only (*S,S*)-hydroxybupropion, but not (*R,R*)-hydroxybupropion is pharmacologically active. Accordingly, (*S,S*)-hydroxybupropion is now considered the most active metabolite of bupropion, and the mediator of pharmacological effect, although it is quantitatively the minor enantiomer of hydroxybupropion. Of note, changes in clinical (*S,S*)-hydroxybupropion concentrations would not be revealed by an achiral assay.

Achiral LC-MS/MS bioanalytical methods which simultaneously measure bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion in whole blood [11],

plasma and urine [12–15], and breast milk[16], from patients receiving oral bupropion, have been previously described. Additionally, stereoselective LC-UV analytical methods were developed for bupropion and hydroxybupropion enantiomers utilizing achiral-chiral chromatography [17], with ovomucoid [18], cyclobond I 2000 [9], cellulose [19], and α_1-acid glycoprotein[20] columns, electrokinetic chromatography [21], and isothiocyanate-based chiral derivatization [22]. Previously, our group utilized an α_1-acid glycoprotein chiral column in a validated LC-MS/MS assay for bupropion and hydroxybupropion enantiomers in plasma [5]. Nevertheless, erythrohydrobupropion and threohydrobupropion, like hydroxybupropion, exist as enantiomeric pairs and are pharmacologically active [23]. Since there is not an assay reported for erythrohydrobupropion and threohydrobupropion enantiomers in plasma, their clinical disposition, pharmacogenetics, and overall impact on bupropion clinical pharmacology remain unknown. Therefore, a stereoselective bioanalytical method for bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion in human plasma was developed and validated.

Materials and Methods

2.1 Materials

Standard methanolic solutions of *rac*-bupropion HCl and *rac*-hydroxybupropion were from Cerilliant Corporation (Round Rock, TX). *rac*-bupropion-d$_9$ hydrochloride, *rac*-hydroxybupropion-d$_6$, *rac*-threohydrobupropion and *rac*-erythrohydrobupropion maleate 1:1 mixture, *rac*-erythrohydrobupropion-d$_9$, and *rac*-threohydrobupropion-d$_9$, (*R*)-bupropion tartaric acid, and (*S*)-bupropion tartaric acid were from Toronto Research Chemicals (Toronto, ON, Canada). Acid citrate dextrose human plasma was obtained from the American Red Cross (St. Louis, MO). HPLC-grade methanol, trichloroacetic acid, ammonium formate, formic acid, and sodium borohydride were from Sigma Aldrich (St. Louis, MO). All solutions were prepared in ultrapure water (18.2 M ·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 analyte, 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. Three working stock concentrations of bupropion (*R* and *S*) were prepared at 50, 500, and 5,000 ng/mL and two working stock concentrations of hydroxybupropion (*R,R* and *S,S*) were prepared at 500 and 50,000 ng/mL and immediately stored at −80°C. The *rac*-erythrohydrobupropion and *rac*-threohydrobupropion (1:1) powder was accurately weighed and prepared as a 1.0 mg/mL stock solution containing 500 µg/mL *rac*-erythrohydrobupropion and 500 µg/mL *rac*-threohydrobupropion in DMSO. Two working stock solutions were prepared in water at 5,000 ng/mL and 500 ng/mL erythrohydrobupropion (*1R,2S*) and (*1S,2R*) and threohydrobupropion (*1S,2S*) and (*1R,2R*). Deuterated internal standards bupropion-d$_9$ (*R* and *S)*, hydroxybupropion-d$_6$ (*R,R* and *S,S*), erythrohydrobupropion-d$_9$ (*1R,2S*) and (*1S,2R*), and threohydrobupropion-d$_9$ (*1S,2S*) and (*1R,2R*) were individually prepared as 500 µg/mL DMSO stock solutions and stored at −80°C. A working stock of 5,000 ng/mL bupropion-d$_9$ (*R* and *S*), 25,000 ng/mL

hydroxybupropion-d_6 (*R,R* and *S,S*), 12,500 ng/mL erythrohydrobupropion-d_9 (*1R,2S*) and (*1S,2R*), and 12,500 ng/mL threohydrobupropion-d_9 (*1S,2S*) and (*1R,2R*) was divided into 50 μL aliquots which were stored at −80°C. On the day of an experiment, a fresh working stock of 200 ng/mL bupropion-d_9 (*R* and *S*), 1000 ng/mL hydroxybupropion-d_6 (*R,R* and *S,S*), 500 ng/mL erythrohydrobupropion-d_9 (*1R,2S*) and (*1S,2R*), and 500 ng/mL threohydrobupropion-d_9 (*1S,2S*) and (*1R,2R*) was prepared.

Initially, calibration standards containing the same analyte enantiomeric concentration in human plasma were prepared at 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 ng/mL along with quality control standards (QC) at 2.5, 50, 500 ng/mL to evaluate instrument performance on AB Sciex 3200, 4000, and 6500 mass spectrometers. For the subsequent validation using analyte-specific concentrations, all calibrators and QC samples at the enantiomeric concentrations described in Table 1 were prepared in individual 50 mL volumetric flasks. Each calibration or QC was subsequently aliquoted (0.25 mL) into 1.5 mL tubes and stored at −80°C until analysis. These calibrator and QC samples were used for the final assay validation on the AB Sciex 3200 mass spectrometer.

2.3 Sample Preparation

Calibration standards, QCs, and patient samples were thawed at room temperature, vortexed, and transferred (200 μL) to a 1.0 mL DeepWell 96-well plate (Nunc, Thermofisher, Pittsburgh, PA). Freshly prepared internal standard aqueous solution (10 μL) containing 200 ng/mL bupropion-d_9 (*R* and *S*), 1000 ng/mL hydroxybupropion-d_6 (*R,R* and *S,S*), 500 ng/mL erythrohydrobupropion-d_9 (*1R,2S*) and (*1S,2R*), and 500 ng/mL threohydrobupropion-d_9 (*1S,2S*) and (*1R,2R*) was added to each well. Next, 40 μL of 20% aqueous trichloroacetic acid was added and the plate shaken for 5 min. The plate was centrifuged at 6,100*g* at 4°C for 15 min, and the supernatant (100 μL) was transferred to a MicroWell 96-well plate (Nunc, Thermofisher, Pittsburgh, PA) containing 10 μL of a 3M aqueous ammonium formate solution, pH 6.9. The final sample pH was 3.

2.4 Syntheses of erythrohydrobupropion and threohydrobupropion from racemic bupropion and individual bupropion enantiomers

rac-bupropion HCl, (*R*)-bupropion tartaric acid, and (*S*)-bupropion tartaric acid (1 mg, 0.004 mmol 1.0 equiv) were individually dissolved in methanol (150 μL) in 1 dram vials and stirred in an ice bath at 0°C for 10 min. Subsequently, sodium borohydride (0.291 mg 2.0 mmol, 2 equiv) was added to the reaction mixture by adding 153 μL of a 1.9 mg/mL sodium borohydride methanolic solution, and the reaction mixture stirred for 30 min at 0°C. The ice bath was removed and the mixture was allowed to warm to room temperature. An aliquot of each reaction mixture was diluted in 20 mM ammonium formate, pH 5.0 and subsequently injected on the LC-MS/MS system with the chromatographic method described in section 2.6.

2.5 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 (Gerstal, 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.

AB Sciex 6500—LC-MS/MS analysis was performed on a Shimadzu HPLC system composed of two LC-20AD XR pumps, DGU20A5R degasser, CBM-20A system controller, CTO-20C column oven, FCV-11AL solvent selection valve, and a SIL-20AC XR temperature regulated autosampler. The LC system was coupled to an API 6500 triple quadrupole tandem mass spectrometer operated with Analyst 1.6.2.

