RESEARCH ARTICLE

MS^M, an Efficient Workflow for Metabolite Identification Using Hybrid Linear Ion Trap Orbitrap Mass Spectrometer

Robert Cho,¹ Yingying Huang,² Jae C. Schwartz,² Yan Chen,² Timothy J. Carlson,¹ Ji Ma¹

¹Department of Pharmacokinetics and Drug Metabolism, Amgen Inc., South San Francisco, CA 94080, USA

²Thermo Fisher Scientific, San Jose, CA 95134, USA

Abstract

Identification of drug metabolites can often yield important information regarding clearance mechanism, pharmacologic activity, or toxicity for drug candidate molecules. Additionally, the identification of metabolites can provide beneficial structure-activity insight to help guide lead optimization efforts towards molecules with optimal metabolic profiles. There are challenges associated with detecting and identifying metabolites in the presence of complex biological matrices, and new LC-MS technologies have been developed to meet these challenges. In this report, we describe the development of an experimental approach that applies unique features of the hybrid linear ion trap Orbitrap mass spectrometer to streamline in vitro and in vivo metabolite identification experiments. The approach, referred to as MS^M, utilizes multiple collision cells, dissociation methods, mass analyzers, and detectors. With multiple scan types and different dissociation modes built into one experimental method, along with flexible post-acquisition analysis options, the MS^M workflow offers an attractive option to fast and reliable identification of metabolites in different kinds of in vitro and in vivo samples. The MS^M workflow was successfully applied to metabolite identification analysis of verapamil in both in vitro rat hepatocyte incubations and in vivo rat bile samples.

Key words: MS^M, Wide isolation width HCD MS², Accurate mass filter, Orbitrap, Metabolite identification, LC-MS

Introduction

The characterization of metabolites is a vital activity at various stages of drug discovery and development. In early discovery, metabolite identification leads to the determination of labile sites on candidate molecules, and this information helps guide medicinal chemists in structural optimization with improved pharmacokinetic properties. Additionally, early biotransformation studies can reveal bioactivation to relevant reactive intermediates, whose formation should be minimized because of potential safety concerns. During preclinical development, an understanding

of the in vivo metabolic profile of a compound is essential since metabolites can contribute to pharmacologic action or have toxicological consequences. Investigation of the metabolic fate of candidates in various preclinical species and in human in vitro models can guide selection of appropriate animal species for toxicology studies. The goals of these studies include demonstrating that metabolites formed in humans are also present in animals chosen to perform safety assessment, and to identify metabolites with potential toxicity concerns [1–5].

Liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS) is the established analytical method of choice for metabolite identification due to its selectivity and sensitivity. With minimal preanalysis sample cleanup, complex mixtures can be chromatographically

separated and characterized by full scan MS and product ion scan MS/MS. Ion trap mass spectrometry is another established technology for metabolite identification due to its unique features, including data-dependent MSⁿ capability as well as better sensitivity and speed for full scan MS and MSⁿ relative to triple quadrupole tandem mass spectrometry. Supplementing low-resolution MS with accurate mass measurement in metabolite identification strategies can provide valuable information in distinguishing isobaric molecular ions, eliminating matrix interference, and providing unequivocal proof of correct fragmentation patterns, resulting in increased confidence in structural assignments.

Combining Orbitrap with linear ion trap technologies enables rapid, sensitive, reliable detection and identification of small molecules with high resolution and mass accuracy [6–8]. The hybrid linear ion trap Orbitrap mass spectrometer combines the ion trapping capacity and MSⁿ scanning capability of the linear ion trap with accurate mass measurements of the Orbitrap. It has been used to identify 58 metabolites of carvedilol, using data-dependent scanning triggered by an accurate mass parent list [9]. The approach utilized chemical formulas from high resolution accurate mass measurements to confirm the structures of product ions of the parent compound, and was subsequently used as a template for metabolite identification. Peterman and co-workers used this technology to obtain accurate mass full scan MS and MS/MS in a data-dependent fashion to eliminate the reliance on a parent mass list [10]. Such data from a single LC-MS experimental run enabled the characterization of the biotransformation profile of nefazodone. Combining a mass defect filter (MDF) with the hybrid linear ion trap Orbitrap mass spectrometry has also been successfully demonstrated for metabolite identification [11–13]. Another metabolite identification approach was evaluated using the hybrid system along with multiple post-acquisition data processing techniques, including mass defect filtering, extracted-ion chromatogram, product-ion filter, and neutral loss filter techniques [14].

