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Stereoselective analysis of bupropion and hydroxybupropion in human plasma and urine by LC/MS/MS

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Abstract

A sensitive, stereoselective assay using solid phase extraction and LC–MS–MS was developed and validated for the analysis of (*R*)- and (*S*)-bupropion and its major metabolite (*R*,*R*)- and (*S*,*S*)-hydroxybupropion in human plasma and urine. Plasma or glucuronidase-hydrolyzed urine was acidified, then extracted using a Waters Oasis MCX solid phase 96-well plate. HPLC separation used an α_1-acid glycoprotein column, a gradient mobile phase of methanol and aqueous ammonium formate, and analytes were detected by electrospray ionization and multiple reaction monitoring with an API 4000 Qtrap. The assay was linear in plasma from 0.5 to 200 ng/ml and 2.5 to 1000 ng/ml in each bupropion and hydroxybupropion enantiomer, respectively. The assay was linear in urine from 5 to 2000 ng/ml and 25 to 10,000 ng/ml in each bupropion and hydroxybupropion enantiomer, respectively. Intra- and inter-day accuracy was >98% and intra- and inter-day coefficients of variations were less than 10% for all analytes and concentrations. The assay was applied to a subject dosed with racemic bupropion. The predominant enantiomers in both urine and plasma were (*R*)-bupropion and (*R*,*R*)-hydroxybupropion. This is the first LC–MS/MS assay to analyze the enantiomers of both bupropion and hydroxybupropion in plasma and urine.

Keywords: Bupropion; Hydroxybupropion; Mass spectrometry; Chiral analysis; LC–MS–MS

1. Introduction

Bupropion is commonly used for the treatment of depression or smoking cessation and has been studied for a number of other uses such as bipolar disorder, attention-deficit hyperactivity disorder and weight loss [1–3]. Bupropion is extensively metabolized, with <0.5% reported to be recovered intact in urine [4]. Three active metabolites have been identified: hydroxybupropion, resulting from cytochrome P450-catalyzed oxidation, and erythrohydrobupropion and threohydrobupropion, reduced diastereomers formed via non-P450-dependent pathways. Although all three metabolites are pharmacologically active, their potency and toxicity relative to bupropion have not been fully characterized. According to *in vivo* studies using the mouse anti-tetrabenazine model of depression, the activity of hydroxybupropion is approximately 50% of the parent drug, and the activity of both erythrohydrobupropion and threohydrobupropion is approximately 20% of bupropion [5–9]. The average elimination half-lives are 8, 19, 35 and 19 h for bupropion, hydroxybupropion, erythrohydrobupropion and threohydrobupropion, respectively [10].

Bupropion is formulated and used clinically as a racemic mixture. The enantiomers have been shown to rapidly racemize in phosphate buffer (pH 7.4, 25 °C): 42%, 62%, and >94% in 2, 4, and 24 h, respectively [11]. When hydroxylated on the *N*-*t*-butyl carbon, bupropion rapidly undergoes ring closure, forming hydroxybupropion with two chiral centers (Fig. 1). However, only the (*R*,*R*)- and (*S*,*S*)-hydroxybupropion diastereomers are observed, possibly because the (*R*,*S*)- and (*S*,*R*)-diastereomers are sterically hindered [11]. Hydroxybupropion also racemizes in phosphate buffer (pH 7.4, 25 °C), albeit much more slowly: approximately 0.1% and 2% in 2 and 24 h, respectively [11]. Due to this slower rate of racemization, plasma concentrations of hydroxybupropion enantiomers accurately reflect stereospecific formation and elimination without confounding by racemization.

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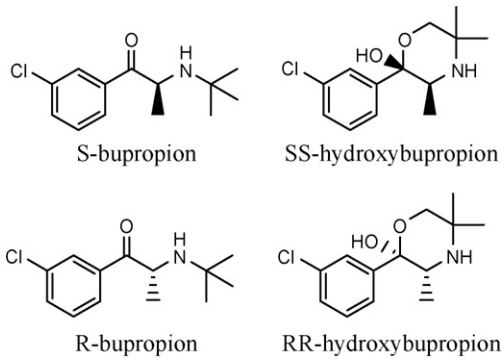

Fig. 1. Structures of bupropion and hydroxybupropion enantiomers.

Stereochemistry may be important for bupropion therapeutic effects. The potency of bupropion enantiomers *in vitro* does not differ from that of the racemate, although racemization at physiological conditions may explain the lack of stereoselectivity [12]. Racemic hydroxybupropion potency is similar to that of racemic bupropion, but (*S*,*S*)-hydroxybupropion is significantly more potent than both (*R*,*R*)-hydroxybupropion and racemic bupropion in a mouse depression model and in antagonism of acute nicotine effects in mice [13].

