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Identification of Non-Reported Bupropion Metabolites in Human Plasma

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Running Title

Running Title: Non-reported Bupropion Metabolites

Abstract

Bupropion and its three active metabolites exhibit clinical efficacy in the treatment of major depression, seasonal depression, and smoking cessation. The pharmacokinetics of bupropion in humans is highly variable. It is unknown if there are any non-reported metabolites formed in humans in addition to the three known active metabolites. In this paper, we report newly identified and non-reported metabolites of bupropion in human plasma samples. Human subjects were dosed with a single oral dose of 75 mg of an immediate release bupropion HCl tablet. Plasma samples were collected and analyzed by LC-MS/MS at 0, 6, and 24 hours. Two non-reported metabolites (M1 and M3) were identified with mass-to-charge (m/z) ratio of 276 (M1, hydration of bupropion) and 258 (M3, hydroxylation of threo/erythrohydrobupropion) from human plasma in addition to the known hydroxybupropion, threo/erythrohydrobupropion, and the glucuronidation products of the major metabolites (M2 & M4-M7). These new metabolites may provide new insight and broaden the understanding of bupropion's variability in clinical pharmacokinetics.

Keyword: Bupropion, Metabolite Identification, LC-MS/MS

Introduction

Bupropion is prescribed for the treatment of major depressive disorder, seasonal affective disorder, and smoking cessation [1-3]. It is reported that bupropion is a non-selective inhibitor of the dopamine and norepinephrine transporter as well as an antagonist of the neuronal nicotinic acetylcholine receptor [4, 5].

Bupropion is extensively metabolized to three known active metabolites, hydroxybupropion and the diasteroisomers threohydrobupropion and erythrohydrobupropion. Using an animal model it was shown that these metabolites may exhibit as much as 25-50% of potency compared to the parent drug [6, 7]. The metabolite hydroxybupropion is formed by cytochrome P450 2B6 (CYP2B6) while threo/erythrohydrobupropion are formed by carbonyl reductase (CR) [8-10]. It is reported that there are multiple enzymes involved in the CR pathway including 11-β HSD and AKR7 family [8, 11, 12]. Both bupropion and its known major active metabolites display long half-life's; bupropion ~ 21, hydroxybupropion ~20 hours, and threo/erythrohydrobupropion ~33-36 hours [13]. Additionally, the C_{max} of threohydrobupropion and hydroxybupropion is ~3 and ~4-5 times higher in plasma than

that of bupropion, respectively [13]. Recent reports show that the major metabolites (hydroxybupropion and threo/ erythrohydrobupropion) undergo glucuronidation [14].

About 87% of bupropion and its metabolites are excreted in the urine where only 0.5% is excreted unchanged, and 10% of bupropion is eliminated in feces where only 0.1% is unchanged in humans after an oral dose [15, 16]. However, one study investigated elimination and recovery of bupropion after a single oral dose in urine from humans, where the data indicated that bupropion consisted of 0.6% and hydroxybupropion consisted of 2.8% of the total recovery; in total these two analytes constituted less than 3.4% of total drug recovered [17]. Although threohydrobupropion or erythrohydrobupropion were not quantified in this study, their abundance is usually lower than hydroxybupropion [13, 18]. The low recovery of bupropion and hydroxybupropion suggests that other metabolites might be formed. In addition, the pharmacokinetics and efficacy of bupropion is highly variable in clinical studies [18, 19]. It is suspected that other non-reported metabolites may be formed. Since the three known major metabolites are considered active, understanding the metabolic pathway and discovery of new metabolites may shed light on the variability of pharmacokinetics and efficacy of bupropion.

Healthy human subjects were administered a single dose of immediate release bupropion HCl (75 mg). Plasma samples were collected at 0, 6, and 24 hours for analysis. To identify additional metabolites, the enhanced mass spectrometry (EMS),

multiple ion monitoring (MIM), and precursor ion scans were used to analyze the plasma samples. In all four subjects, M1 to M7, were identified in addition to the three major known active metabolites. M1 is formed from the hydration of the benzene ring of bupropion and M3 results from further oxidation of threo/ erythrohydrobupropion or reduction of hydroxybupropion. Both M1 and M3 have unknown activity although it is possible that they could be active. These data provide new insight on bupropion's metabolism *in vivo,* which may contribute to its variability in pharmacokinetics and efficacy. Further studies are warranted to assess the pharmacological activity of these metabolites.

Methods and Materials

Reagents

Bupropion HCl and venlafaxine HCl (internal standard; IS) were purchased from Sigma (St. Louis, MO). Hydroxybupropion was purchased from Cayman Chemicals (Ann Arbor, MI). Threohydrobupropion and Erythrohydrobupropion were purchased from Torando Chemical Company (Torando, Canada). Methanol, LC-MS/MS grade, was purchased from Fischer Sciences (Hampton, New Hampshire).

Plasma Sample Extraction

All plasma samples were obtained in accordance to University of Michigan Institutional Review Board (HUM00081894) and the Food & Drug Administration Institutional Review

Board/ Research Involving Human Subjects Committee (RIHSC #13-087D). Healthy participants voluntarily took a single dose of an immediate release 75 mg of bupropion HCl tablet. Blood samples were collected in an EDTA K2 pretreated tubes from four participants at 0, 6, and 24 hours, then immediately centrifuged at 4°C for 10 minutes at 14,000 rpm. The plasma (supernatant) was then transferred to another cryogenic tube and stored at -80°C until analysis. Plasma was diluted 1:3 in methanol and vortexed for one minute. Samples were centrifuged at 14,000 rpm for 10 minutes at 4° C. The supernatant was transferred to another tube and evaporated using nitrogen gas and reconstituted in 120 µL of 20% methanol in water. Samples were then analyzed by LC-MS/MS to monitor metabolites.