The introduction of higher energy collision-induced dissociation (HCD) to the hybrid linear ion trap Orbitrap mass spectrometer allows generation of typical triple quadrupole-like fragmentation patterns with high efficiency and spectra quality [15–17]. This capability adds to existing ion trap-based fragmentation methods, like collision induced dissociation (CID) [18] and pulse Q dissociation (PQD) [19]. Unlike the ion trap-based CID method that generally has a low mass cut off at approximately one-third of the precursor mass, HCD experiments offer MS/MS spectra with a full range of fragment ions regardless of the m/z value of the precursor ion.

In this paper, we describe a novel experimental design for the hybrid linear ion trap-Orbitrap mass spectrometer, which extends its metabolite identification capabilities. Key features include a wide isolation width of up to 600 m/z values

in the linear ion trap, and the employment of HCD excitation on this range of precursor ions prior to Orbitrap scanning. The experimental design allowed for the acquisition of a large and diverse amount of mass spectral information in a single run, including multiple data-dependent MS² scans with both the ion trap and Orbitrap, as well as full MS and wide range accurate mass determinations. The workflow was developed for both in vitro soft spot analysis and in vivo definitive metabolite profiling. Using verapamil as a model compound, the potential of the workflow was demonstrated through analysis of rat hepatocyte incubations and rat bile samples.

Experimental

Materials

HPLC grade acetonitrile, methanol, and water were purchased from Burdick and Jackson (Morristown, NJ, USA). Verapamil, NADPH, and reagent grade formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cryopreserved rat hepatocytes were purchased from Celsis (Baltimore, MD, USA).

In Vitro Rat Hepatocyte Incubations with Verapamil

Cryopreserved rat hepatocytes were used for incubation at ~1 million cells/mL. Verapamil was incubated at final concentrations of 1 μ M at 37 °C in an incubator filled with carbogen (95/5% O₂/CO₂) for up to 4 h. The samples were quenched with three volumes of acetonitrile containing 3% of formic acid. After 15 min centrifugation at ~2200 $\times g$, the supernatants were dried down and reconstituted with 2:1 H₂O:MeOH for LC-MS analysis.

In Vivo Rat Bile Duct Cannulated Study of Verapamil

Verapamil was administered by intravenous bolus injection to male Sprague-Dawley rats ($n=3$) at a dose of 1.0 mg/kg. Bile was collected via an implanted cannula over 24 h. Bile samples were prepared for LC-MS analysis by quenching with two volumes of acetonitrile containing 3% of formic acid. Following centrifugation at ~1500 $\times g$ for 15 min, the clarified supernatants were transferred into 2 mL glass autosampler vials for LC-MS analysis.

LC-MS Analysis of Rat Hepatocyte Incubation and Rat Bile Samples of Verapamil

Analysis of verapamil rat hepatocyte extracts were performed on a Thermo Scientific hybrid LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). An Agilent 1200 Rapid Resolution binary pump

and a LEAP HTS autosampler were connected in line with the mass spectrometer.

Liquid chromatographic separation was achieved with a Thermo Scientific Hypersil Gold column (1.9 μm , 2.0 $\times$ 100 mm, Thermo Fisher Scientific, Waltham, MA, USA) using a binary gradient of 0.1% formic acid aqueous solution (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). In a typical run, mobile phase B was initially held at 5% for 2 min, then increased linearly to 40% over 17 min. The solvent composition was then increased linearly to 100% of mobile phase B over 1.5 min, and was held for an additional 2 min. Subsequently, the mobile phase was returned to the initial conditions for 2.5 min. The LC flow rate was set at 400 $\mu\text{L}/\text{min}$, with 100% of the flow directed into the mass spectrometer. A typical sample injection volume was 20 μL .