The primary enzyme in bupropion metabolism to hydroxybupropion is cytochrome P4502B6 (CYP2B6) [14–17]. Racemic bupropion hydroxylation has been used as an *in vitro* probe for the activity of CYP2B6 in liver microsomes [18–24]. More recently, racemic bupropion clearance has been used as an *in vivo* probe for the activity of hepatic CYP2B6, and for assessing the effects of CYP2B6 single nucleotide polymorphisms and CYP2B6 drug interactions [25–32]. We recently found that bupropion hydroxylation catalyzed by CYP2B6 and human liver microsomes is stereoselective, with the rate of (*S*)-bupropion hydroxylation about three times that of (*R*)-bupropion (submitted). Clearance of the bupropion enantiomers may provide a better *in vivo* probe for CYP2B6 activity. Nevertheless, there is no information regarding plasma concentrations of bupropion enantiomers.

The use of bupropion as an *in vivo* CYP2B6 probe is not straightforward. The non-stereoselective assays used to date have shown that the half-life of hydroxybupropion exceeds that of bupropion, suggesting that hydroxybupropion concentrations are elimination rate-limited rather than formation rate-limited. Therefore, the ratio of hydroxybupropion/bupropion area under the plasma concentration versus time curves does not accurately reflect the rate of bupropion hydroxylation *in vivo*. An alternative potential for assessing bupropion hydroxylation, and CYP2B6 activity, is the urine formation clearance of hydroxybupropion. Nonetheless, there is no information on urine hydroxybupropion formation clearance, or that of the enantiomers.

Several methods have been published for the stereoselective analysis of bupropion in plasma using coupled achiral–chiral liquid chromatography, an ovomucoid column, or an α_1-acid glycoprotein column [33–35]. Recently, a method was published for the stereoselective analysis of hydroxybupropion in plasma using a Cyclobond I 2000 column [36]. All of these methods, however, utilize ultraviolet detection and none analyze both bupropion and hydroxybupropion in a single assay. Published methods for the detection of bupropion and its metabolites by tandem mass spectrometry are available, but none are chiral [26,37]. In addition, there are no published assays for either chiral or achiral analysis of bupropion and its metabolites in urine. There is no information regarding the formation clearance of the hydroxybupropion enantiomers. Therefore, the purpose of this investigation was to develop and apply a stereoselective LC–MS–MS assay for the analysis, in human plasma and urine, of bupropion enantiomers and hydroxybupropion diastereomers in a single assay.

2. Experimental

2.1. Chemicals and reagents

All standards were racemic and of >98% chemical purity. (±)-Hydroxybupropion was purchased from BD Gentest (San Jose, CA) and the internal standards (±)-bupropion-d_9 and (±)-hydroxybupropion-d_6 were purchased from Toronto Research Chemicals (Ontario, Canada). Oasis MCX 96-well solid phase extraction (SPE) plates were supplied by Waters (Milford, MA). Crude ß-glucuronidase (type HP-2), (±)-bupropion and all other reagents were ACS or reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Instrumentation

Analysis was performed on an API 4000 Q-trap triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an electrospray source operated at 650 °C. The HPLC system consisted of two LC-20AC pumps with a CTO-20A oven, SIL-20A autosampler, DGU-20A3 degasser, FCF-11AL valve and a CBM 20A controller (Shimadzu, Columbia, MD) The chromatographic separation was performed on a chiral α_1-acid glycoprotein (AGP) column (100 × 2 mm, 5 μm) with a chiral AGP guard cartridge (10 × 2 mm, 5 μm) (ChromTech, Apple Valley, MO). The injection volume was 10 μl and the oven temperature was 28 °C. Before each injection, the needle was washed with methanol. Mobile phase (0.22 ml/min) was (A) 20 mM formate buffer, pH 5.7 and (B) methanol using the following program: 10% B for 0.5 min, linear gradient to 30% B between 0.5 and 1.0 min, held at 30% until 5 min, linear gradient to 50% until 8 min, held at 50% B until 15 min, then re-equilibrated to initial conditions between 15 and 20 min. Under these conditions, retention times for (*R*)- and (*S*)-bupropion were 8.4 and 10.1 min, and those for (*S*,*S*)- and (*R*,*R*)-hydroxybupropion were 9.3 and 13.5 min, respectively (Table 1). Both Q1 and Q3 quadrupoles were optimized to unit mass resolution, and the mass spectrometer conditions were optimized for each analyte. The instrument was operated in positive-ion mode with an ion spray voltage of 5500 V, entrance potential of 10 V and exit potential of 10 V. The curtain gas was set at 25, ion source gas 1 at 30, ion source gas 2 at 20 and the collision gas on high. Transitions monitored for each analyte, along with the analyte-specific parameters, are listed in Table 1.

Table 1
Multiple-reaction monitoring transitions and compound specific settings

Compound	Retention time (min)[a]	Resolution between enantiomers[b]	Mass transition (*m/z*)	Declustering potential (V)	Collision energy (V)
Bupropion	8.4, 10.1	3.0	240.0 > 184.0	56	19
Bupropion-d_9	8.3, 10.0	3.2	249.2 > 184.0	61	27
Hydroxybupropion	9.3, 13.5	7.3	256.1 > 238.1	51	17
Hydroxybupropion-d_6	9.2, 13.4	6.1	262.2 > 244.4	56	19

[a] Retention time for each enantiomer.
[b] Resolution = $1.18(t_b - t_a)/(W_{1/2a} + W_{1/2b})$.

