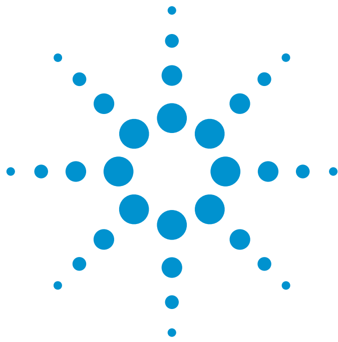

Drug Metabolite Purification and Identification

The Agilent Analytical-scale Purification System, Agilent Automated Purification Software, and Agilent 6540 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system

Application Note

Drug Discovery

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Abstract

Drug metabolism and pharmacokinetics (DMPK) studies require the identification of drug metabolites usually by mass spectrometry (MS), and in later stages of drug development, confirmation of metabolite structures by NMR. To achieve this, purification of metabolites may be required. In this Application Note, purification of bupropion drug metabolites was achieved using Agilent Automated Purification Software in conjunction with the Agilent 1260 Analytical-scale Purification System. The metabolites were identified using high-resolution accurate mass MS and MS/MS data acquired using an Agilent Accurate Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system and Mass-MetaSite Software.

Agilent Technologies

Introduction

Confident identification of the chemical structures of metabolites is crucial to drug compound evaluation in drug discovery. In the discovery stage, typically high-resolution accurate mass data together with MS/MS fragmentation patterns can be used to generate elemental formulae and provide insight about putative metabolite structures. The structural identification of metabolites is facilitated using metabolite structure prediction software. In the later stages of drug discovery, structural confirmation may be required using Nuclear Magnetic Resonance (NMR). Additionally, preclinical toxicological studies on major drug metabolites needs to be performed. Therefore, isolation of the metabolites from complex biological samples is required. The Agilent Automated Purification Software together with Agilent 1260 Infinity Analytical-scale Purification System supports the scale-up from analytical to semipreparative columns. To isolate metabolites, fraction collection can be performed efficiently either by time-based or peak-based UV/MS detection. The time-based method is used to collect fractions periodically in specified retention time windows, irrespective of detector sensitivity, and is possibly preferred to minimize sample losses due to compound concentrations below the threshold. The peak-based method is useful to isolate metabolites based on their UV/MS signal and is preferable for the isolation of the most abundant metabolites[1]. MS triggered fractionation is preferred over UV to isolate trace levels of known metabolites from complex samples using the more sensitive single ion monitoring detection mode with a single quadrupole mass spectrometer.

This Application Note demonstrates the purification and identification of bupropion metabolites obtained by *in vitro* liver microsomal incubations. We used time-based and peak-based triggering for fraction collection. The possible structures of the metabolites derived from accurate mass LC/MS/MS data in Mass-MetaSite software is also discussed.

Experimental

Workflow

The general workflow for the metabolite purification and identification is shown in Figure 1. In this study, the metabolites were generated by incubating bupropion with human liver microsomes. The metabolites were profiled using a generic gradient run on the analytical column. A generic gradient profile was used, as the requested metabolites have different polarities. Purification of the metabolites was performed using the Agilent 1260 Infinity Analytical-scale Purification System connected to an Agilent 6150 Single Quadrupole Mass Spectrometer. Concomitantly, the identification of metabolites was performed on an Agilent 6540 Q-TOF LC/MS system and Mass-MetaSite Software (Molecular Discovery). The scale-up process from the analytical 4.6-mm id column to the 9.4-mm id column was performed fully automated by the Agilent Automated Purification Software[2]. This could be achieved by generating either a focused or a linear gradient profile based on scaling up the analytical gradient profile. A focused gradient represents a gradient profile designed to purify a specific metabolite.