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

2.6 Chromatographic Conditions

Chiral chromatographic separation of analytes and respective internal standards was achieved utilizing a chiralpak α_1-acid glycoprotein (AGP) column (100 x 2.0 mm, 5μm, Chiral Technologies, Westchester, PA) equipped with a chiral AGP guard cartridge (10 x 2.0 mm, Sigma Aldrich, St. Louis, MO). 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 20 mM aqueous ammonium formate, pH 5.0 (A) and methanol (B). The time program to achieve the separation was as follows: 10% B for 0.5 min, linear gradient to 20% B until 1 min, held at 20% B until 5 min, linear gradient to 50% B until 8 min, and then re-equilibrated to initial conditions until 12 min. The column oven was at ambient temperature and the autosampler was at 4°C. Flow was directed into the mass spectrometer at 2.5 min and diverted to waste at 11.5 min. Under these conditions, retention times (min) for the analytes were: (*R*)-bupropion 3.83, (*S*)-bupropion 4.85, (*R,R*)-hydroxybupropion 7.52, (*S,S*-hydroxybupropion) 4.10, (*1S,2S*)-threohydrobupropion 6.11, (*1R,2R*)-threohydrobupropion) 6.90, (*1R,2S*)-erythrohydrobupropion 7.87, and (*1S,2R*)-erythrohydrobupropion 8.75 (Figure 2).

2.7 Mass Spectrometry Conditions

AB Sciex 3200—The mass spectrometer was operated with a turbo spray ion source in the positive mode (ESI+) with multiple reaction monitoring (MRM). Global parameters were optimized across all analytes: curtain gas 30 psig, ion spray voltage 5000 V, source temperature 650°C, Gas 1 40 psig, and Gas 2 40 psig. Dwell times were 500 msec.

Optimized analyte- and internal standard-specific MRM transitions, declustering potential, entrance potential, collision energy, and exit potential are described in Table 2.

AB Sciex 4000—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 500 msec.

AB Sciex 6500—Global parameters were optimized across all analytes: curtain gas 20 psig, ion spray voltage 5000 V, source temperature 600°C, Gas 1 40 psig, and Gas 2 40 psig. Dwell times were 500 msec.

2.8 Method Validation

Validation experiments and run acceptance criteria were based on the United States Food and Drug Administration Bioanalytical Method Validation Guidance [24].

2.8.1 Accuracy and precision—The accuracy and precision for each analyte in pooled blank human plasma 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.

2.8.2 Selectivity—Individual lots of human plasma 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. Eight individual lots of blank human plasma 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.

2.8.3 Ion suppression—To investigate any loss of signal due to matrix effect on ion suppression, a solution of all analytes and internal standards 200 ng/mL bupropion (*R* and *S*), 200 ng/mL bupropion-d$_9$ (*R* and *S*), 5,000 ng/mL hydroxybupropion (*R,R* and *S,S*), 5,000 ng/mL hydroxybupropion-d$_6$ (*R,R* and *S,S*), 500 ng/mL erythrohydrobupropion (*1R, 2S*) and (*1S,2R*), 500 ng/mL erythrohydrobupropion-d$_9$ (*1R,2S*) and (*1S,2R*), 500 ng/mL threohydrobupropion (*1S,2S*) and (*1R,2R*), and 500 ng/mL threohydrobupropion-d$_9$ (*1S,2S*) and (*1R,2R*) was infused into the mass spectrometer while simultaneously injecting a processed blank human plasma sample.

2.8.4 Stability—Stability experiments utilizing low and high QC samples were performed after 3 subsequent freeze/thaw cycles at −80°C, processed as described in section 2.3, and analyzed by LC-MS/MS. Additionally, the stability of the analytes in solution were assessed by storing fresh processed samples (no freeze/thaw) in the autosampler at 4°C over a period of 48 hours.

2.9 Clinical Samples

The validated bioanalytical method was applied to clinical study approved by the Washington University in St Louis institutional review board. Venous blood samples were obtained immediately before and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, and 24 hr after a daily steady-state dose of 300 mg extended release bupropion. Samples were centrifuged to obtain plasma, which was stored at −80°C until analysis.

3. Results and Discussion

3.1 Method Development

The stereoselective chromatographic separation of bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion was initially attempted with a lux cellulose-3 column (250 x 4.6 mm 5 μM, Phenomenex, Torrence, CA). The method involved an isocratic elution with a mobile phase consisting of 62% 5 mM ammonium bicarbonate, pH 9.0–9.2, 25% methanol, and 13% acetonitrile at a flow rate of 1.0 mL/min. Under these conditions, there was adequate resolution of bupropion (*R* and *S*), erythrohydrobupropion enantiomers, and threohydrobupropion enantiomers; however hydroxybupropion enantiomers could not be resolved. Separation was next attempted with a previously published stereoselective chromatographic method for bupropion and hydroxybupropion enantiomers using an α_1-acid glycoprotein column [5]. However, incomplete resolution was observed with erythrohydrobupropion and threohydrobupropion enantiomers. The previous method utilized a 20 mM ammonium formate buffer at pH 5.7, which is above the buffering capacity of ammonium formate. By incrementally lowering the pH of the 20 mM ammonium formate buffer, the erythrohydrobupropion and threohydrobupropion enantiomers began to resolve while the bupropion and hydroxybupropion enantiomer retention times significantly decreased and retained baseline resolution. The optimal pH for chromatographic resolution of all the analytes ranged from pH 4.8–5.1, and required optimization for each individual HPLC column.

Plasma sample preparation was initially performed according to the previously published method [5], using solid phase extraction with Oasis MCX 96 well plates (Waters, Milford, MA). Samples were eluted with 80:18:2 methanol:water:ammonium hydroxide and subsequently acidified with 2 M ammonium formate, pH 4. This method was suboptimal due to inadequate chromatographic separation of erythrohydrobupropion and threohydrobupropion and their enantiomers, due primarily to the high percentage of organic solvent. Therefore, the goal was to develop a sample preparation method which ultimately yielded a predominantly aqueous injectate. Because bupropion is chemically unstable and racemizes (42%, 62%, and >94% after 2, 4, and 24 hours, respectively, in phosphate buffer pH 7.4 at 25°C, and also at pH 4 and 5) [25,26], but does not undergo racemization at low pH (pH 1.2 at 25°C for 50 days) [26], trichloroacetic acid was chosen to precipitate plasma proteins. However, injecting a sample directly in an acidic pH abolished chromatographic resolution. Therefore, 3 M aqueous ammonium formate, pH 6.9 was added to increase the final sample pH to 3.

3.2 Peak identification of erythrohydrobupropion and threohydrobupropion enantiomers

Stereoselective chromatographic separation of *rac*-erythrohydrobupropion and *rac*-threohydrobupropion produced four distinct peaks, two enantiomers for *rac*-erythrohydrobupropion and two enantiomers for *rac*-threohydrobupropion (Figure 2D). Since authentic standards for each erythrohydrobupropion enantiomer and each threohydrobupropion enantiomer were not commercially available, each corresponding enantiomer peak was stereochemically identified. The assignment of each individual enantiomer peak was determined by sodium borohydride reduction of (*R*)- and (*S*)- bupropion and comparing their respective product retention times to the product retention times of a *rac*-bupropion sodium borohydride reduction. Carbonyl reduction of *rac*-bupropion yields both *rac*-erythrohydrobupropion and *rac*-threohydrobupropion metabolites as expected (Figure 3A). Individual reduction of (*R*)-bupropion or (*S*)-bupropion will yield one erythrohydrobupropion enantiomer and one threohydrobupropion enantiomer because the chiral center at C2 is fixed, and sodium borohydride reduction of ketone functional groups is not stereoselective. The reaction mixtures from the small scale syntheses were diluted in 20 mM ammonium formate, pH 4.8 and analyzed by LC-MS/MS. It was determined that reduction of (*R*)-bupropion produced the second threohydrobupropion and second erythrohydrobupropion peaks, which structurally correspond to (*1R,2R*)- threohydrobupropion and (*1S,2R*)-erythrohydrobupropion (Figure 3B). The (*S*)-bupropion reduction produced the first threohydrobupropion and first erythrohydrobupropion peaks, which structurally correspond to (*1S,2S*)-threohydrobupropion and (*1R,2S*)- erythrohydrobupropion (Figure 3C).