2.3. Plasma analysis

Solid phase extraction of plasma samples was performed by adding 25 μl of internal standard (5 ng of (±)-bupropion-d_9 and 25 ng of (±)-hydroxybupropion-d_6), 250 μl plasma, and then 1 ml of 4% phosphoric acid to each well of a 96-well plate. The plate was capped and vortexed. Oasis MCX 96 well SPE plates were conditioned with 1 ml methanol, then 1 ml of water. Samples were transferred to the SPE plate using a multi-channel pipette. The SPE plate was washed with 1 ml 0.1 N HCl, 1 ml methanol and 1 ml 50:48:2 methanol:water:ammonium hydroxide. The SPE plate was dried at full vacuum for 2 min, then the samples were eluted with 0.5 ml 80:18:2 methanol:water:ammonium hydroxide. After elution the samples were acidified by adding 100 μl of 2 M ammonium formate, pH 4.0 to each well. A 200 μl aliquot of each sample was transferred to an autosampler plate and 10 μl was injected. Calibrators and quality control (QC) samples were prepared along with patient samples.

2.4. Urine analysis

Solid phase extraction of urine samples was performed by pipetting 25 μl of internal standard (5 ng of (±)-bupropion-d_9 and 25 ng of (±)-hydroxybupropion-d_6), 25 μl of urine, then 0.5 ml of ß-glucuronidase (5000 units/ml in 0.1 M acetate buffer, pH 5.0) to each well of a 96-well plate. The plate was capped, vortexed, then incubated at 37 °C for 16 h. SPE plates were

Table 2
Accuracy and precision[a] (CV) of plasma validation samples and dilutions

Compound	Analyte concentration (ng/ml)					Dilutions	
	0.5	1	10	100	200	200 × 10	200 × 20
(*R*)-Bupropion							
Mean ± S.D.	0.52 ± 0.03	1.03 ± 0.05	10.3 ± 0.4	104 ± 5	202 ± 6	19.9 ± 0.2	10.1 ± 0.2
Accuracy (%)	104	103	103	104	101	100	101
Within run CV (%)	5	3	2	3	2	1	2
Between run CV (%)	3	4	3	4	2		
Total CV (%)	6	5	4	5	3		
(*S*)-Bupropion							
Mean ± S.D.	0.50 ± 0.02	1.05 ± 0.06	10.5 ± 0.6	105 ± 9	213 ± 15	20.2 ± 0.3	10.0 ± 0.1
Accuracy (%)	100	105	105	105	107	101	100
Within run CV (%)	3	2	4	3	2	1	
Between run CV (%)	3	5	5	8	7		
Total CV (%)	5	6	6	9	7		
Compound	Analyte concentration (ng/ml)					Dilutions	
	2.5	5	50	500	1000	1000 × 10	1000 × 20
(*S*,*S*)-Hydroxybupropion							
Mean ± S.D.	2.58 ± 0.15	5.35 ± 0.36	52.4 ± 2.4	493 ± 23	1000 ± 39	106 ± 1	53.5 ± 1.5
Accuracy (%)	103	107	105	99	100	106	107
Within run CV (%)	4	6	4	4	3	1	3
Between run CV (%)	4	4	2	3	2		
Total CV (%)	6	7	5	5	4		
(*R*,*R*)-Hydroxybupropion							
Mean ± S.D.	2.58 ± 0.19	4.95 ± 0.31	51.6 ± 1.6	498 ± 37	1039 ± 54	100 ± 2	51.3 ± 0.3
Accuracy (%)	103	99	103	100	104	100	103
Within run CV (%)	2	6	3	4	4	2	1
Between run CV (%)	7	2	1	6	4		
Total CV (%)	7	6	3	7	5		

[a] $n = 3$ replicates for within run CV and $n = 3$ batches for between run CV.