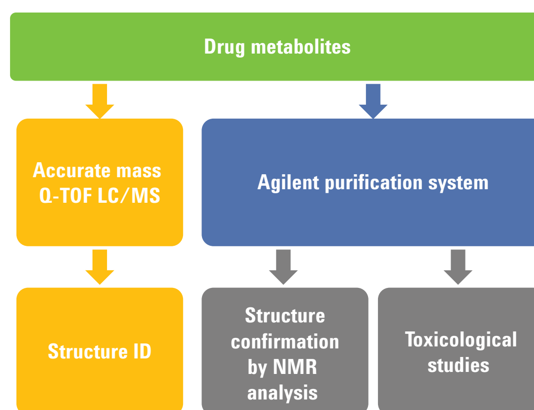

Figure 1. Workflow for metabolite purification and identification.

Metabolite generation

A 10 mM stock solution of bupropion (Sigma-Aldrich) was prepared in a 20:80 (acetonitrile:water) solution and diluted to a final incubation concentration of 250 µM in phosphate buffer (0.1M, pH 7.4). Human liver microsomes (HMMC-3A, Life Technologies) and NADPH (N5130, Sigma-Aldrich) were combined to a final incubation concentration (2 mg and 2.4 mM respectively) in phosphate buffer[3]. The reaction was incubated at 37.5 °C at 150 rpm for 120 minutes. The reaction was stopped by adding an equal volume of 6 % formic acid in acetonitrile, and centrifuged at 11,000 rpm for 10 minutes. The supernatant was analyzed using a LC/MS Q-TOF System. The supernatant was vacuum evaporated and reconstituted in 300 µL for LC-UV/MS SQ fraction collection experiments.

Reagents and materials

All solvents and additives used were LC/MS grade. Purified water was obtained from a Milli-Q water purification system (Millipore, USA).

Table1. Instrument and method parameters for the Agilent Analytical-scale Purification System.

Agilent 1200 Series Analytical-scale Purification System	
Parameter	**Set value**
Agilent 1260 Infinity Quaternary Pump (G1311B)	
Mobile phase A	Water with 0.1 % formic acid and 0.01 % TFA
Mobile phase B	100 % Acetonitrile with 0.1 % formic acid and 0.01 % TFA
Mobile phase gradient	Analytical: 0–21 minutes: 5–90 % B; 23–28 minutes: 5 % B Preparative: Based on Automated Purification Software
Flow rate	Analytical: 1.0 mL/min Preparative: 3.0 mL/min
Agilent 1260 Infinity Autosampler (G1367B)	
Injection volume	Analytical: 10 µL Preparative: 90 µL
Agilent 1260 Infinity Column Compartment (G1316C) having 2-pos/6-port valve	
Temperature	25 °C
Column	Analytical: Agilent ZORBAX SB-C18, 4.6 × 75 mm, 3.5 µm (p/n 866953-902) Preparative: Agilent ZORBAX SB-C18, 9.4 × 50 mm, 5.0 µm (p/n 846975-202)
Agilent 1260 Infinity Variable Wavelength Detector, High Pressure Cell (G1314-60082)	
Wavelength	252 nm
Agilent 1260 Infinity Fraction Collector analytical scale (G1364C)	
	Automated delay volume calculation using Lab Advisor Software
Instrumentation for mass-based fraction collection system	
Parameter	**Set value**
Agilent 6150 Single Quadrupole LC/MS System (G6150B) with AJS	
Range	100–700, Peak width: 0.1 minutes
Mode	Positive
Drying gas flow	10 L/min
Nebulizer pressure	30 psig
Drying gas temperature	300 °C
Sheath gas temperature	350 °C
Sheath gas flow	10 L/min
Capillary cap	3,500 V
Nozzle Voltage	1,000 V
Fragmentor/Peak width	100/0.05 minutes
Makeup pump: Agilent 1260 Infinity Isocratic Pump (G1310B)	
Flow rate	0.9 mL/min having acetonitrile-water (70–30) with 0.1 % formic acid
Agilent OpenLab CDS ChemStation Edition	
For data acquisition and data analysis	Rev. C.01.05
Agilent Openlab CDS ChemStation Automated Purification Software	
Version A.01.01 [066]	