Liquid Chromatography- Mass Spectrometry method

The LC-MS/MS analysis was conducted using an Shimadzu SIL-20ACHT HPLC system coupled with an API 4500 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada), equipped with an API electrospray ionization (ESI) source. The LC-MS/MS (or LC-MS) was operated at positive ESI ionization mode. The Supelco C18 (150 x 4.6 mm I.D., 5 µm) column was purchased from Sigma-Aldrich and used for separation. The LC separation condition was optimized with a blank plasma sample spiked with bupropion and its three known metabolites. The mobile phases consisted of 0.04% formic acid in purified water (A) and 0.04% formic acid in methanol (B). The gradient was ran at 0-2 minutes 2% (B), an increase to 32% (B) from 2-32 minutes, followed by 90% (B) at 32-

36 minutes, and finally decreased back to 2% (B) at 36-39.5 minutes with a flow rate of 1mL/min. EMS, precursor ion scan, and MIM scan were used as survey scan to identify metabolites of bupropion. Either information dependent acquisition (IDA) was used to collect fragmentation of metabolite candidates or single experiment of enhanced product ion scan (EPI) was applied to acquire fragmentations of metabolites after running the survey scan.

The ion spray voltage of QTrap (ABI) was set at 5500 V with a spray temperature of 600°C, curtain gas of 30 psi, gas 1 and gas 2 of 60 psi. The declustering potential (DP) was at 51 V. The collision energy was kept at 5 eV for survey scan of EMS & MIM and 25 eV for precursor ion scan and EPI fragmentation.

Additionally, a more thorough analysis was performed as described below to examine the isotopic patterns of the newly identified metabolites. High resolution LC-MS analyses were performed using an HPLC system (1200 series, Agilent Technologies) coupled to a 6520 Accurate-Mass Q-TOF (Agilent Technologies) operated in a positive electrospray ionization mode. ESI conditions were gas temperature 325 °C, drying gas 5 L/min, nebulizer 30 psig, fragmentor 150 V, and skimmer 65 V. The instrument was set to acquire over the m/z range 50 to 3200 with an acquisition rate of 2 spectra/s.

Data Analysis

The analytical data were processed by the software Analyst version 1.6.2 (Applied Biosystems, Foster City, CA). The data was also analyzed in Lightsight version 2.3.0 (Applied Biosystems). ChemBioDraw Ultra version 13.0 (CambridgeSoft) was used to draw structures.

Results

Bupropion and Its Metabolites

Figure 1 highlights the structures of both currently known metabolites of bupropion [hydroxybupropion and threo/erythrohydrobupropion] and 7 metabolites identified in this study from human plasma. Bupropion is metabolized by both CYP2B6 and CR to form hydroxybupropion and threo/erythrohydrobupropion, respectively [8-10]. It is also known that threo/erythrohydrobupropion can further undergo glucuronidation [20]. In addition to these metabolites, M1 and M3 were detected in human plasma in this study and are shown as non-reported metabolites. **Figure 2** illustrates the daughter ions of bupropion (2A) and the daughter ions of three known metabolites; hydroxybupropion (2B), threohydrobupropion (2C), and erythrohydrobupropion (2C) that were acquired by direct MS infusion using authentic standards. Threohydrobupropion and erythrohydrobupropion have same product ion spectra and fragments. The fragmentation of bupropion and its three metabolites were proposed based upon their product ion spectra. The characteristic daughter ions included m/z ratios of 103, 131,

139, 151, 166, 167, 168, 184, and 238, which served as precursor ions for the survey scan of precursor ion scan.

Identification of Non-reported Bupropion's Metabolites

Figure 3 indicates the extracted ion chromatograms of all metabolites detected in human plasma after a single oral dose of bupropion. Figure 3A shows the relative intensity and retention times for all metabolites detected, including bupropion and the previous three known metabolites in one MIM method after all survey scans were inspected. Figure 3B-E shows the extracted ion chromatograms of seven metabolites detected in plasma, which corresponded to m/z ratios of (B) M1: 276, (C) M2: 432, (D) M3: 258, (E) M4-M7: 418. The M1 metabolite elution was quite early. M2 and M3 were eluted right after hydroxybupropion. Multiple peaks with the same m/z=418 were observed (Figure 3E) and represented by M4, M5, M6 and M7. These four peaks all had the same product ion spectra, suggesting that stereochemistry might be involved. Together, the extracted ion chromatogram results suggested seven metabolites in addition to hydroxybupropion and threo/erythrohydrobupropion.

Structure Determination of New Bupropion Metabolites

To determine the structures of the newly identified metabolites, the product ion spectra were evaluated **(Figure 4).** M1's m/z ratio (m/z=276) increased 36 Daltons (Da) compared to that of bupropion (m/z=240). As shown in Figure 4A, the fragments of the

characteristic ions that were consistent with bupropion were m/z ratios of 167, 184 and 240. The M1 metabolite was further investigated with a high resolution mass spectrometer. The accurate mass (m/z) of M1 that was measured was 276.1356 as shown in **Figure 5**. This molecular ion has also shown the characteristic isotopic pattern of chlorine atoms. The monoisotopic ion of 276 has an intensity three times that of isotopic ion 278 as shown in **Figure 5**, confirming the metabolite was indeed real and not an artifact. The predicted formula for this metabolite was $C_{13}H_{22}ClNO_3$ with a mass accuracy of 2 ppm, which contains four more hydrogen atoms and two more oxygen atoms than bupropion. These additional atoms were assigned to the benzyl ring of bupropion as shown in proposed structure of Figure 4(A).