Table 3
Accuracy and Precision[a] (CV) of urine validation samples and dilutions

Compound	Analyte concentration (ng/ml)					Dilutions
	5	10	100	1000	2000	2000 × 10
(*R*)-Bupropion						
Mean ± S.D.	5.0 ± 0.2	10.1 ± 0.2	99 ± 2	1015 ± 30	2004 ± 48	202 ± 8
Accuracy (%)	101	101	99	102	100	101
Within run CV (%)	1	1	2	2	2	4
Between run CV (%)	3	2	1	2	1	
Total CV (%)	3	2	2	3	2	
(*S*)-Bupropion						
Mean ± S.D.	5.0 ± 0.1	10.0 ± 0.2	100 ± 2	1011 ± 28	1992 ± 34	200 ± 6
Accuracy (%)	99	100	100	101	100	100
Within run CV (%)	1	2	2	3	2	3
Between run CV (%)	2	1	1	1	1	
Total CV (%)	2	2	2	3	2	
Compound	Analyte concentration (ng/ml)					Dilutions
	25	50	500	5000	10000	10000 × 10
(*S*,*S*)-Hydroxybupropion						
Mean ± S.D.	24.9 ± 1.0	50.6 ± 1.6	500 ± 13	4992 ± 109	9855 ± 176	1052 ± 18
Accuracy (%)	100	101	100	100	99	105
Within run CV (%)	4	2	2	2	1	2
Between run CV (%)	2	2	1	1	1	
Total CV (%)	4	3	3	2	2	
(*R*,*R*)-Hydroxybupropion						
Mean ± S.D.	24.9 ± 0.5	50.0 ± 2.0	494 ± 9	4980 ± 158	10284 ± 297	1075 ± 16
Accuracy (%)	100	100	99	100	103	2
Within run CV (%)	2	4	2	2	2	
Between run CV (%)	1	1	1	3	2	
Total CV (%)	2	4	2	3	3	

[a] $n = 3$ replicates for within run CV and $n = 3$ batches for between run CV.

conditioned with 1 ml methanol, then 1 ml of water. Samples were centrifuged at 2500 rpm for 5 min, then the supernatant was transferred to the SPE plate using a multi-channel pipette. The SPE plate was washed with 1 ml 0.1N HCl, 1 ml methanol and 1 ml 50:48:2 methanol:water:ammonium hydroxide. The plate was dried at full vacuum for 2 min, then the samples were eluted with 0.5 ml 80:18:2 methanol:water:ammonium hydroxide. After elution the samples were acidified by adding 100 μl of 2 M ammonium formate, pH 4.0 to each well. A 200 μl aliquot of each sample was transferred to an autosampler plate and 10 μl was injected. Calibrators and QCs were prepared along with patient samples.

2.5. Calibrators, quality control and internal standard samples

Methanolic solutions at 1 mg/ml were prepared of each analyte, bupropion, hydroxybupropion, bupropion-d_9, and hydroxybupropion-d_6 taking into account salt concentrations and purity. Dilutions from these stock standards were prepared and used to make calibrator and quality control (QC) samples in plasma and urine. Plasma calibrators contained 1, 2, 4, 10, 20, 40, 100, and 400 ng/ml racemic bupropion (0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/ml of each enantiomer) and 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml racemic hydroxybupropion (2.5, 5, 10, 25, 50, 100, 250, 500, 1000 of each enantiomer). Plasma QCs were made at 1, 10 and 100 ng/ml in each bupropion enantiomer and 5, 50 and 500 ng/ml in each hydroxybupropion enantiomer. Urine calibrators and controls were made at 10 times the plasma levels. The internal standard was made in water at 0.1 μg/ml of each bupropion-d_9 enantiomer and 0.5 μg/ml of each hydroxybupropion-d_6 enantiomer.

2.6. Assay validation

Accuracy and precision (coefficient of variation, %CV) were evaluated at five different concentrations each in plasma and urine. These samples were extracted in triplicate in three different batches. Dilutions (10- and 20-fold) were also evaluated by extracting a high concentration sample in triplicate. The assay was considered acceptable if the variation and deviation were <15% for all samples.

Recovery and ion suppression were calculated by preparing a sample in elution solvent without extraction (neat), and comparing the peak area counts to a sample at the same concentration extracted from water (extraction recovery), a blank plasma sample and blank urine sample that was extracted and then spiked with the same concentration post-extraction (ion suppression) and samples at the same concentration extracted from plasma and urine (total recovery: extraction plus ion suppression) [38].

Table 4
Analyte recovery and ion suppression

	Urine				Plasma			
	ng/ml	Total recovery[a] (%)	Extraction recovery (%)	Ion suppression[b] (%)	ng/ml	Total recovery[a] (%)	Extraction recovery (%)	Ion suppression (%)
(*R*)-Bupropion	100	74	76	104	10	74	72	96
(*R*)-Bupropion-d_9	100	70	72	103	10	75	70	96
(*S*)-Bupropion	100	73	80	102	10	59	75	77
(*S*)-Bupropion-d_9	100	70	77	105	10	60	73	75
(*S*,*S*)-Hydroxybupropion	500	48	59	105	50	65	72	96
(*S*,*S*)-Hydroxybupropion-d_6	500	48	58	104	50	64	70	97
(*R*,*R*)-Hydroxybupropion	500	53	61	110	50	84	75	109
(*R*,*R*)-Hydroxybupropion-d_6	500	51	58	109	50	74	73	107

Results are the mean of three determinations.
[a] Extraction recovery plus ion suppression.
[b] Expressed as a percentage of the neat sample with no matrix effect.

Extraction recovery is determined from water to assess the SPE efficiency alone. Total recovery from plasma or urine is determined to assess matrix effects on SPE efficiency. The recovery and ion suppression are expressed as a percentage of the neat sample, indicating the change from 100% due to extraction loss or ion suppression/enhancement.