3.3 Method Validation

3.3.1 General development on AB Sciex 3200, 4000, and 6500 mass spectrometers—Analytical method validation was initially conducted on three mass spectrometers (AB Sciex 3200, 4000 QTRAP, and 6500) to assess linearity and sensitivity. Initially, calibrator concentrations and QCs were prepared over a broad range and analyzed on each instrument. 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.

Evaluation of performance across the instruments was investigated by comparing intra-assay (n=3) and inter-assay (n=5) accuracy and precision, as well as sensitivity and linearity. Calibration curves were linear for all analytes on all three instruments (typical r^2 values were 0.994 for the 3200 (0.5–1,000 ng/mL), 4000 (0.25–1,000 ng/mL), and 6500 (0.25–1,000 ng/mL) instruments, respectively). At each of the QC concentration investigated, accuracy and precision were within 15% (Tables 3–5). The validation results on all three instruments exemplify the robustness of the analytical assay. The sensitivity of each instrument at the lowest calibrator (0.25 ng/mL) is illustrated in Figure 4. Bupropion (*R* and *S*) was below the limit of detection on the 3200, although readily quantifiable on the 4000 and 6500 instruments, and for this reason were not used in the calibration curve for each instrument. The 6500 instrument response for bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion enantiomers was roughly 65-fold and 8-fold higher than the 3200

and 4000 instruments, respectively. Additionally, the 4000 sensitivity was roughly 9-fold higher than the 3200.

3.3.2 Analyte specific validation on an AB Sciex 3200 mass spectrometer—

Analyte specific calibrator and QC concentrations (Table 1) were based on a preliminary analysis of plasma samples from a clinical investigation in which patients received 300 mg extended release bupropion once daily, and from previously published clinical studies [12]. Sensitivity down to 0.25 ng/mL was not needed for any analyte, and the upper concentration ranges for bupropion and hydroxybupropion were decreased and increased, respectively. The 3200 instrument was chosen for the final assay development and full validation because it had the requisite sensitivity and the greater sensitivity of the 4000 and 6500 instruments was not needed.

The final assay validation met all acceptance criteria. Accuracy and precision data for each analyte and the QC concentrations are shown in Table 6. For each analyte at each respective concentration, the accuracy and precision was within 12%, including at the limit of quantification. Figure 5 and Figure 6 show the extracted ion chromatograms from a processed plasma sample devoid of analytes or internal standards, and at the lowest calibrator concentration for each analyte, respectively. Extracted ion chromatograms from ion suppression experiments are shown in Figure 7. In each of the chromatograms, ion suppression from matrix components only occurred from 1–3 min and did not interfere with the respective analyte signals. Analyte stability following three subsequent freeze thaw cycles at −80°C and autosampler storage 4°C for 48 hr was evaluated (Table 7). Analyte stability in human plasma ranged from 93–113%, 94–104%, and 88–110% of the nominal concentration after the 1st, 2nd, and 3rd subsequent −80°C freeze thaw cycles, respectively. Additionally, analyte concentrations ranged from 92–115% of the nominal concentration when stored in the autosampler at pH 3 and 4°C for 48 hr. Analyte racemization was also assessed in the autosampler for 21 days at pH 3 at 4°C. All analytes were stable and did not undergo any racemization during the storage and freeze thaw cycles (data not shown). The results of these experiments show that bupropion and metabolite enantiomers are stable after freeze/thaw cycles and storage at pH 3 at 4°C for an extended period of time.

3.4 Method Application

The validated method was successfully used to quantify plasma concentrations of bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion enantiomers from a patient who received 300 mg oral extended release bupropion once daily (Figure 8). Concentrations were within the calibration ranges. This is the first accurate quantification or erythrohydrobupropion enantiomers and threohydrobupropion enantiomers in human plasma. There was a 1.5- to 2-fold difference in enantiomer concentrations for both erythrohydrobupropion and threohydrobupropion. These differences substantiate the requirement for a stereoselective assay for all bupropion metabolites.

3.5 Method comparison

This is the first stereoselective bioanalytical method to quantify enantiomers of bupropion, and the principle metabolites hydroxybupropion, erythrohydrobupropion, and

threohydrobupropion in human plasma. It therefore represents an advance over previous methods, which either analyzed these analytes achirally [3,11–15,27,28], or analyzed only bupropion and hydroxybupropion stereoselectively [5,9,17]. In addition, sample preparation (acid precipitation), enabled in part by changing the chromatographic method, was simpler than the previously reported solid phase extraction [5].

There are three published bioanalytical methods for the stereoselective analysis of bupropion and/or hydroxybupropion enantiomers in plasma from clinical investigations [5,9,17]. Each of the previous methods utilized either liquid-liquid extraction (1.5% v/v isoamyl alcohol in *n*-heptane) or solid-phase extraction (mixed-mode cation exchange) for sample cleanup. The current method utilizes 20% trichloroacetic acid for plasma protein precipitation, which vastly improved assay efficiency and throughput. One full 96-well plate of samples takes approximately 1 hr to prepare. Total run times for the previous methods ranged from 15–30 min and they only analyzed hydroxybupropion (*R,R* and *S,S*) and/or bupropion (*R* and *S*) enantiomers. The current improved method analyzes bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), threohydrobupropion (*1S,2S* and *1R,2R*), and erythrohydrobupropion (*1R,2S* and *1S,2R*) within 12 min. Previous limits of quantification for bupropion enantiomers were 12.5 ng/mL when analyzed by LC-UV at 254 nm and 0.5 ng/mL when analyzed by LC-MS/MS. For hydroxybupropion enantiomers, previous limits of quantification were either 12.5 and 62.5 ng/mL when analyzed by LC-UV at 214 nm or 2.5 ng/mL when analyzed by LC-MS/MS. In this assay, limits of quantification were 0.5 ng/mL, 2 ng/mL, 1 ng/mL and 1 ng/mL for bupropion (*R* and *S*), hydroxybupropion (*R,R* and *S,S*), threohydrobupropion (1*S*,2*S* and 1*R*,2*R*), and erythrohydrobupropion (1R,2S and 1*S*,2*R*), respectively. The sensitivity for bupropion and hydroxybupropion enantiomers is comparable to the previously published LC-MS/MS assay, although no attempt was made to maximize sensitivity.

4. Conclusion

The overall goal was to develop and validate a short high-throughput and completely stereoselective method for bupropion and its primary major metabolites. This is the first stereoselective bioanalytical method to quantify enantiomers of bupropion and the principle metabolites hydroxybupropion, erythrohydrobupropion and threohydrobupropion in human plasma, and is therefore an advance over previous methods, The validated method is within full compliance with FDA guidelines for assay validation. As a result of this work, erythrohydrobupropion and threohydrobupropion enantiomers can be resolved chromatographically and the enantiomers of bupropion and major metabolites can all be quantified using a single method.

Acknowledgments

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Highlights

- A stereoselective analytical method for bupropion and the principle metabolites was developed and validated on three AB Sciex triple quadrupole mass spectrometers (3200, 4000 QTRAP, and 6500).

- Bupropion, hydroxybupropion, erythrohydrobupropion and threohydrobupropion enantiomers were resolved utilizing a α_1-acid glycoprotein chiral column.

- Each erythrohydrobupropion and threohydrobupropion enantiomer peaks were stereochemically assigned by sodium borohydride reduction of enantiopure (*R*)-bupropion and (*S*)-bupropion.