Results and Discussion

LC-UV/MS detection of bupropion metabolites

Detection of bupropion metabolites was performed using an analytical column on an Agilent Analytical-scale Purification system at 252 nm in positive mode (Table 1). The UV analysis showed that bupropion metabolites were formed. Mass spectrometry analysis, using extracted ion chromatograms (EIC) of known metabolites[4], showed the presence of hydroxybupropion (*m/z* 256) and erythro/threo (*m/z* 242) bupropion metabolites (Figure 2). Control samples from microsomal incubations performed without the drug were also analyzed in parallel to confidently identify metabolite formation from the bupropion incubations (data not shown). Concomitantly, an accurate Mass Q-TOF system was also used to detect trace level metabolites.

The *m/z* ions: 256.7479 and 242.7644 were detected by Q-TOF analysis. Metabolite erythro/threo bupropion are stereoisomers, elute close to the parent drug peak, and are suitable for a time-based fraction collection for highest recovery. The hydroxybupropion metabolite showed enhanced MS and UV sensitivity compared to other metabolites, and thus is most suited for peak-based fraction collection.

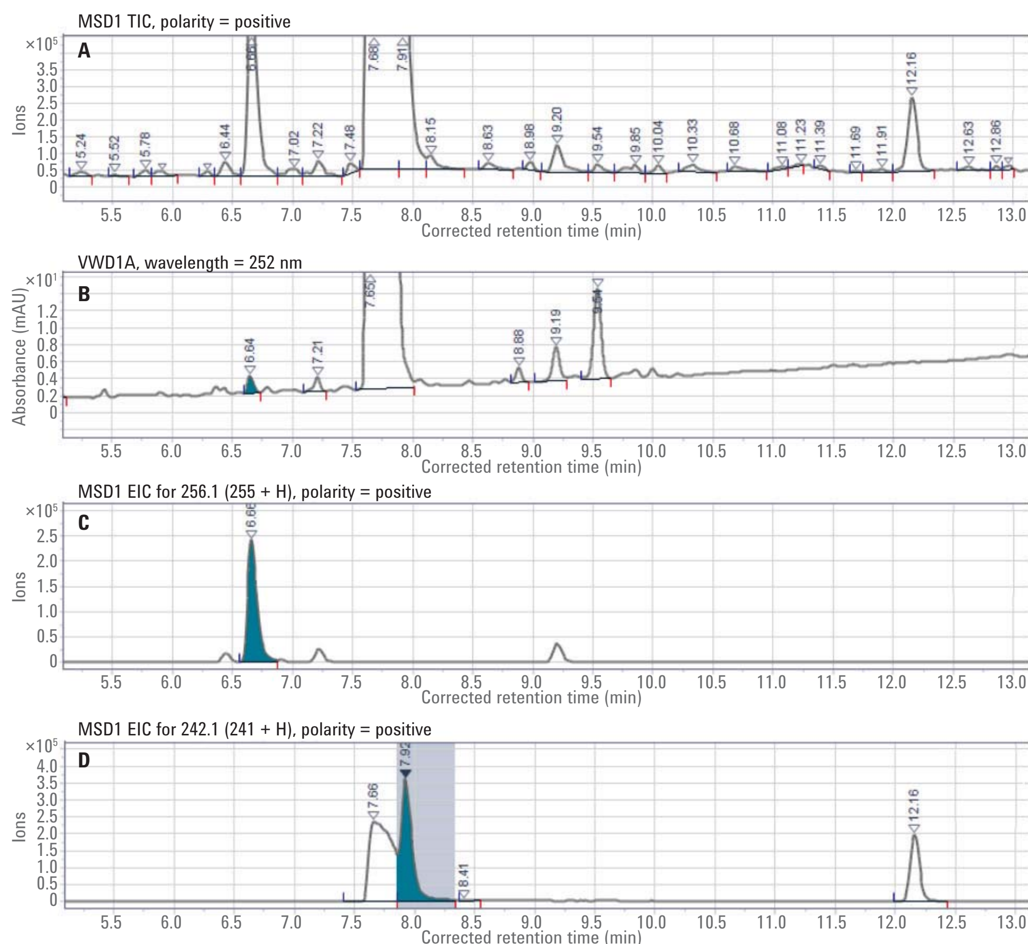

Figure 2. UV and MS profiles of the bupropion liver microsomal incubation mixture. A) Total Ion Chromatogram (TIC), B) UV at 252 nm, C) EIC of *m/z* 256 representing hydroxybupropion, and D) EIC of *m/z* 242 representing erythro/threo bupropion.

MS peak-based fraction collection