Ion suppression was further investigated by infusing a solution at 1 μg/ml in all analytes and internal standards and injecting extracted blank matrix samples. Both blank urine and blank plasma were extracted by the described method. For comparison, a blank plasma sample was also extracted without the third wash step of the SPE method (50:48:2 methanol:water:ammonium hydroxide) and by protein precipitation with acetonitrile.

Experiments were conducted to determine the optimal conditions for hydrolysis of hydroxybupropion conjugates. Urine (25 μl) plus internal standard was incubated in triplicate with 2500 units of ß-glucuronidase for 2 and 24 h at both 37 and 60 °C, then extracted and analyzed as described. Total hydroxybupropion concentration was calculated to determine the conditions for maximum hydrolysis. The enantiomeric excess was also compared to ensure that the hydrolysis was not causing excessive racemization.

Sample stability was evaluated in two ways. First, 11 plasma samples and 4 urine samples were extracted initially, and after being stored at −20 °C for 45 days to evaluate the stability of the enantiomers in matrix. Second, three plasma and three urine samples were subjected to three freeze/thaw cycles. Frozen samples were left at room temperature for 3 h then refrozen and this process repeated on two consecutive days. The stability was acceptable if the bias was within 15% of the initial result.

Both bupropion and hydroxybupropion racemize readily, causing possible errors in quantitation. Racemization in the final extract solution was evaluated by extracting 11 patient samples and re-injecting them after storage at various temperatures for 1, 4 and 7 days.

Carryover was assessed by injecting a low positive sample in triplicate, then a high sample followed by a single injection of the low positive sample. Carryover was defined as the difference between the average of the first three injections and the single injection after the high sample. This method was repeated for increasing high samples.

2.7. Method application

This method was applied to samples obtained from a clinical investigation of bupropion disposition. The study was approved by the Washington University Institutional Review Board and performed after obtaining written consent from the subject. The subject received 150 mg oral immediate release racemic bupropion, and venous blood samples were obtained at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after dosing and all urine was collected for 48 h. Plasma and urine samples were stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Mobile phase optimization

The LC method used in this paper was based initially on a previously published method for bupropion using an AGP column with UV detection [35]. When the ammonium acetate (pH 5.0) eluent from that method was applied to mass spectrometry, it resulted in poor ionization of bupropion and hydroxybupropion.

Table 5
Stability of extracted plasma samples

Temperature (°C)	Days stored	Enantiomeric excess (%)	
		Bupropion	Hydroxybupropion
10	0	35 ± 6	89 ± 4
	1	39 ± 4	85 ± 4
	4	34 ± 8	80 ± 5
	7	20 ± 16	71 ± 6
4	1	35 ± 4	89 ± 4
	4	33 ± 6	86 ± 4
	7	21 ± 9	84 ± 4
−20	1	36 ± 3	90 ± 4
	4	33 ± 4	90 ± 4
	7	ND[a]	90 ± 4

Eleven patient plasma samples were extracted and analyzed after storage under various conditions. Stability was assessed as the enantiomeric excess. Results are shown as the mean (±S.D.) of the 11 samples.
[a] ND = not determined (due to column interference).

By switching to ammonium formate (pH 5.7), the signal intensity was increased 100-fold. However, at pH 5.7, ammonium formate is not an ideal buffer and was not adequately buffering the sample, resulting in considerable retention time shifts between injections. To stabilize the retention time and neutralize the ammonium hydroxide in the SPE elution solvent, the sample was buffered prior to injection by adding 2 M ammonium formate at pH 4.0.

3.2. Peak identification

Because no analyte was available in an optically pure form, the enantiomer peaks were initially labeled as bupropion 1 and 2 and hydroxybupropion 1 and 2. After the patient samples were evaluated, the hydroxybupropion peaks were labeled as (*S*,*S*)-hydroxybupropion eluting first, followed by the larger (*R*,*R*)-hydroxybupropion peak, based upon previous reports that (*R*,*R*)-hydroxybupropion is the predominant enantiomer in human plasma [33,36]. Bupropion peaks were identified by incubating individual bupropion enantiomers (obtained by preparative chiral HPLC) with CYP2B6 under standard conditions [26], and the resulting hydroxybupropion peaks were analyzed (results not shown). The first eluting bupropion enantiomer was hydroxylated to (*R*,*R*)-hydroxybupropion, identifying it as *R*-bupropion. The second eluting bupropion enantiomer was hydroxylated to (*S*,*S*)-hydroxybupropion, identifying it as *S*-bupropion.