M2 showed an m/z ratio of 432, which had 176 Da more than hydroxybupropion (Figure 4B). In addition, its fragments contained the same characteristic ions of 166, 184 and 256 as seen in hydroxybupropion, suggesting glucuronidation of hydroxybupropion.

M3 (m/z=258) increased in 16 Da compared to threo/erythrohydrobupropion and 18 Da more than bupropion. The fragments of M3 (Figure 4C) had the same characteristic ions of as seen in threo/erythrohydrobupropion; 151, 168, and 186. The fragment of 186 corresponded to loss of tert-butyl group and therefore no modification could occur the fragmented structure of 186. This suggested that 16 Da affected the tert-butyl group of threo/erythrohydrobupropion. The product ion of m/z 240 of M3

confirmed this position. The 16 Da increase was proposed as hydroxylation to threo/erythrohydrobupropion. In figure 3D, in addition to the peak for M3, a large peak was observed with the same parent ion of 258. This peak eluted out earlier than M3 and at same retention time of hydroxybupropion (22min). The product ion spectrum of this peak confirmed that it is the hydroxybupropion isotopic ion containing ^{37}Cl.

Finally, the last metabolite m/z=418, had 176 Da more than threo/erythrohydrobupropion, and it also had characteristic ions of m/z 151, 168 and 186 as seen in threo/erythrohydrobupropion, suggesting glucuronidation (figure 4D). This observation was consistent with Figure 3E producing four peaks. It can be postulated that two of these peaks were from the conjugation of chiral β-D glucuronic acid to hydroxyl group of two threohydrobupropion enantiomers respectively and the other two were from the β-D glucuronic acid conjugation of two erythrohydrobupropion enantiomers respectively. The conjugation of chiral glucuronic acid introduced another chiral center to the enantiomers and resulted in four diasteroisomers. These conjugates were able to be separated on the column showing four distinct peaks.

The isotopic distributions of three other metabolites through EMS full scans with a narrow mass range was also examined. As shown in **Supplemental Figure 1**, mass spectra of M2, M3 and M4-M7 all have relative isotopic abundance of ^{35}Cl and ^{37}Cl, which further confirmed these three metabolites came from bupropion.

Relative Quantification of New Metabolites in Human Plasma

To assess each metabolite formation in each subject at the two time points of 6 and 24 hours, we compared the peak areas (counts) **(Figure 6).** The peak area for each metabolite for the four subject samples are illustrated in Figure 6 by the m/z ratio. The time points of 6 vs. 24 hours were compared in order to assess the relative amount of metabolite formed. Over time, the majority of the metabolites had a higher peak area at 6 hours compare to at 24 hours (Figure 6A to 6G). There were variations in peak area for each metabolite formation between subjects, with M1 showing the largest variability. However, these data suggest that all seven metabolites form *in vivo*.

Discussion

Bupropion's biotransformation involves multiple enzymes that are expressed at different levels in both the gastrointestinal tract and the liver [21, 22]. Since bupropion HCl is clinically available in multiple formulations (immediate release, sustained release, or extended release) at various dose (strengths75- 300 mg), it is likely that these factors may influence the amount of metabolites formed in liver and gastrointestinal (GI) tract. Therefore, we intended to investigate the complete biotransformation of bupropion *in vivo*.

In this study, we analyzed human plasma after a single oral dose of 75 mg bupropion HCl tablet. We identified seven metabolites including two non-reported

metabolites for bupropion; hydration of bupropion (M1) and oxidation of threo/erythrohydrobupropion (M3). Hydration of bupropion was assigned to the benzyl ring of bupropion since only the ring has unsaturated bonds for addition of hydrations (Figure 4A). The fact that the retention time was much shorter compared to the other metabolites of bupropion provided evidence of hydration of bupropion since more polar compounds typically results in shorter retention times. Much to our surprise, we only observed one peak for hydroxylation of threo/erythrohydrobupropion (M3). One explanation is that the second peak was too low to observe, which seems sensible considering how low the abundance of erythrohydrobupropion is in human plasma. The M4-M7 metabolites share the same m/z ratio and fragmentation pattern, which indicates stereochemistry selective that is expected from the diastereoisomers of threo/erythrohydrobupropion through conjugation to β-D glucuronic acid. The isotopic pattern of chlorine was used to distinguish the metabolite candidates that may have been detected but were not a result of bupropion's metabolism [23]. Bupropion contains one chlorine atom on the benzyl ring, which assists in the identification of metabolites from complex plasma samples. The isotopic pattern of chlorine atom in Figure 5 and supplemental Figure 1 clearly demonstrated that the new metabolites are produced via biotransformation of bupropion.

Gufford et al. has recently proposed several UGT enzymes that might be responsible formation of glucuronidation metabolite for bupropion, these same

glucuronidation products were consistent with what was observed in this study [14]. However, Gufford et al study analyzed urine and not plasma. Therefore, M1 or M3 may not have been excreted in urine or these metabolites may not have been identified using only multiple reaction monitoring (MRM). In addition to the MRM scanning method Gufford et al. used, samples in this study also were tested using precursor ion scan, MIM, and EMS, allowing for the detection of M1 and M3.