The molecular weight information (m/z of MH^+ ions) gathered from the initial analytical experiments were used to isolate target metabolites. The hydroxybupropion metabolite (MH^+ m/z 256) is the most abundant metabolite of bupropion, and was easily detected by UV and MS, so it was collected using the peak-triggered mode. A focused gradient was determined by the purification software and enabled the isolation of the peak at m/z 256 (Figure 3). Mass-triggered fractions (m/z 256) were collected as shown. The MS spectral profile of fraction A1/B6 shows a predominant peak at m/z 256. The hydroxybupropion metabolite was 0.2 % of the parent, based on the UV response from the analytical run. With a semiprep LC analysis, approximately 0.05 µg of hydroxybupropion was isolated from a single injection of 90 µL.

Time-based fraction collection

Isolation of minor abundant metabolites was obtained by collecting fractions at discrete time slices. Figure 4 shows the method used for the time-based fraction collection. The scale-up calculation was determined by the purification software based on the analytical settings. A linear gradient rather than a focused gradient was used, as the compounds of interest showed a wider polarity range. The fraction collected at plate location A3 showed the MS profile of erythro/threo bupropion metabolites (m/z 242).

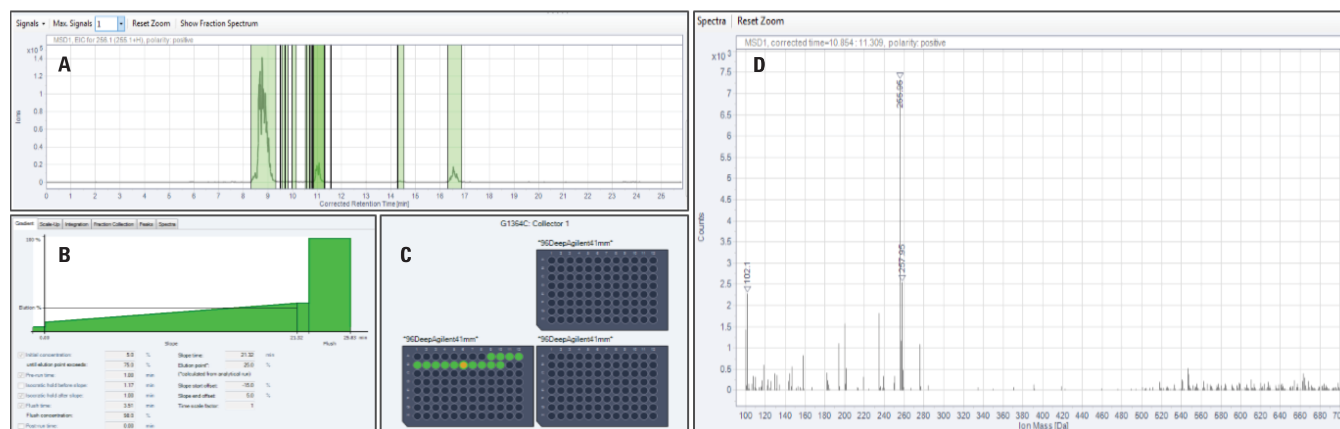

Figure 3. Peak-based mass triggered fraction collection for m/z 256. A) TIC indicating the threshold based collection, B) the focused gradient used, C) the fraction collection window indicating the location of the collected fraction, and D) the MS spectra of the collected fraction.