3.3. Assay validation

Sample chromatograms of the low and high calibration samples are shown in Fig. 2. Accuracy and precision of the validation

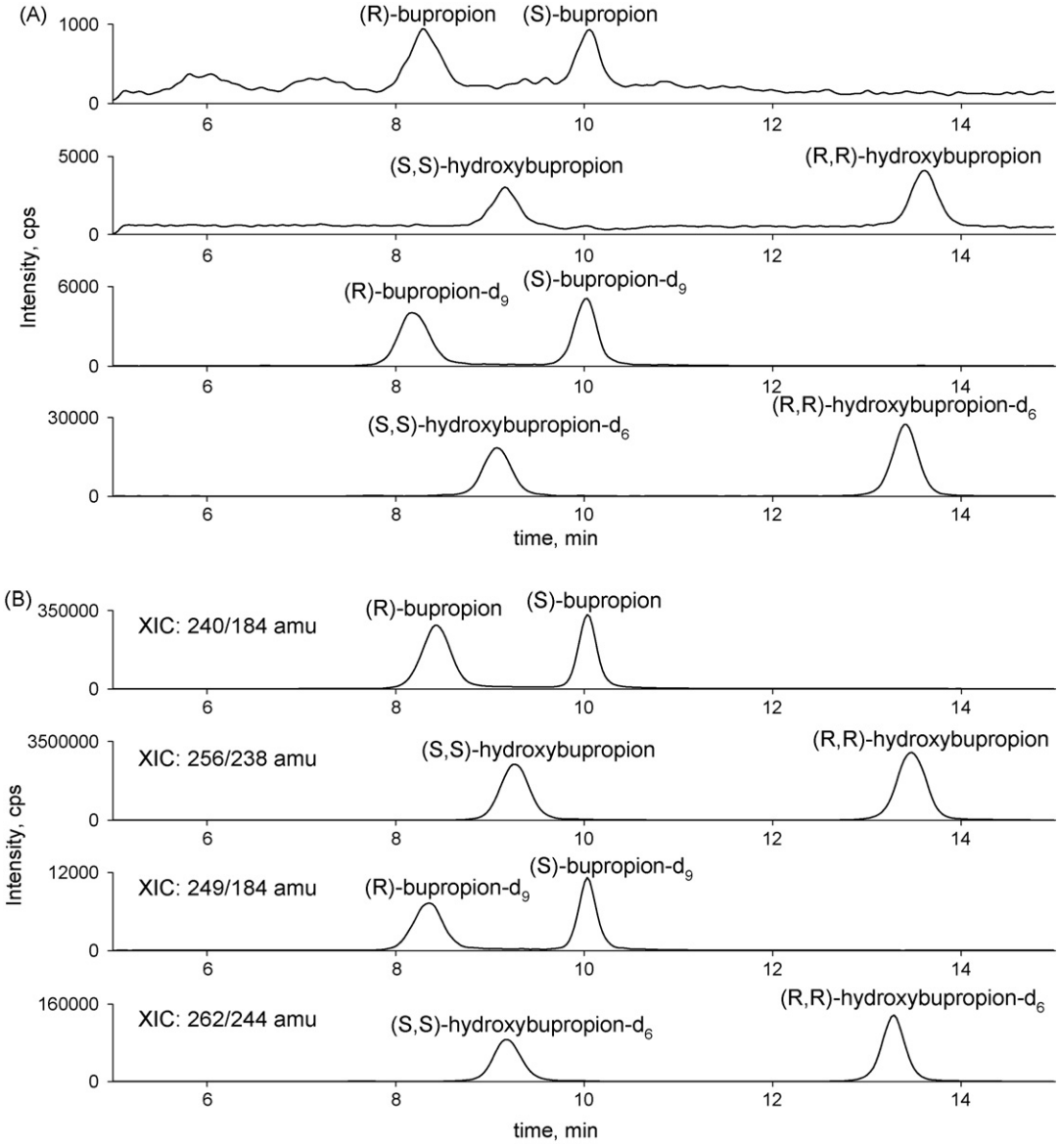

Fig. 2. Sample chromatograms. (A) Low calibrator (0.5 ng/ml each bupropion enantiomer and 2.5 ng/ml each hydroxybupropion enantiomer). (B) High calibrator (200 ng/ml each bupropion enantiomer and 1000 ng/ml each hydroxybupropion enantiomer). All samples also contained 10 ng/ml each bupropion-d_9 enantiomer and 50 ng/ml each hydroxybupropion-d_6 enantiomer.

samples and dilutions are listed in Tables 2 and 3. Within run precision was <6% and the between run precision was <8% for both bupropion and hydroxybupropion enantiomers. Accuracy was >98% for all analytes and all concentrations. Dilutions of the high validation samples was within 10% of expected concentrations with a CV <3% for both analytes and enantiomers. The assay was linear in plasma for bupropion enantiomers from 0.5 to 200 ng/ml and from 2.5 to 1000 ng/ml for hydroxybupropion enantiomers. The assay was linear in urine for bupropion enantiomers from 5 to 2000 ng/ml and from 25 to 10,000 ng/ml for hydroxybupropion enantiomers. No attempt was made to attain greater sensitivity.