While all seven metabolites formed in four different individuals, the peak area of these metabolites varied quite a bit from individual to individual. The known primary enzymes responsible for conversion of bupropion to form hydroxybupropion and threo/erythrohydrobupropion are highly polymorphic. CYP2B6 can have a 20-250 fold variability in terms of expression and activity and in addition is expressed at different levels in multiple tissues, including the GI tract and the liver [19, 21, 24-27]. Carbonyl reductase enzymes have also been found to be highly polymorphic with varying activity [28, 29]. Similarly, studies have shown the Uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes, which are responsible for glucuronidation reactions, have high variability in terms of the activity, which might lead to a functional impact [30, 31]. Therefore, it is reasonable to suspect that polymorphs can explain the intersubject variability observed amongst these four subjects.

Previous *in vitro* results have suggested other possible phase I metabolism enzymes might be involved in the biotransformation of bupropion and major

metabolites, specifically hydroxybupropion including: CYP2E1 CYP2C19, and CYP3A4 *in vitro [25, 32, 33]* , however this has not been explored *in vivo*. These phase I metabolism enzymes may also be candidates for forming these new metabolites. The metabolites identified in this study may exhibit activity however, further investigations would be necessary to determine if these metabolites are indeed active. Nevertheless, the identification of M1 to M7 may shed light for further variability of bupropion in terms of pharmacokinetics and efficacy.

Conclusion

Seven metabolites of bupropion in human plasma samples were detected after a single dose of bupropion HCl (IR 75 mg). Two non-reported metabolites (M1 and M3) were identified with mass-to-charge (m/z) ratio of 276 (M1, hydration of bupropion) and 258 (M3, hydroxylation of threo/erythrohydrobupropion) in addition to the known hydroxybupropion, threo/erythrohydrobupropion, and the glucuronidation products of the major metabolites (M2 & M4-M7).

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Figure legends

Figure 1. **Bupropion and its Metabolites Found in Human Plasma.** Bupropion has been reported to be metabolized by both CYP2B6 and carbonyl reductase to form hydroxybupropion and threo/erythrohydrobupropion respectively. Seven other metabolites were identified in human plasma after a single oral dose of bupropion; hydration of bupropion (M1), glucuronidation of hydroxybupropion (M2), hydroxylation of threo/ erythrohydrobupropion (M3) and glucuronidation of threo/erythrohydrobupropion (M4-M7).

Figure 2. **Proposed Fragmentation of Bupropion and its Metabolites.** Authentic standards were directly infused to acquire product ion spectra of (A) Bupropion (m/z 240); (B) Hydroxybupropoin (m/z=256); (C) Threo/Erythrohydrobuproprion (m/z=242). The structure indicates how these fragmentations occur on each molecule.

Figure 3. **Extracted Ion Chromatograms for Bupropion's Metabolites Found from Human Plasma.** (A). All metabolites observed including; hydroxybupropion (Hbup), threo/ erythrohydrobupropion (TBUP/EBUP), and M1-M7; (B) M1 (m/z=276); (C) M2 (m/z=432); (D) M3 (m/z=258); (E) M4-M7 (m/z=418). For the metabolites M4-M7, the same m/z value of 418 were observed.

Figure 4. **Proposed Fragmentation of Bupropion's Metabolites Found from Human Plasma.** The spectra indicated the fragmentation of each of bupropion's metabolites detected. Their structures were proposed; (A) M1, hydration of bupropion (m/z=276); (B) M2,glucuronidation of hydroxybupropion (m/z=432); (C) M3, oxidation of threo/erythrohydrobupropion (m/z=258); (D) M4, glucuronidation of threo/erythrohydrobupropion (m/z=418). M4 to M7 have same product ion spectra, therefore only one spectrum was shown here.

Figure 5. **High Resolution Mass and Isotopic Pattern of Newly found Metabolite, m/z= 276**. The predicated formula of this metabolite is C_{13} H_{22} Cl N O_3 with a mass accuracy of 2 ppm. The isotopic pattern of ^{35}Cl and ^{37}Cl offers strong evidence that this metabolite is a product of bupropion.

Figure 6. **Relative Intensity of Metabolites Newly Found in four Human Subjects**. For comparison purposes, the peak area of each new metabolite that formed in the plasma sample for each subject is shown at 6 hours and 24 hours. (A) is M1 with m/z of 276; (B) is M2 with m/z of 432; (C) is M3 with m/z of 258 and (D) is the sum of M4-M7 peak areas with m/z of 418.

Supplemental figure 1. **Isotopic Distribution of Metabolites Newly Found in Human Plasma**. (A) M2 (m/z=432), (B) M3 (m/z=258), and (C) M4 (m/z=418). M4 to M7 have same isotopic pattern.

Conflict of Interest

There is no conflict of interest with the authors.