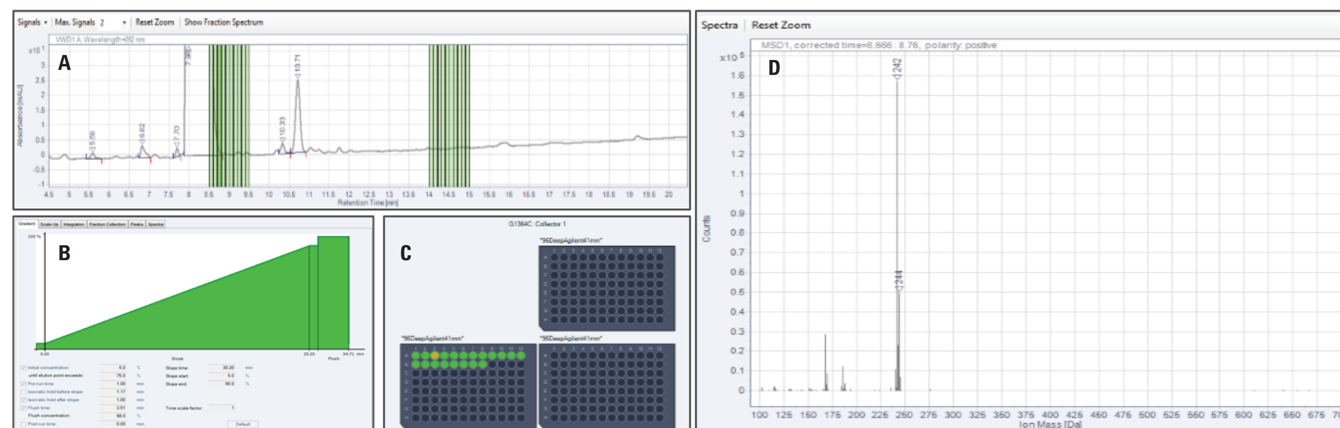

Figure 4. Time-based fraction collection enabled collection of erythro/threo-bupropion. A) TIC indicating the time slices of 0.1 minutes at two time segments, B) the linear gradient used, C) the fraction collection window indicating the location of the collected fraction, and D) the MS spectra of the collected fraction.

Identification of the hydroxybupropion metabolite

The structure of hydroxybupropion was confirmed using high-resolution accurate mass MS/MS information. Analysis of MS/MS fragmentation data (*m/z* 166 and 238) arises from cleavage of the C-N bond and hydroxyl group, respectively, as indicated in Figure 5.

Mass-MetaSite software was used to confirm these structural assignments based on predictive metabolism and the accurate mass MS/MS data. Using the liver microsomal model, the possible structures for hydroxybupropion suggested that the hydroxyl group was located on either on the nitrogen atom, or the methyl group, or the tert-butyl group (see structures A, B, and C in Figure 6)[4,5].