Recovery and ion suppression for all analytes and internal standards are listed in Table 4. Recovery was >70% in plasma and >55% in urine for all analytes. Recovery in urine was lower, possibly due to the difficulty in transferring all the supernatant without disturbing the ß-glucuronidase pellet. The signal response loss due to ion suppression was negligible for all analytes except (*S*)-bupropion in plasma, where the response was 75% of that without matrix effect. The ion suppression infusion study (Fig. 3) confirms that the only significant ion suppression is at the retention time of (*S*)-bupropion in plasma. The recovery of all analytes was slightly better without the third SPE wash step (50:48:2 methanol:water:ammonium hydroxide), but ion suppression was significantly higher and decreased the sensitivity for (*S*)-bupropion. Although protein precipitation alone, without extraction, is commonly used for triple quad analysis, the ion suppression was too great to achieve the desired sensitivity using only protein precipitation, especially with (*S*,*S*)-hydroxybupropion and (*S*)-bupropion.

Hydrolysis was optimized by incubation with ß-glucuronidase at 37 and 60 °C for 2 and 24 h (Fig. 4). Urine hydroxybupropion concentrations tripled with hydrolysis

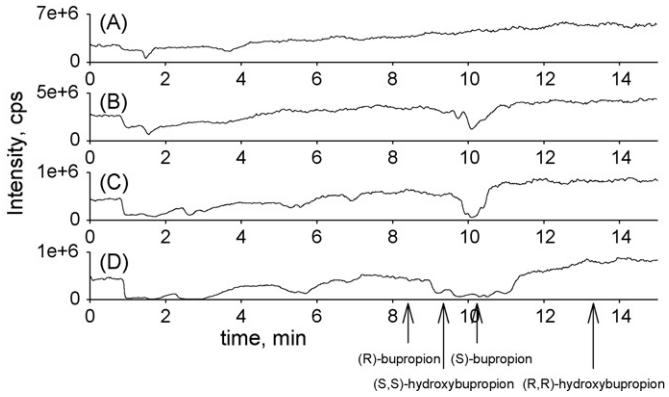

Fig. 3. Evaluation of ionization suppression. Shown is the total ion chromatogram from all of the ion transitions monitored, while continuously infusing a solution containing all analytes and internal standards (1 μg/ml each). Then injected were (A) an SPE-extracted blank urine sample, (B) an SPE-extracted blank plasma sample, (C) an SPE-extracted plasma sample without the third wash step, and (D) a plasma sample with protein precipitation only.

at 37 °C for 2 h, confirming excretion as the glucuronide conjugate. Increasing the incubation time increased the hydrolysis. Increasing the temperature did not increase hydroxybupropion concentrations but it did cause substantial racemization. Based on these experiments, the final incubation conditions used were 37 °C for 24 h. Bupropion does not form a glucuronide conjugate and total urine concentrations remained stable with and without hydrolysis, except at 60 °C, at which both bupropion and its internal standard degraded. Nevertheless, bupropion racemization occurred after only 2 h at 37 °C. Therefore, accurate quantification of bupropion enantiomers in urine requires analysis without hydrolysis, and thus separate assays for urine bupropion and hydroxybupropion are needed.

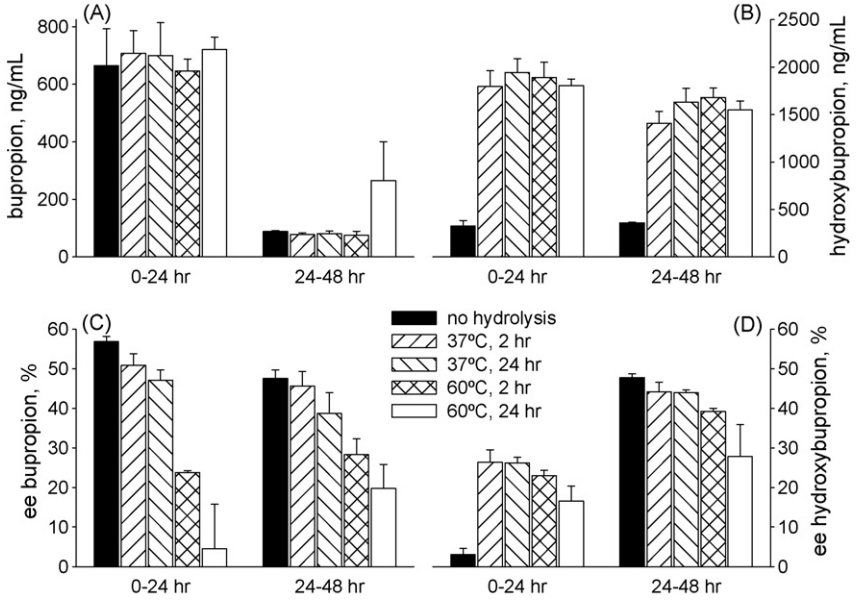

Fig. 4. Optimization of urine hydrolysis with ß-glucuronidase. Urine samples (0–24 h total collection and 24–48 h total collection) from a subject who received 150 mg oral bupropion were incubated with ß-glucuronidase at 37 °C or 60 °C for the indicated period of time. The top panels show total bupropion (A) and hydroxybuprion (B) concentrations. The bottom two panels show the enantiomeric excess for bupropion (C) and hydroxybuprion (D). Each bar is the mean ± S.D. of three replicate analyses.