- The validated assay was applied to determine plasma concentrations of (*R*)-bupropion, (*S*)-bupropion, (*R,R*)-hydroxybupropion, and (*S,S*)-hydroxybupropion, (*1S,2S*)-threohydrobupropion, (*1R,2R*)-threohydrobupropion, (*1R,2S*)-erythrohydrobupropion, and (*1S,2R*)-erythrohydrobupropion from a patient who received extended release (*R,S*)-bupropion.
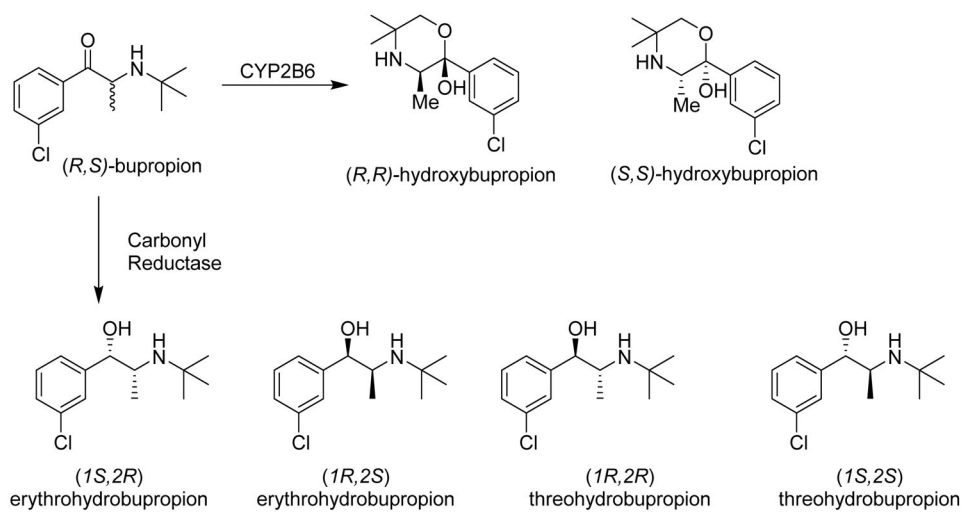
Figure 1.
Oxidative and reductive metabolic pathways of (*R,S*)-bupropion
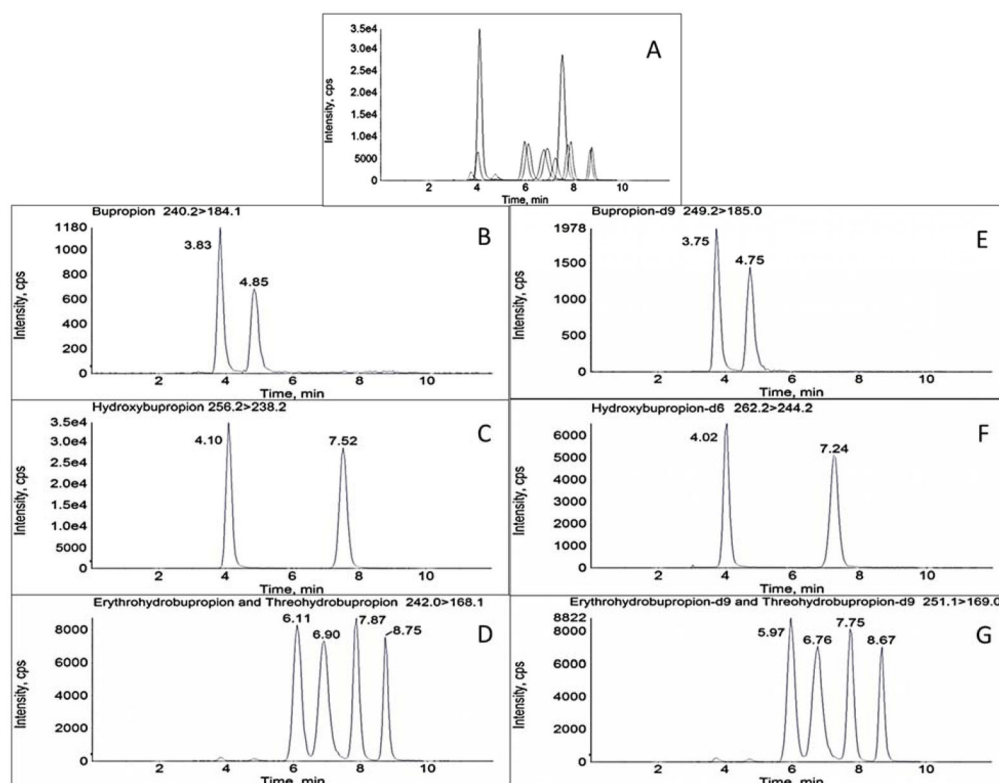
Figure 2.
Chromatographic separation of bupropion, hydroxybupropion, erythrohydrobupropion, and threohydrobupropion enantiomers and respective deuterated standards in processed plasma on an AB Sciex 3200 mass spectrometer. Numbers in parenthesis refer to retention times in min. A: Total ion chromatogram. B–G: Representative MRM chromatograms B: (*R*)-bupropion (3.83), (*S*)-bupropion (4.85). C: (*R,R*)-hydroxybupropion (7.52), (*S,S*)-hydroxybupropion (4.10). D: (*1S,2S*)-threohydrobupropion (6.11), (*1R,2R*)-threohydrobupropion) (6.90), *(1R,2S)*-erythrohydrobupropion (7.87), and *(1S,2R)*-erythrohydrobupropion (8.75). E: (*R*)-bupropion-d$_9$ (3.75), (*S*)-bupropion-d$_9$ (4.75). F: (*R,R*)-hydroxybupropion-d$_6$ (7.24), (*S,S*)-hydroxybupropion-d$_6$ (4.02). G: (*1S,2S*)-threohydrobupropion-d$_9$ (5.97), (*1R,2R*)-threohydrobupropion-d$_9$) (6.76), (*1R,2S*)-erythrohydrobupropion-d$_9$ (7.75), and *(1S,2R)*-erythrohydrobupropion-d$_9$ (8.67). Concentrations correspond to Calibration level 6 (10 ng/mL *R*- or *S*-bupropion; 250 ng/mL (*R,R*)- or (*S,S*)-hydroxybupropion; 50 ng/mL (*1R,2S*)- or (*1S,2R*)-erythrohydrobupropion; 50 ng/mL (*1S,2S*)- or (*1R,2R*)-threohydrobupropion)
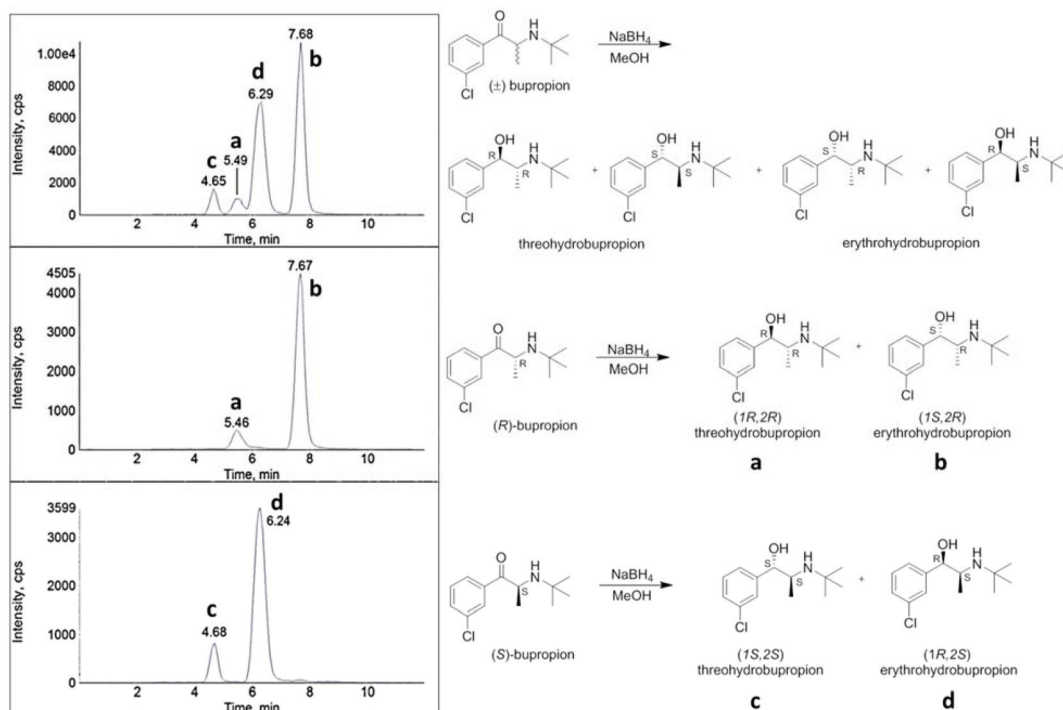
Figure 3.