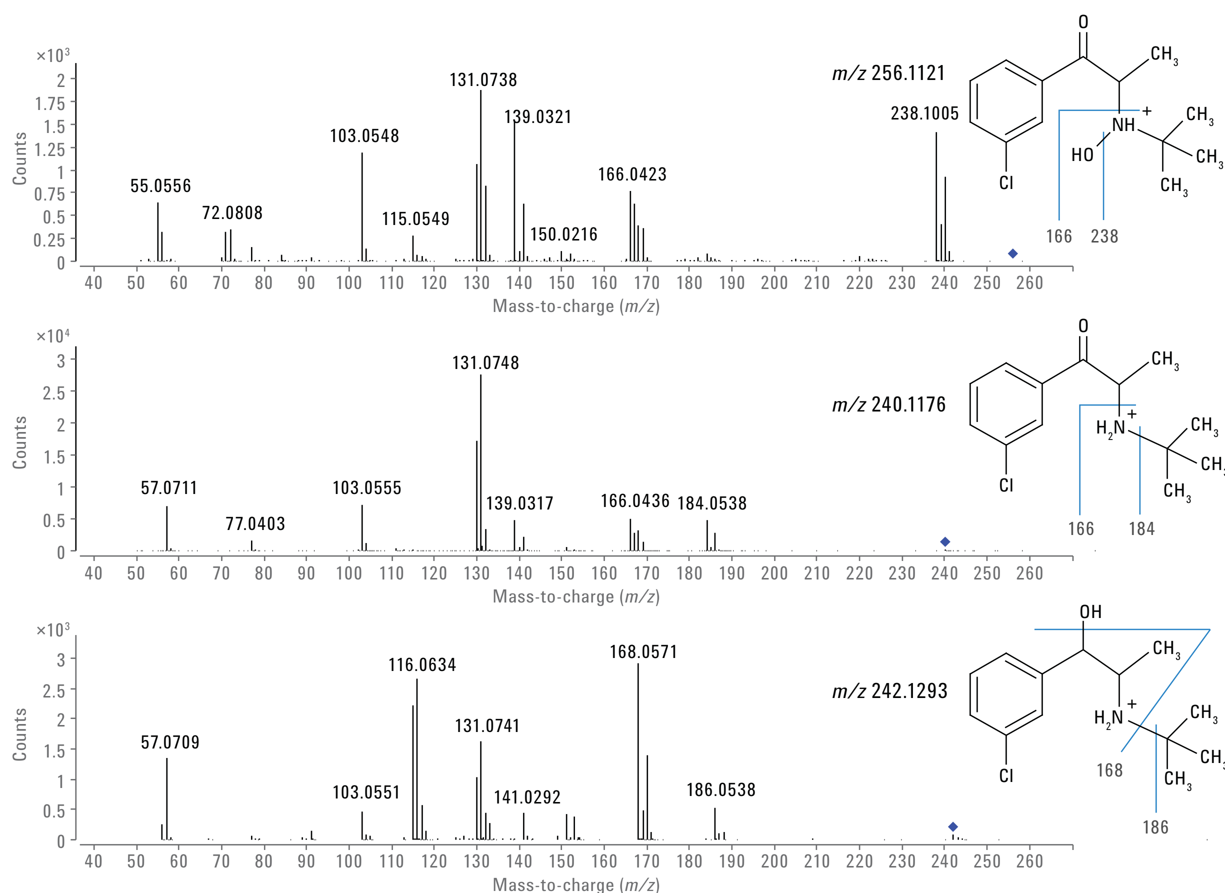

Figure 5. The LC/MS/MS fragmentation pattern of hydroxybupropion (MH⁺ *m/z* 256.1121), bupropion (MH⁺ *m/z* 240.1176), and erythro/threo bupropion (MH⁺ *m/z* 242.1293). The fragmentation of the metabolite compared with the fragment pattern from the parent bupropion.