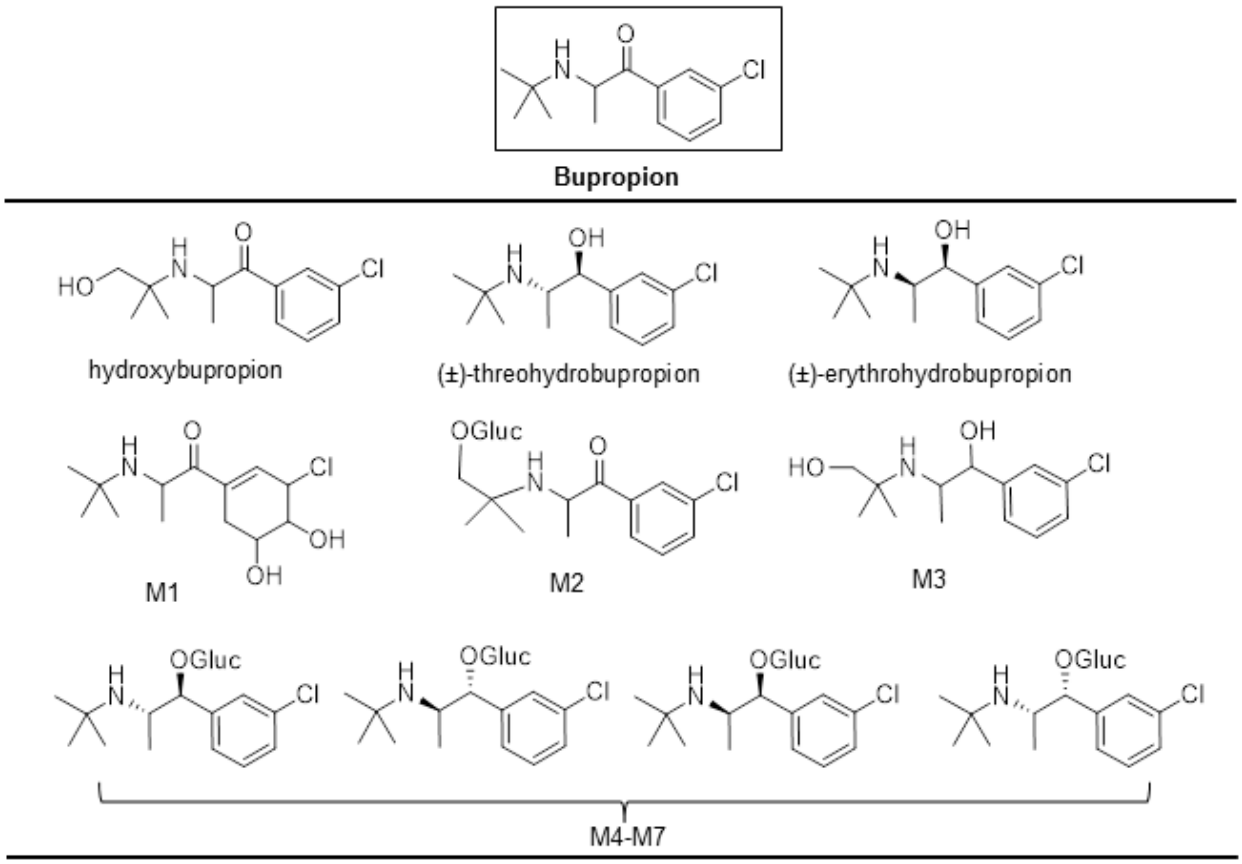

Figure 1. **Bupropion and its Metabolites Found in Human Plasma.** Bupropion has been reported to be metabolized by both CYP2B6 and carbonyl reductase to form hydroxybupropion and threo/erythrohydrobupropion respectively. Seven other metabolites were identified in human plasma after a single oral dose of bupropion; hydration of bupropion (M1), glucuronidation of hydroxybupropion (M2), hydroxylation of threo/ erythrohydrobupropion (M3) and glucuronidation of threo/erythrohydrobupropion (M4-M7).