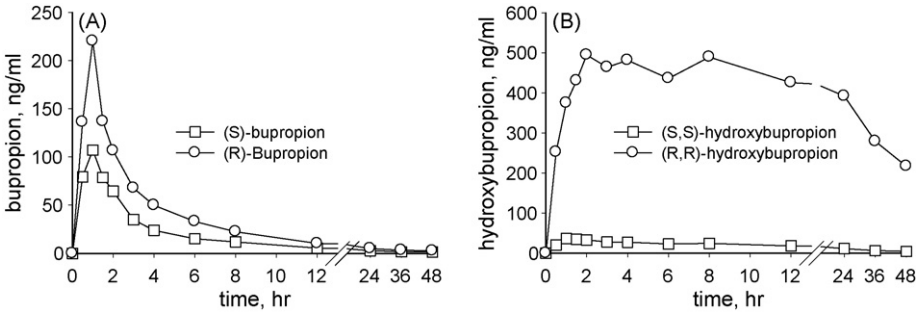

Fig. 5. Plasma bupropion (A) and hydroxybupropion (B) concentrations from a research subject who received 150 mg oral immediate release bupropion. Plasma was analyzed using the validated the chiral SPE–LC–MS–MS assay.

All 11 plasma samples and 4 urine samples were within 15% of the initial value for both bupropion enantiomers and both hydroxybupropion enantiomers when stored at −20 °C. After three freeze/thaw cycles, all analyte concentrations in all three plasma and urine samples were within 15% of the initial value.

Potential racemization of analytes was a particular consideration with this assay. The average enantiomeric excess (ee%, defined as the difference between the concentration of the two enantiomers divided by the total concentration × 100) of 11 extracted samples when stored at various temperatures and reinjected after 1, 4 and 7 days are displayed in Table 5. When the extracted samples were stored in the autosampler at 10 °C, both bupropion and hydroxybupropion began to racemize within 24 h. The autosampler temperature was lowered to 4 °C and the enantiomeric ratio remained stable for 24 h but started to racemize by 4 days. When the extracted samples were stored in the freezer at −20 °C before analysis, the enantiomeric ratio remained constant for at least 7 days.

Carryover was less than 0.01 ng/ml (<0.0004%) for both bupropion enantiomers and less than 0.8 ng/ml (<0.032%) for both hydroxybupropion enantiomers after injecting a 2500 ng/ml sample. Carryover did not become significant until a 25,000 ng/ml sample was evaluated, after which carryover was 0.8, 0.0, 1.27 and 12.5 ng/ml for (*R*)-bupropion, (*S*)-bupropion, (*S*,*S*)-hydroxybupropion and (*R*,*R*)-hydroxybupropion, respectively. Since is it unlikely that any plasma or urine samples will be evaluated above 2500 ng/ml, this carryover was considered acceptable.

3.4. Method application

Fig. 5 presents plasma results from a subject who received 150 mg oral immediate release racemic bupropion. In plasma, 65% of total bupropion was (*R*)-bupropion and 95% of the total hydroxybupropion was (*R*,*R*)-hydroxybupropion. Less than 3.4% of the dose was recovered in urine as bupropion or hydroxybupropion (0.6% and 2.8%, respectively). From the 75 mg (*R*)-bupropion portion of the dose, 0.9% and 3.6% were recovered as (*R*)-bupropion and (*R*,*R*)-hydroxybupropion, respectively. From the (*S*)-bupropion portion of the dose, 0.4% and 2.0% were recovered as (*S*)-bupropion and (*S*,*S*)-hydroxybupropion. In urine, 65% of the total bupropion was (*R*)-bupropion and 72% of the total hydroxybupropion was (*R*,*R*)-hydroxybupropion. Plasma and urine samples were analyzed with the method validated in this paper.

This method provides a robust, sensitive and selective method for the analysis of bupropion and hydroxybupropion enantiomers. Despite the reported rapid racemization of bupropion, patient samples analyzed by this method were between 27 and 37% ee. Prevention of racemization during assay was achieved by acidifying the solution and keeping the sample stored at −20 °C until analysis. Hydroxybupropion has a slower rate of racemization, but the low temperatures also help to stabilize the observed high ee (85–97%). The use of tandem mass spectroscopy provides increased selectivity and sensitivity over other published method using UV detection [33,36,39]. The only other published method using tandem mass spectroscopy is not enantioselective [37].

4. Conclusion

This paper presents a novel LC–MS–MS method to analyze the enantiomers of both bupropion and hydroxybupropion in plasma and urine. The assay is linear in plasma from 0.5 to 200 ng/ml and 2.5 to 1000 ng/ml in each bupropion and hydroxybupropion enantiomer, respectively. The assay is linear in urine from 5 to 2000 ng/ml and 25 to 10,000 ng/ml in each bupropion and hydroxybupropion enantiomer, respectively. The predominant enantiomers in both urine and plasma were (*R*)-bupropion and (*R*,*R*)-hydroxybupropion.

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