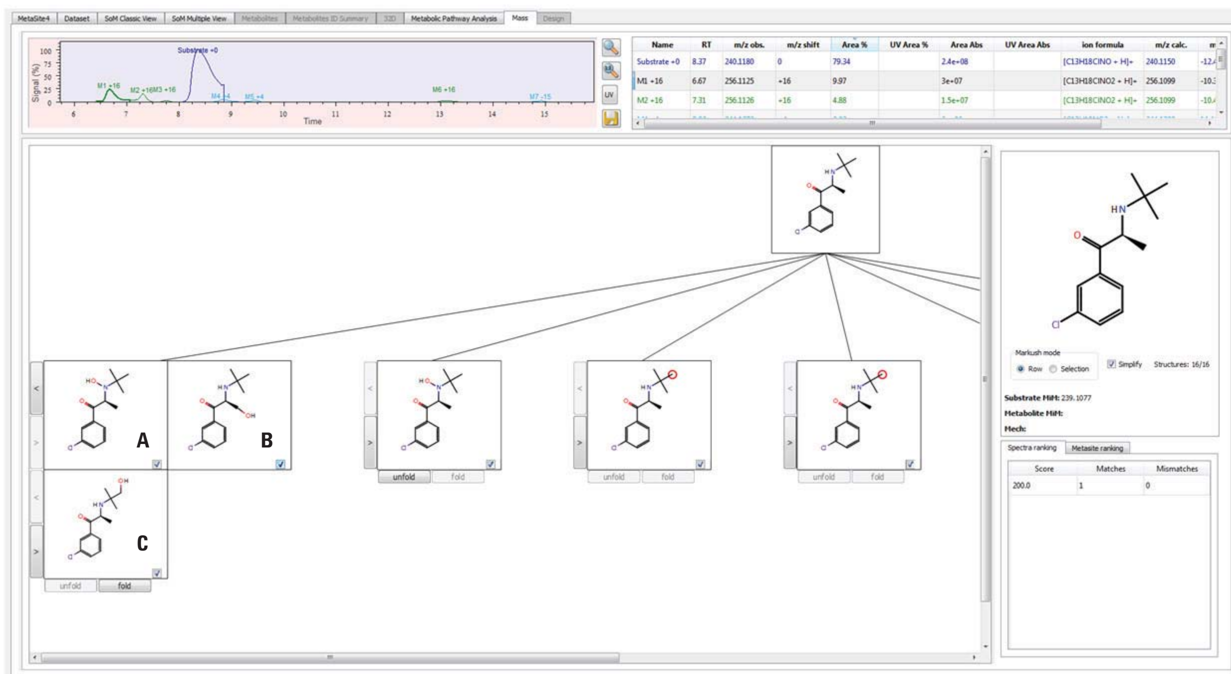

Figure 6. The Mass-MetaSite analysis of the possible structures for hydroxyl bupropion. A) Hydroxyl group on nitrogen, B) hydroxyl group on methyl group, C) hydroxyl group on tert-butyl group.

Conclusion

We have demonstrated a practical approach to the purification and identification of bupropion metabolites using several Agilent platforms. We have also shown that the HPLC method for the purification of bupropion metabolites can be scaled up from an analytical column to a semipreparative column using the Agilent Automated Purification Software. This method enabled the isolation of metabolites on a larger scale using both time-based and peak-based fraction collection modes. The Agilent 6540 Q-TOF LC/MS system was used to illustrate the metabolite structures using high-resolution accurate mass data from MS and MS/MS analysis. Mass-MetaSite software worked seamlessly to confirm these structural assignments. The flexibility and versatility of the purification allowed easy scalable capabilities for the preparative collection of metabolites to support additional experiments such as structural elucidation by NMR or drug preclinical toxicity studies.

References

1. Peak-based fraction collection with the Agilent 1100 Series purification system AS – Influence of delay volumes on recovery, *Agilent Technologies Technical Note*, publication number 5988-5746EN.

2. Analytical to Preparative HPLC Method Transfer, *Agilent Technologies Technical Note*, publication number 5991-2013EN.

3. Barros Jr., A; *et.al*. Development and evaluation of a multiple-plate fraction collection for sample processing: Application to radioprofiling in drug metabolism studies. *J. Pharm. and Biomed. Anal.* **2011**, *54*, pp 979-986.

4. Faucette, S. R; *et.al*. Evaluation of the contribution of cytochrome P450 3A4 to Human Liver Microsomal Bupropion hydroxylation. *Drug Metabolism and Disposition* **2001**, *29*, pp 1123-1129.

5. Wang, X; *et.al*. Simultaneous quantitative determination of bupropion and its three major metabolites in human umbilical cord plasma and placental tissue using high-performance liquid chromatography–tandem mass spectrometry. *J. Pharm. and Biomed. Anal.* **2012**, *70*, pp 320-329.

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