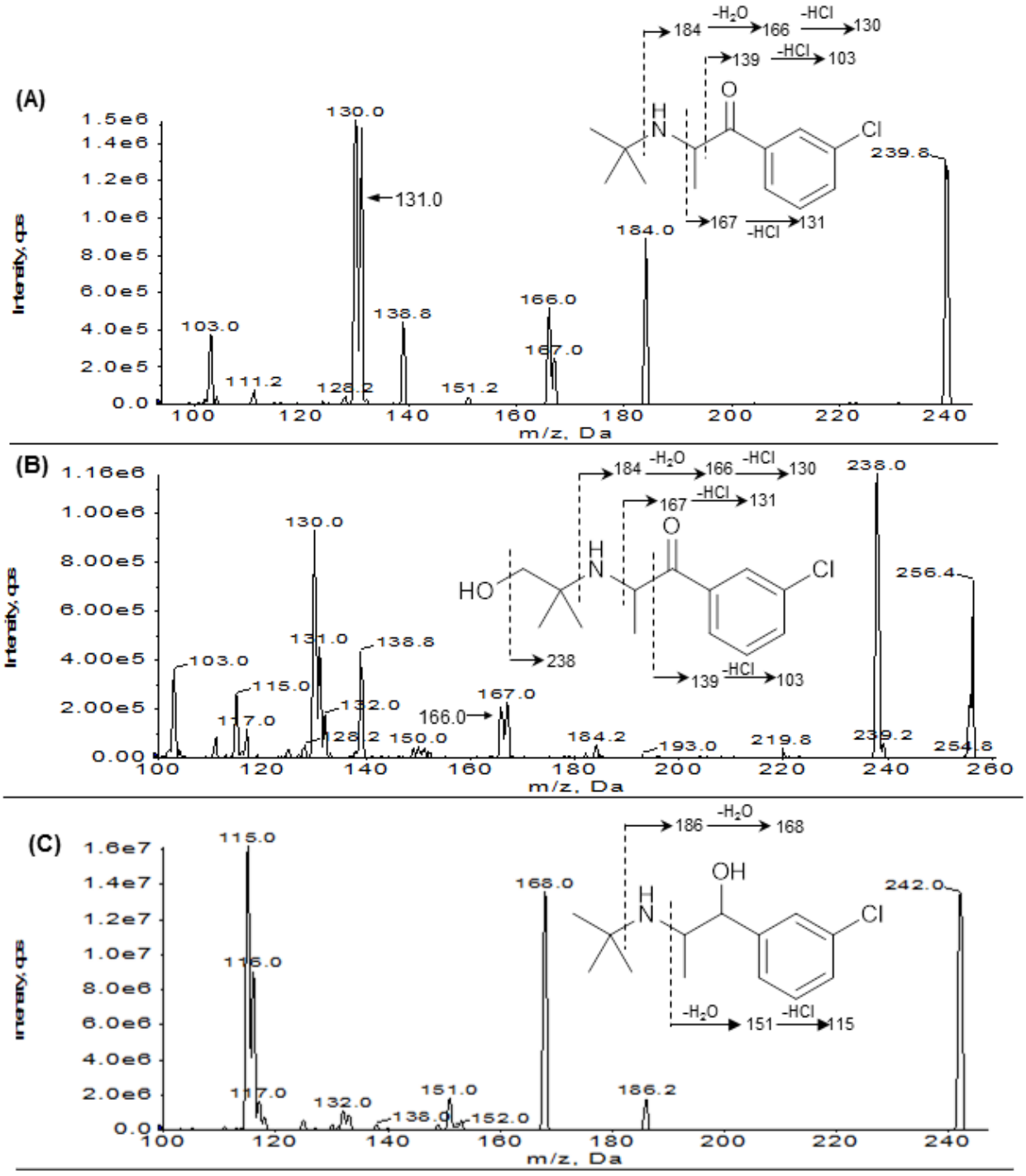

Figure 2. **Proposed Fragmentation of Bupropion and its Metabolites.** Authentic standards were directly infused to acquire product ion spectra of (A) Bupropion (m/z 240); (B) Hydroxybupropoin (m/z=256); (C) Threo/Erythrohydrobuproprion (m/z=242). The structure indicates how these fragmentations occur on each molecule.

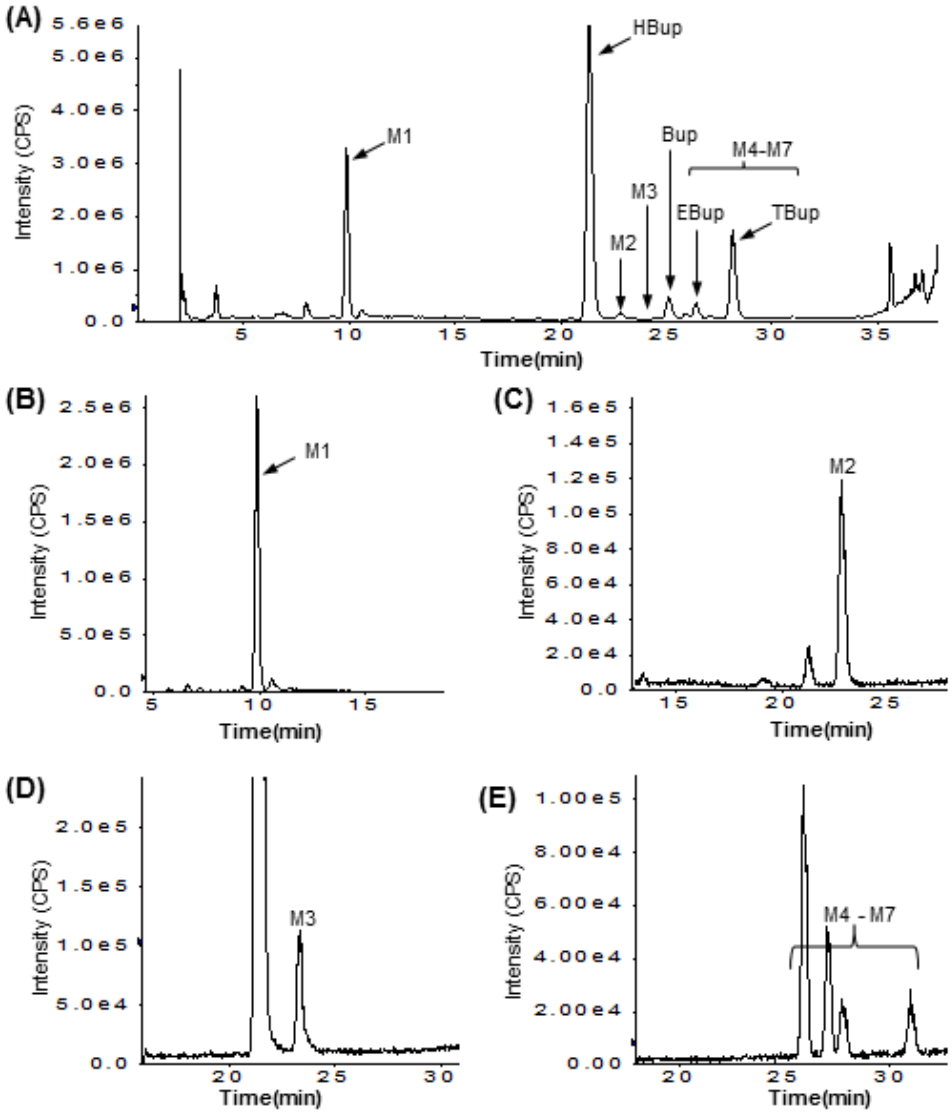

Figure 3. **Extracted Ion Chromatograms for Bupropion's Metabolites Found from Human Plasma.** (A). All metabolites observed including; hydroxybupropion (Hbup), threo/ erythrohydrobupropion (TBUP/EBUP), and M1-M7; (B) M1 (m/z=276); (C) M2 (m/z=432); (D) M3 (m/z=258); (E) M4-M7 (m/z=418). For the metabolites M4-M7, the same m/z value of 418 were observed.