Identification of erythrohydrobupropion enantiomer peaks and threohydrobupropion enantiomer peaks. Representative MRM chromatograms from the sodium borohydride reduction of: A: racemic (*R,S*)-bupropion, B: (*R*)-bupropion, and C: (*S*)-bupropion. Experiments were performed utilizing an AB Sciex 3200 instrument. Threohydrobupropion enantiomers elute at 4.65 (c) and 5.49 (a) min, respectively. Erythrohydrobupropion enantiomers elute at 6.29 (d) and 7.68 (b) min, respectively. To accurately identify each of the enantiomers, retention times of the peaks in panel A (a–d) were aligned with the retention times of the peaks in panel B (a and b) and C (c and d), respectively.
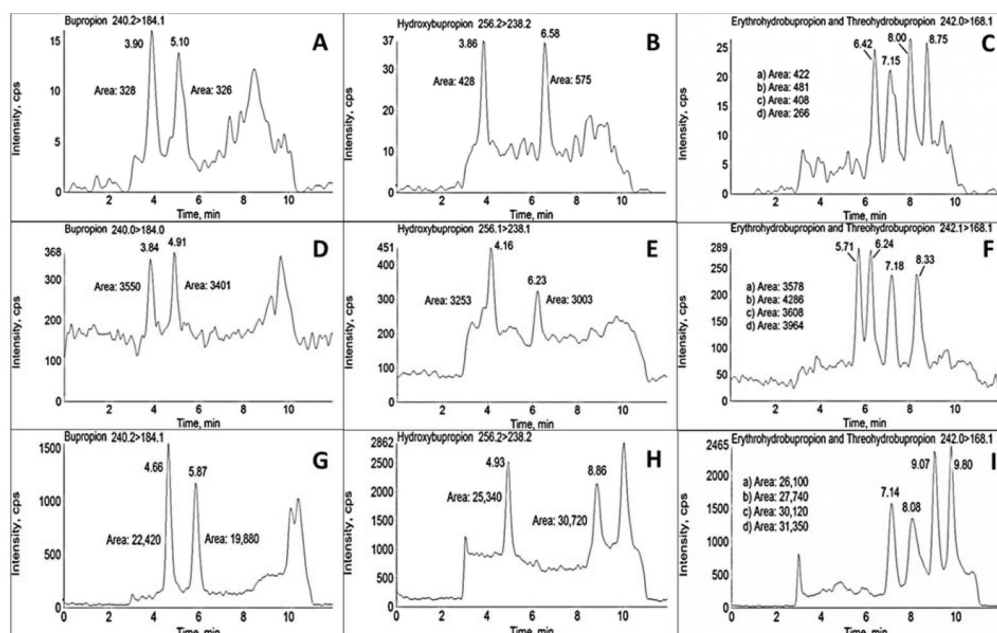
Figure 4.
Evaluation of instrument sensitivity at 0.25 ng/mL analyte concentrations. Analysis was performed on AB Sciex 3200 (A–C, top), 4000 QTRAP (D–F, middle), and 6500 (G–I, bottom) instruments. Analytes were (*R*)- and (*S*)-bupropion (A,D,G), (*R,R*)- and (*S,S*)-hydroxybupropion (B,E,H), (*1S,2S*)- and (*1R,2R*)-threohydrobupropion and (*1S,2R*)- and (*1R,2S*)-erythrohydrobupropion (C,F,I). Lowercase letters a-d (panels C,F,I) refer to (*1S,2S*)-threohydrobupropion, (*1R,2R*)-threohydrobupropion, *(1R,2S)*-erythrohydrobupropion, and *(1S,2R)*-erythrohydrobupropion, respectively. Peak areas are shown on each panel. Chromatograms are shown to maximize peak areas; note however the difference in peak intensity (Y axis) ranges. There were small differences in retention times between the three instruments.
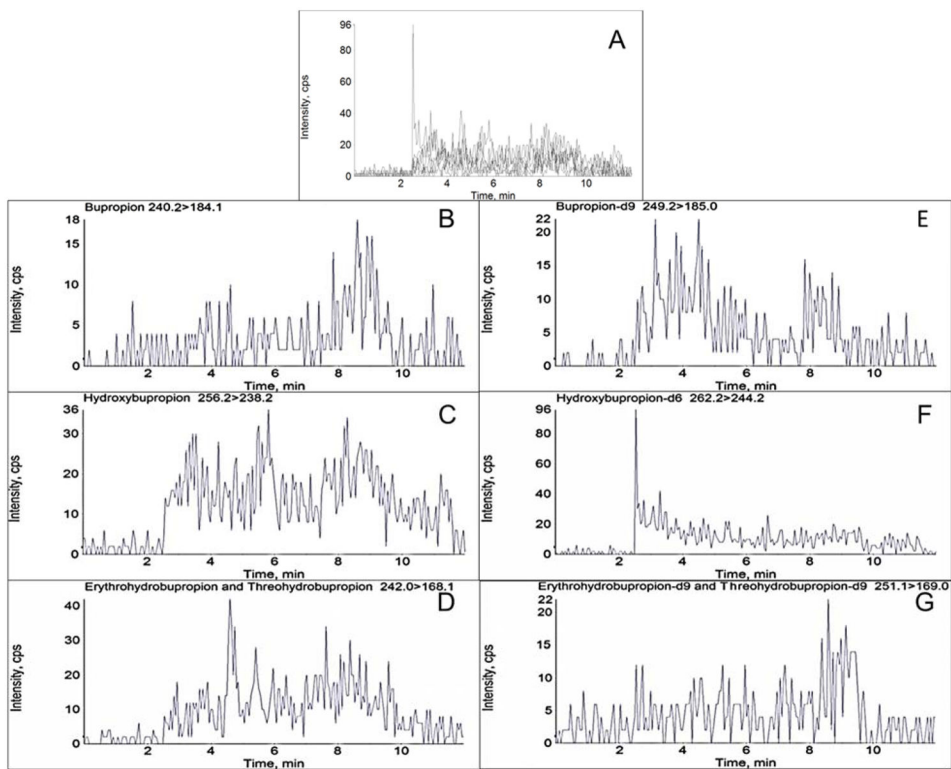
Figure 5.