Instrument settings included a capillary temperature of 300 $^{\circ}\text{C}$, source heater temperature at 300 $^{\circ}\text{C}$, sheath gas flow at 42 arbitrary units, aux gas flow at 5 arbitrary units, sweep gas flow at 1 arbitrary unit, and the ion source voltage at 5000 V. An established generic tune setting was used for all experiments with an S-lens RF level at 68%, exit lens offset at 0, multiple rf at 400, multiple 00 offset at -4.25, lens 0 voltage at -4.50, multiple 0 offset at -5.00, lens 1 voltage at -23, gate lens offset at -66, multiple 1 offset at -8, and front lens at -6.75 V.

For the MS^M experiment, the scan cycle started with a full scan mass spectrum (MS) at a resolving power of 30,000 (at m/z 400) using the Orbitrap (Figure 1). Based on pre-scan information from the full scan MS, a parallel data-dependent CID MS² scan using the linear ion trap was performed on the most intense precursor ion. The third scan event was a data-dependent CID MS³ scan using the linear ion trap on the most intense product ion from the previous MS² scan event. In the global data-dependent setting, both the “FT master scan preview” and “predict ion injection time” options were enabled to reduce the overall cycle time. The precursor ion mass width was set at ± 10 ppm. The dynamic exclusion feature was also enabled with a repeat count of 1 and repeat duration of 15 s. For the CID MSⁿ scans, the minimal signal threshold was set at 5000 ion counts, and the normalized collision energy was set to 40 arbitrary units. The stepped collision energy feature was enabled with a collision energy width of 10 arbitrary units and number of collision energy steps of 3. A precursor ion list that was populated with predicted metabolites of verapamil was imported into the data-dependent MSⁿ scan setting to allow

automatic selection of precursor ions of interest. The second part of the scan cycle began with a wide isolation width (m/z 250–850) MS² scan using the higher energy collision-induced dissociation cell (HCD) and Orbitrap mass analysis (resolving power was 30,000 at m/z 400). Two additional linear ion trap data-dependent scans (MS² and MS³) on the second most intense ions from the pre-scan event were performed in parallel. The last part of the scan cycle consisted of two data-dependent HCD MS² scans at a resolving power of 15,000 (at m/z 400) on the same top two precursor ions. The microscan count for both linear ion trap and Orbitrap scans were set to 1, with maximum ion injection time at 20, 50, and 50 ms for linear ion trap MSⁿ scan, Orbitrap full scan MS, and MSⁿ scan, respectively.

Data Analysis

MS^M data analysis was facilitated by Xcalibur 2.1 and Networks 1.3 from Thermo Scientific (San Jose, CA, USA).

Results and Discussion

Wide Isolation Width HCD MS² Experiment on Hybrid Linear Ion Trap Orbitrap Mass Spectrometer

The key differentiating instrumental settings of the new experimental method versus a classic MS² method are the wide isolation width utilized in the linear ion trap, as well as the subsequent HCD excitation and Orbitrap scanning of the resultant wide range of ions (Figure 2). The steps of a classic MS² experiment using the HCD cell first involve ion trapping and precursor ion isolation in the linear ion trap. The precursor ions rapidly pass through the c-trap into the HCD collision cell for ion dissociation. All fragment ions are then transmitted to the Orbitrap for high resolution mass analysis after an intermediate step of c-trap trapping and ion cooling. In such a classic MS² experiment, the isolation width of the precursor ion is normally in the range of 0.75–2 m/z . However, with improvements on the hybrid linear ion trap Orbitrap instrumental control software, the isolation width of precursor ion can be increased up to a value of 600 m/z without significant loss of ion trapping efficiency. This is demonstrated by the comparable signal intensities observed between the high resolution full scan MS and the high resolution wide isolation width HCD MS² scan with a

Orbitrap:	Full MS		Wide Range HCD MS ²		DD* 1 st HCD MS ²	DD 2 nd HCD MS ²
LIT:	DD 1 st CID MS ²	DD 1 st CID MS ³	DD* 2 nd CID MS ²	DD 2 nd CID MS ³		

* **Orbitrap**: high resolution orbitrap scan; **LIT**: linear ion trap scan; **DD**: Data Dependent MSⁿ scan with full MS orbitrap scan as referenced scan event.