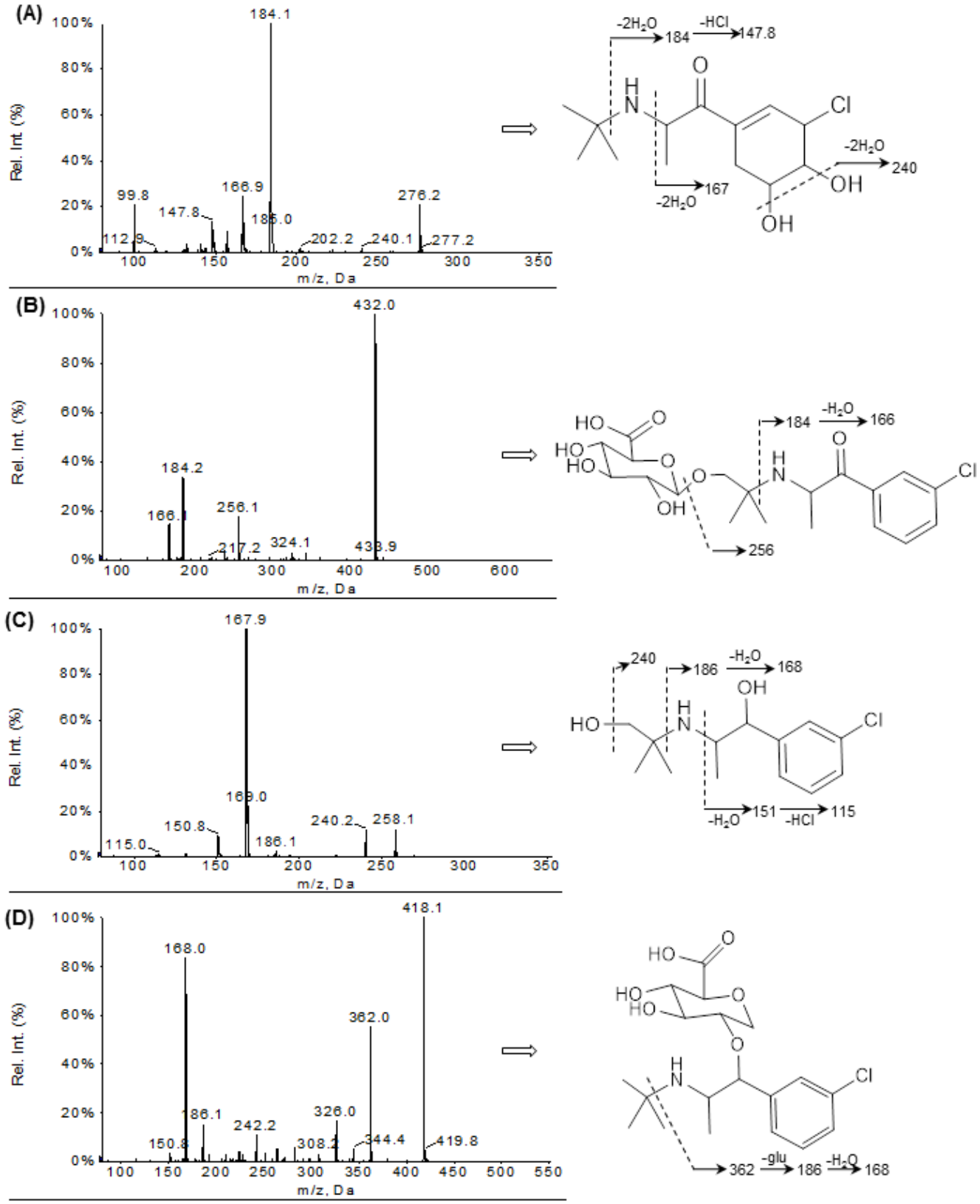

Figure 4. **Proposed Fragmentation of Bupropion's Metabolites Found from Human Plasma.** The spectra indicated the fragmentation of each of bupropion's metabolites detected. Their structures were proposed; (A) M1, hydration of bupropion (m/z=276); (B) M2,glucuronidation of hydroxybupropion (m/z=432); (C) M3, oxidation of threo/erythrohydrobupropion (m/z=258); (D) M4, glucuronidation of threo/erythrohydrobupropion (m/z=418). M4 to M7 have same product ion spectra, therefore only one spectrum was shown here.