Analysis of analyte- and internal standard free human plasma on an AB Sciex 3200 mass spectrometer. (A) Representative total ion chromatogram and representative MRM chromatograms of: (B) bupropion, (C) hydroxybupropion, (D) erythrohydrobupropion and threohydrobupropion, (E) bupropion-d_9, (F) hydroxybupropion-d_6, (G) erythrohydrobupropion-d_9, and threohydrobupropion-d_9.
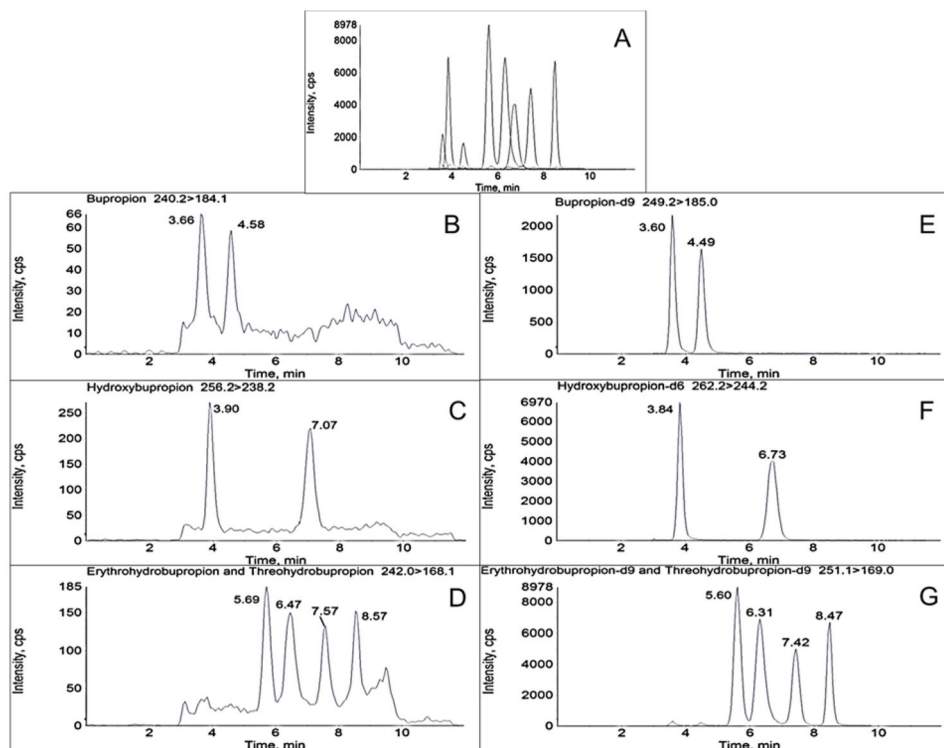
Figure 6.
Representative MRM chromatograms of a plasma calibration sample at the limit of quantification on an AB Sciex 3200 mass spectrometer (A) Total ion chromatogram, (B) 0.5 ng/mL bupropion (*R* and *S*), (C) 2 ng/mL hydroxybupropion (*R,R* and *S,S*) (D) 1 ng/mL threohydrobupropion (*1S,2S* and *1R,2R*) and erythrohydrobupropion (*1S,2R* and *1R,2S*). Panels E-G show the deuterated internal standards (E) 10 ng/mL (R)- and (S)-bupropion-d$_9$, (F) 50 ng/mL (*R,R)*- and (*S,S*)- hydroxybupropion-d$_6$, (G) 25 ng/mL (*1S,2S)*- and *(1R,2R)*- threohydrobupropion-d$_9$ and (*1S,2R)*- and (*1R,2S*)-erythrohydrobupropion-d$_9$.
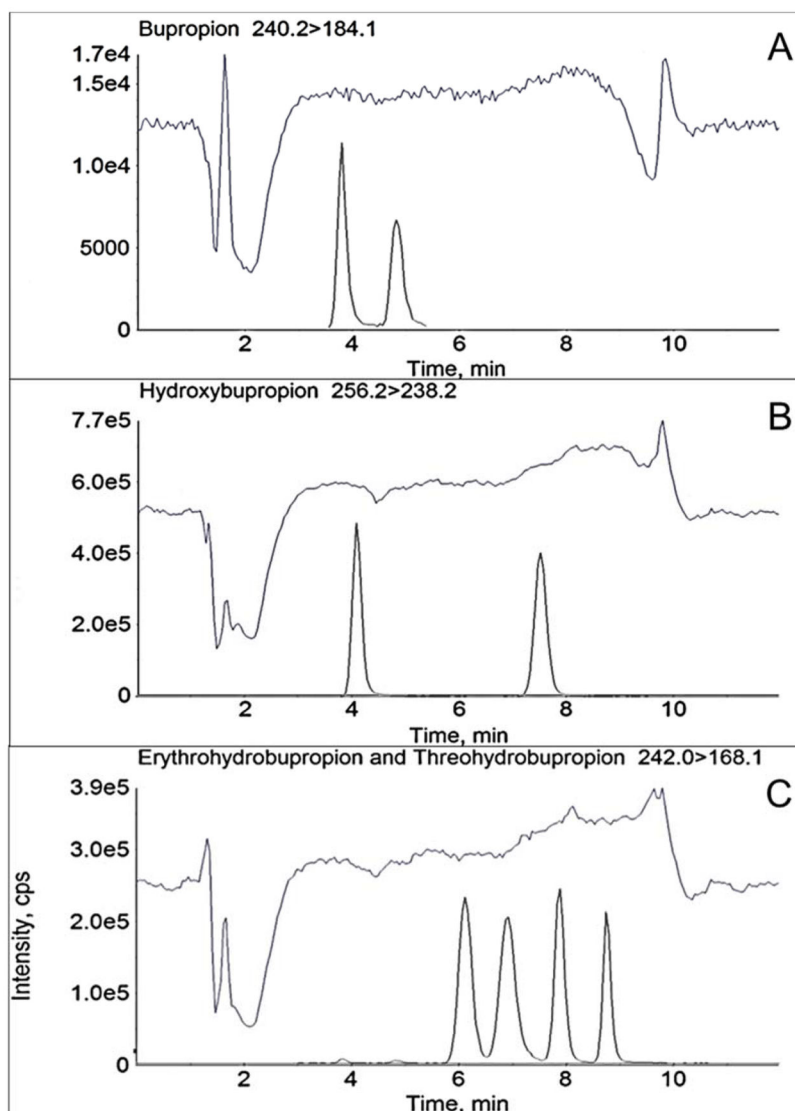
Figure 7.
Evaluation of ionization suppression. Extracted ion chromatograms were acquired after injecting analyte- and internal standard free human plasma while simultaneously infusing a solution of analytes and internal standards into the AB Sciex 3200 mass spectrometer: (A) bupropion, (B) hydroxybupropion, and (C) erythrohydrobupropion and threohydrobupropion. Peak overlays are from a calibration standard to show the respective retention time of each analyte.
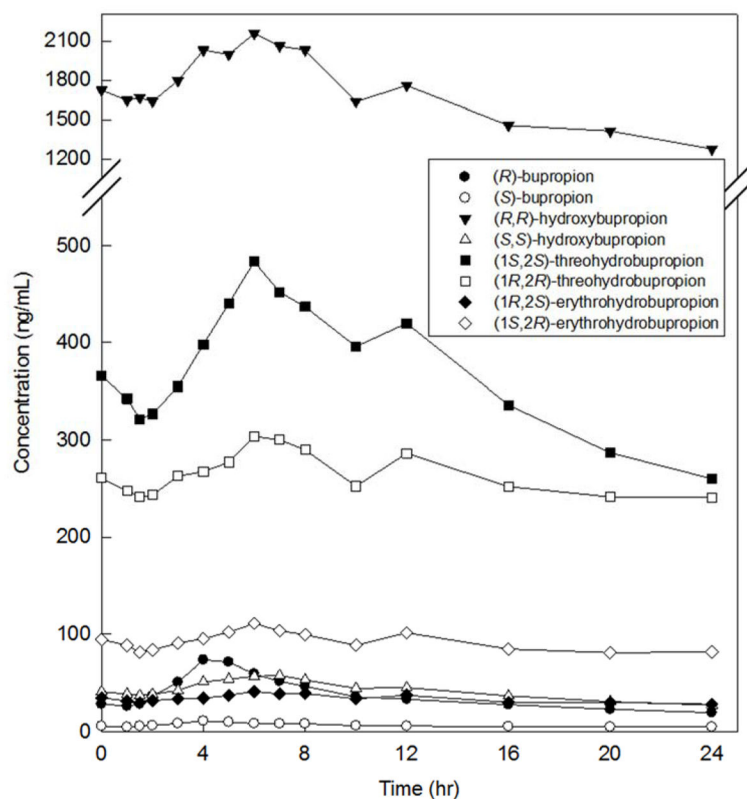
Figure 8.
Plasma concentrations of (*R*)-bupropion, (*S*)-bupropion, (*R,R*)-hydroxybupropion, (*S,S*)-hydroxybupropion, (*1S,2S*)-threohydrobupropion, (*1R,2R*)-threohydrobupropion, *(1R,2S)*-erythrohydrobupropion, and *(1S,2R)*-erythrohydrobupropion from a research subject who received 300 mg oral extended release bupropion each morning.

Table 1

Final 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
bupropion (*R* and *S*)	0.5, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100	2.5	10	25	50	
hydroxybupropion (*R,R* and *S,S*)	2, 5, 10, 25, 50, 250, 500, 750, 1000, 1250, 1500, 2000, 2500, 3000	5	25	250	1000	2000
threohydrobupropion (*1S,2S)* and (*1R,2R*)	1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 400, 600	2.5	10	50	200	
erythrohydrobupropion (*1R,2S* and (*1S,2R*)	1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 400, 600	2.5	10	50	200	

[a] Analyte-specific calibrator and QC concentrations chosen based on a preliminary analysis of patient plasma from a clinical study in patients receiving 300 mg oral bupropion daily at steady-state.

Table 2

Analyte and internal standard MS/MS optimized parameters on AB Sciex 3200, 4000 QTRAP, and 6500 mass spectrometers.

Analyte	MRM transition (*m/z*)	Declustering potential	Entrance potential	Collision energy	Exit potential
AB Sciex 3200					
bupropion	240.2 > 184.1	31	3.5	15	14
bupropion-d9	249.2 > 185.0	36	5.5	17	14
hydroxybupropion	256.2 > 238.2	36	3.5	17	20
hydroxybupropion-d6	262.2 > 244.2	36	5	17	20
erythrohydrobupropion	242.0 > 168.1	31	4	21	14
erythrohydrobupropion-d9	251.1 > 169.0	41	8	25	3
threohydrobupropion	242.0 > 168.1	31	4	21	14
threohydrobupropion-d9	251.1 > 169.0	41	8	25	3
AB Sciex 4000 QTRAP					
bupropion	240.0 > 184.0	56	10	19	28
bupropion-d9	249.3 > 185.1	61	10	19	12
hydroxybupropion	256.1 > 238.1	46	10	17	6
hydroxybupropion-d6	262.3 > 131.1	61	10	43	22
erythrohydrobupropion	242.1 > 168.1	66	10	23	26
erythrohydrobupropion-d9	251.2 > 169.2	106	10	27	28
threohydrobupropion	242.1 > 168.1	66	10	23	26
threohydrobupropion-d9	251.2 > 169.2	106	10	27	28
AB Sciex 6500					
bupropion	240.2 > 184.1	31	3.5	15	14
bupropion-d9	249.2 > 185.0	36	5.5	17	14
hydroxybupropion	256.2 > 238.2	36	3.5	17	20
hydroxybupropion-d6	262.2 > 244.2	36	5.0	17	20
erythrohydrobupropion	242.0 > 168.1	31	4.0	21	14
erythrohydrobupropion-d9	251.1 > 169.0	41	8.0	25	3
threohydrobupropion	242.0 > 168.1	31	4.0	21	14
threohydrobupropion-d9	251.1 > 169.0	41	8.0	25	3

Table 3

Intra-assay and inter- assay accuracy and precision for determination of bupropion and metabolites in human plasma[a] on an AB Sciex 3200 mass spectrometer

Analyte	Nominal concentration (ng/mL)	Intra-assay (n=3)			Inter-assay (n=5)		
		Calculated Concentration (ng/mL)[b]	Accuracy (%)	Precision (%)	Calculated Concentration (ng/mL)[b]	Accuracy (%)	Precision (%)
(R)-bupropion	2.5	2.5 ± 0.1	101	5	2.3 ± 0.2	92	7
	25	25.0 ± 2.5	100	10	22.3 ± 2.0	91	9
	500	572 ± 58	114	10	481 ± 63	96	13
(S)-bupropion	2.5	2.6 ± 0.2	103	9	2.4 ± 0.2	97	8
	25	24.6 ± 1.8	98	7	22.5 ± 1.1	90	5
	500	577 ± 32	115	5	521 ± 47	104	9
(2R)-hydroxybupropion	2.5	2.6 ± 0.1	104	4	2.4 ± 0.2	97	7
	25	24.7 ± 1.2	99	5	23.0 ± 0.7	92	3
	500	519 ± 16	104	3	475 ± 30	95	6
(2S)-hydroxybupropion	2.5	2.2 ± 0.2	89	10	2.3 ± 0.2	90	8
	25	25.3 ± 2.5	101	10	23.1 ± 0.8	92	3
	500	534 ± 16	107	3	481 ± 40	96	8
(1S,2S)-threohydrobupropion	2.5	2.6 ± 0.1	106	5	2.3 ± 0.2	94	11
	25	25.9 ± 3.2	104	12	23.0 ± 0.8	92	4
	500	523 ± 29	105	6	473 ± 38	95	8
(1R,2R)-threohydrobupropion	2.5	2.6 ± 0.1	99	3	2.4 ± 0.2	97	9
	25	24.9 ± 1.9	93	8	22.8 ± 1.3	91	6
	500	538 ± 39	103	7	479 ± 53	96	11
(1R,2S)-erythrohydrobupropion	2.5	2.6 ± 0.2	105	8	2.4 ± 0.3	94	13
	25	25.1 ± 2.1	96	8	22.4 ± 1.4	90	6
	500	544 ± 23	106	4	481 ± 34	96	7
(1S,2R)-erythrohydrobupropion	2.5	2.5 ± 0.3	99	11	2.5 ± 0.4	100	15
	25	24.8 ± 2.7	99	11	23.5 ± 1.0	94	4
	500	531 ± 51	106	10	481 ± 40	96	8

[a] Calibration standards were 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 ng/mL

bMean ± standard deviation

Table 4

Intra- assay and inter- assay accuracy and precision for determination of bupropion and metabolites in human plasma on an AB Sciex 4000 QTRAP mass spectrometer

Analyte	Nominal concentration (ng/mL)	Intra-assay (n=3)			Inter-assay (n=5)		
		Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%)	Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%)
(*R*)-bupropion	2.5	2.5 ± 0.3	99	13	2.4 ± 0.2	94	9
	25	23.0 ± 1.1	92	5	23.1 ± 1.5	92	6
	500	517 ± 39	103	8	510 ± 50	102	10
(*S*)-bupropion	2.5	2.4 ± 0.1	94	2	2.5 ± 0.1	99	3
	25	21.9 ± 0.9	88	4	22.7 ± 1.2	91	5
	500	532 ± 13	106	2	524 ± 44	105	8
(*R,R*)-hydroxybupropion	2.5	2.3 ± 0.2	94	7	2.4 ± 0.3	96	12
	25	22.0 ± 1.1	88	5	22.8 ± 1.2	91	5
	500	481 ± 22	96	5	481 ± 35	96	7
(*S,S*)-hydroxybupropion	2.5	2.4 ± 0.3	98	12	2.3 ± 0.3	92	12
	25	23.0 ± 2.5	92	11	22.9 ± 1.3	92	6
	500	490 ± 43	98	9	471 ± 30	94	6
(1*S*,2*S*)-threohydrobupropion	2.5	2.4 ± 0.1	94	6	2.4 ± 0.3	96	11
	25	24.3 ± 1.1	97	5	23.1 ± 1.5	93	7
	500	512 ± 27	102	5	472 ± 50	94	11
(1*R*,2*R*)-threohydrobupropion	2.5	2.3 ± 0.2	90	8	2.4 ± 0.3	97	14
	25	23.7 ± 2.5	95	11	23.0 ± 1.0	92	4
	500	519 ± 38	104	7	481 ± 42	96	9
(1*R*,2*S*)-erythrohydrobupropion	2.5	2.6 ± 0.3	105	10	2.3 ± 0.3	90	12
	25	23.6 ± 2.1	94	9	21.6 ± 1.3	87	6
	500	480 ± 18	96	4	461 ± 56	92	12
(1*S*,2*R*)-erythrohydrobupropion	2.5	2.3 ± 0.2	94	7	2.4 ± 0.3	95	11
	25	22.9 ± 2.6	91	11	23.3 ± 1.7	93	7
	500	490 ± 39	98	8	487 ± 34	97	7