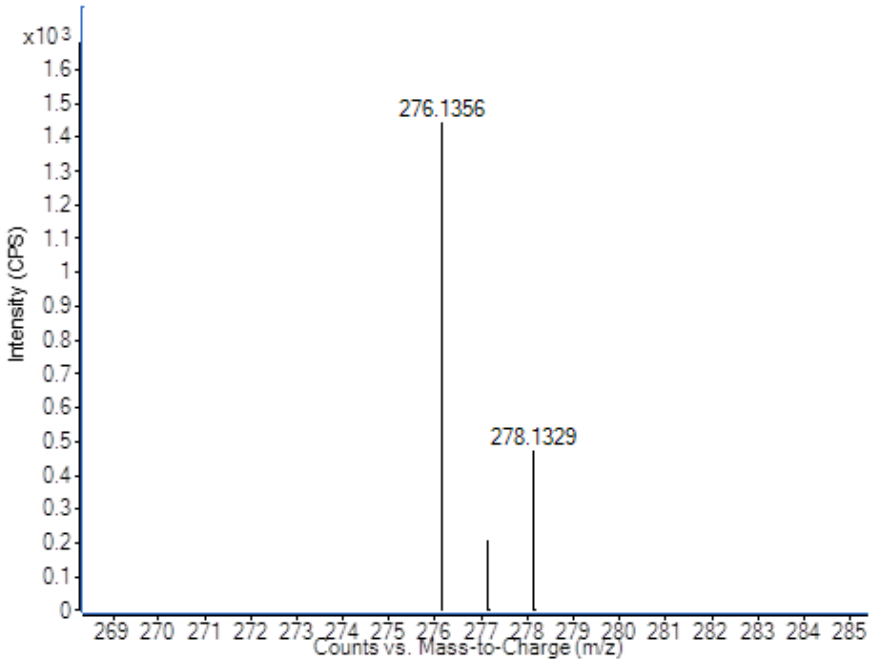

Figure 5. **High Resolution Mass and Isotopic Pattern of Newly found Metabolite, m/z= 276**. The predicated formula of this metabolite is C_{13} H_{22} Cl N O_3 with a mass accuracy of 2 ppm. The isotopic pattern of ^{35}Cl and ^{37}Cl offers strong evidence that this metabolite is a product of bupropion.

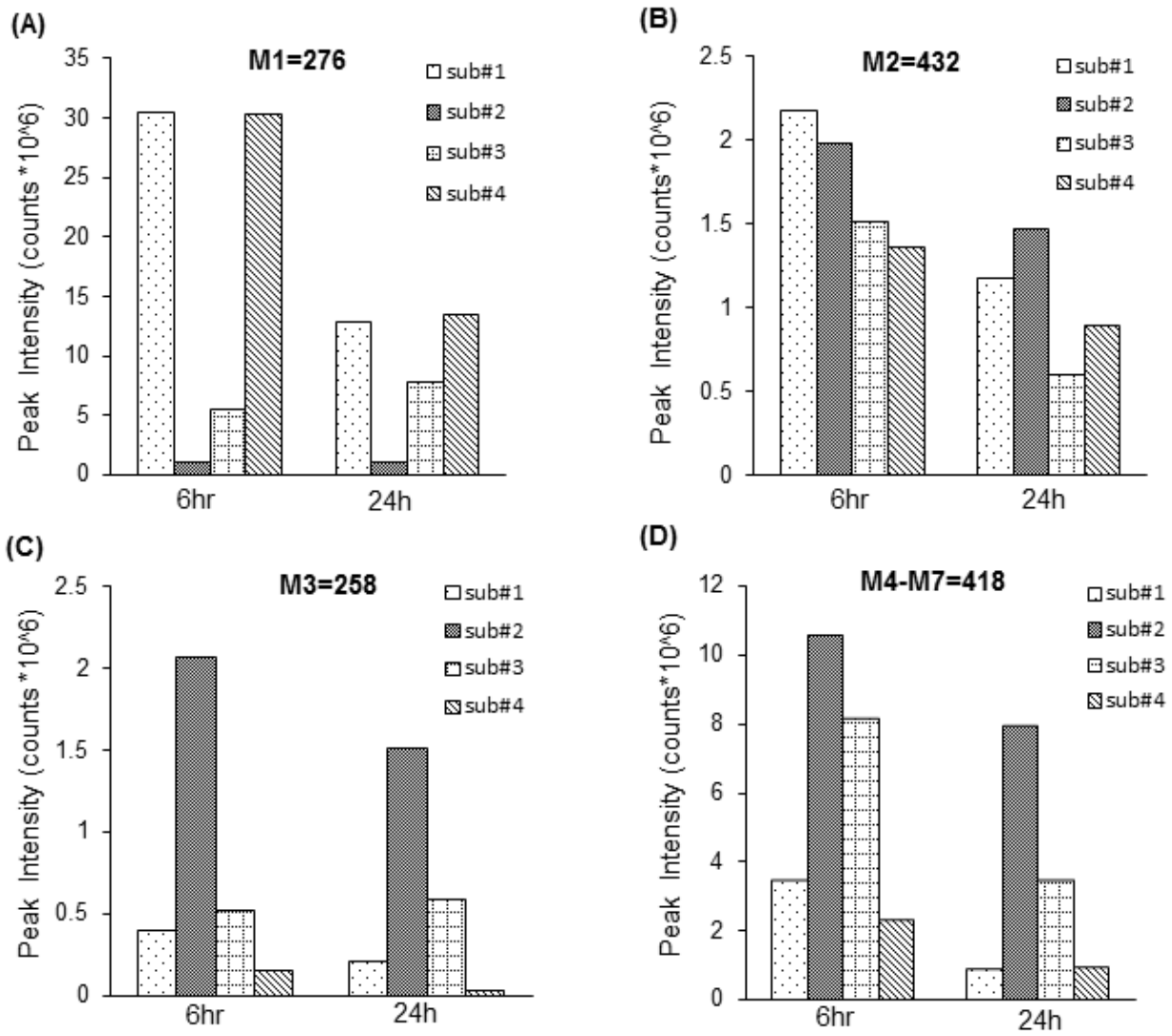

Figure 6. **Relative Intensity of Metabolites Newly Found in four Human Subjects**. For comparison purposes, the peak area of each new metabolite that formed in the plasma sample for each subject is shown at 6 hours and 24 hours. (A) is M1 with m/z of 276; (B) is M2 with m/z of 432; (C) is M3 with m/z of 258 and (D) is the sum of M4-M7 peak areas with m/z of 418.