Results are the mean ± standard deviation

Table 5

Intra-assay and inter-assay accuracy and precision for determination of bupropion and metabolites in human plasma on an AB Sciex 6500 mass spectrometer

Analyte	Nominal concentration (ng/mL)	Intra-assay (n=3)			Inter-assay (n=5)		
		Calculated Concentration (ng/mL)	Accuracy(%)	Precision (%)	Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%)
(R)-bupropion	2.5	2.2 ± 0.1	88	5	2.3 ± 0.3	93	11
	25	21.1 ± 1.1	85	5	22.5 ± 1.5	90	7
	500	494 ± 13	99	3	498 ± 35	100	7
(S)-bupropion	2.5	2.3 ± 0.2	91	7	2.4 ± 0.3	96	11
	25	21.6 ± 1.4	86	7	22.7 ± 1.7	91	8
	500	522 ± 11	104	2	534 ± 52	107	10
(R,R)-hydroxybupropion	2.5	2.1 ± 0.1	84	7	2.3 ± 0.3	91	14
	25	20.6 ± 1.3	82	6	21.9 ± 1.8	88	8
	500	518 ± 23	104	4	534 ± 34	107	6
(S,S)-hydroxybupropion	2.5	2.3 ± 0.0	93	2	2.3 ± 0.3	93	11
	25	20.9 ± 1.7	84	8	21.7 ± 2.0	87	9
	500	485 ± 7	97	1	480 ± 28	96	6
(1S,2S)-threohydrobupropion	2.5	2.4 ± 0.1	94	6	2.4 ± 0.2	96	10
	25	22.2 ± 1.1	89	5	23.0 ± 1.2	92	5
	500	457 ± 43	91	9	485 ± 39	97	8
(1R,2R)-threohydrobupropion	2.5	2.3 ± 0.2	90	7	2.4 ± 0.2	96	9
	25	21.7 ± 0.7	87	3	22.8 ± 1.2	91	5
	500	492 ± 9	98	2	493 ± 36	99	7
(1R,2S)-erythrohydrobupropion	2.5	2.3 ± 0.1	91	6	2.3 ± 0.3	91	14
	25	22.6 ± 0.6	90	3	22.9 ± 1.6	92	7
	500	478 ± 15	95	3	485 ± 49	97	10
(1S,2R)-erythrohydrobupropion	2.5	2.3 ± 0.2	91	7	2.4 ± 0.3	96	12
	25	21.6 ± 1.2	87	6	22.7 ± 1.5	91	7
	500	483 ± 6	97	1	489 ± 42	98	9

Results are the mean ± standard deviation

Table 6

Intra-day and inter-day accuracy and precision for determination of bupropion and metabolites in human plasma with analyte specific calibration ranges[a] on an AB Sciex 3200 mass spectrometer

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	2.5	2.5 ± 0.2	99	6	2.6 ± 0.3	106	10
	10	9.8 ± 0.3	98	3	9.9 ± 0.5	99	5
	25	25.5 ± 1.0	102	4	25.8 ± 1.2	103	5
	50	49.1 ± 2.3	98	5	49.6 ± 3.1	99	6
(S)-bupropion	2.5	2.1 ± 0.1	97	6	2.5 ± 0.1	100	3
	10	7.8 ± 0.5	94	6	9.4 ± 0.4	94	5
	25	20.8 ± 0.9	100	4	25.7 ± 0.9	103	3
	50	41.1 ± 1.7	100	4	49.2 ± 1.9	98	4
(R,R)-hydroxybupropion	5	4.8 ± 0.4	96	9	5.1 ± 0.2	102	3
	25	27.7 ± 2.0	111	7	27.6 ± 1.1	110	4
	250	260 ± 9	104	4	262 ± 12	105	5
	1000	969 ± 38	97	4	964 ± 42	96	4
	2000	1990 ± 151	100	8	2035 ± 121	102	6
(S,S)-hydroxybupropion	5	5.0 ± 0.3	100	7	5.1 ± 0.3	102	5
	25	26.1 ± 1.2	104	5	26.1 ± 0.1	105	0
	250	248 ± 10	99	4	246 ± 5	98	2
	1000	963 ± 33	96	3	955 ± 29	96	3
(1S,2S)-threohydrobupropion	2.5	2.5 ± 0.2	100	9	2.5 ± 0.1	100	5
	10	9.7 ± 0.6	97	6	9.8 ± 0.2	98	2
	50	49.0 ± 2.1	98	4	50.0 ± 2.1	100	4
	200	200 ± 6	100	3	201 ± 3	100	1
(1R,2R)-threohydrobupropion	2.5	2.5 ± 0.2	99	10	2.5 ± 0.0	99	2
	10	9.6 ± 0.5	96	5	9.8 ± 0.5	98	5
	50	49.2 ± 2.4	98	5	50.8 ± 1.8	102	3
	200	200 ± 7	100	4	202 ± 8	101	4
(1R,2S)-erythrohydrobupropion	2.5	2.5 ± 0.3	100	12	2.6 ± 0.2	105	8

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 (%)
	10	9.6 ± 0.7	96	7	9.8 ± 0.3	98	3
	50	48.2 ± 2.5	96	5	50.8 ± 2.0	102	4
	200	197 ± 8	99	4	198 ± 6	99	3
(1S,2R)-erythrohydrobupropion	2.5	2.5 ± 0.2	100	7	2.5 ± 0.1	100	4
	10	9.9 ± 0.5	99	5	9.9 ± 0.3	99	3
	50	48.6 ± 2.0	97	4	50.6 ± 1.7	101	3
	200	201 ± 6	101	3	203 ± 4	101	2

[a]Calibration ranges are provided in Table 1

[b]Mean ± standard deviation

Table 7

Stability of bupropion and metabolites in human plasma

Analyte	Nominal Concentration (ng/mL)	Stability in plasma (%)[a] (n = 3)			
		1st freeze-thaw	2nd freeze-thaw	3rd freeze-thaw	48 hr in 4°C autosampler
(*R*)-bupropion	2.5	93 ± 11	103 ± 11	88 ± 11	102 ± 3
	50	98 ± 2	95 ± 1	97 ± 3	104 ± 2
(*S*)-bupropion	2.5	113 ± 1	104 ± 4	110 ± 8	115 ± 3
	50	96 ± 2	94 ± 1	93 ± 1	101 ± 5
(*R,R*)-hydroxybupropion	5	102 ± 1	104 ± 8	101 ± 9	99 ± 7
	2000	96 ± 2	97 ± 2	96 ± 2	102 ± 2
(*S,S*)-hydroxybupropion	5	101 ± 6	99 ± 5	97 ± 10	92 ± 2
	2000	96 ± 2	98 ± 4	97 ± 6	100 ± 2
(1*S*,2*S*)-threohydrobupropion	2.5	96 ± 7	102 ± 11	99 ± 6	104 ± 6
	200	96 ± 2	96 ± 2	95 ± 2	101 ± 2
(1*R*,2*R*)-threohydrobupropion	2.5	96 ± 7	101 ± 9	97 ± 11	104 ± 10
	200	98 ± 4	100 ± 3	95 ± 1	99 ± 3
(1*R*,2*S*)-erythrohydrobupropion	2.5	100 ± 5	109 ± 8	109 ± 6	112 ± 5
	200	98 ± 3	98 ± 4	99 ± 1	103 ± 3
(1*S*,2*R*)-erythrohydrobupropion	2.5	93 ± 6	102 ± 10	92 ± 9	100 ± 5
	200	97 ± 0	98 ± 1	96 ± 1	102 ± 3

[a]Reported values are the percentage of the initial observed concentration (prepared freshly with no freeze thaw cycles), mean ± standard deviation