Figure 1. Experimental design of MS^M mass spectrometer method using hybrid linear ion trap Orbitrap mass spectrometer

Figure 2. Comparison of ion manipulation steps between classic HCD MS² experiment and wide isolation width HCD MS² experiment using hybrid linear ion trap Orbitrap mass spectrometer

zero collision energy (Figure 3a and b). Optimization of two key HCD parameters, the center mass for the precursor ion isolation and the HCD collision energy, generates reproducible and comprehensive fragmentation for all precursor ions isolated in the targeted range (Figure 3c). Figure 3d and e

demonstrate the differences between the wide isolation width HCD MS² and in-source CID data using the same hybrid linear ion trap Orbitrap platform. While major fragments remain the same, the wide isolation width HCD MS² spectrum exhibits higher fragmentation efficiency as

Figure 3. Mass spectra of six compounds mixture containing 1 μ M each of buspirone (m/z 386.2551), donepezil (m/z 380.2220), glyburide (m/z 494.1511), haloperidol (m/z 376.1474), nefazodone (m/z 470.2317), loperamide (m/z 477.2303), and verapamil (m/z 455.2904): **(a)** high resolution full scan MS with mass range of m/z 40–850, **(b)** high resolution wide isolation width (m/z 250–850) HCD MS² scan and collision energy at 0 eV, **(c)** high resolution wide isolation width (m/z 250–850) HCD MS² scan and collision energy at 40 eV, **(d)** high resolution full scan MS with mass range of m/z 40–850, in-source fragmentation voltage was 40 V, **(e)** high resolution full scan MS with mass range of m/z 40–850, in-source fragmentation voltage was 60 V. The X axis was zoomed to the mass range of m/z 60–600

indicated by the higher intensity of the fragments, resulting in better overall MS² quality.

MS^M Workflow

With incorporation of the optimized wide isolation width HCD MS² scan, the general MS^M experiment method consisting of eight scan events was designed as was briefly described in the [Experimental](#) Section (Figure 1). By taking advantage of the hybrid design of the linear ion trap Orbitrap mass spectrometer, high resolution full scan MS and wide isolation width HCD MS² scan events can be achieved in a parallel fashion with four data-dependent MSⁿ scans in the linear ion trap. To achieve this, the preview data of the high resolution full scan MS enables early selection of the top two most intense precursor ions. After these scan events, the method also incorporated two additional data-dependent high resolution HCD MS² scans of the same top two precursor ions. The overall cycle time in such an experimental method is largely determined by the four high resolution Orbitrap scans. The data-dependent MSⁿ scans, which are performed in parallel in the linear ion trap, have minimal impact on the total cycle time. One concern in conducting multiple high resolution Orbitrap scans in parallel with several data-dependent MSⁿ scans is collection of sufficient data points across a typical chromatographic

peak of 10–14 s. Typically, at least 4–6 data points are needed to allow reliable construction of chromatographic peaks for analytes of interest. To achieve sufficient cycle time of 2–3 s, several key instrument settings were optimized. The maximum ion injection time, number of micro scans per scan event, and automatic gain control (AGC) ion target settings were adjusted to achieve a reasonable balance between scan speed and spectrum quality. The cycle time was approximately 2.7 s with injection amount of 2.0×10^{-8} or 2.0×10^{-5} μmol on column of verapamil (Figure 4a and b) covering concentrations over three orders of magnitude. This data suggests that the optimized instrument settings yield a minimum of five to seven cycles per typical chromatographic peak with peak width in the range of 10–14 s. Figure 4c–j is a set of spectra from one complete scan cycle (scan number 2465–2472) in the LC-MS experiment of 2.0×10^{-8} μmol on column of verapamil (Figure 4a). The precursor ion of verapamil (m/z 455.2902) was detected in the high resolution full scan MS with 0.4 ppm mass accuracy (Figure 4c). As shown Figure 4f, a signature product ion of verapamil of m/z 165.0909 was also observed in the wide isolation width HCD MS² scan with a mass accuracy of 0.6 ppm. Although the first set of data-dependent MSⁿ spectra (Figure 4d, e, and i) was generated on an abundant background ion at m/z 255.9439, good quality spectra were still collected on

Figure 4. LC-MS data of different concentrations of verapamil collected using MS^M experiment method: **(a)** zoomed base peak MS chromatogram with accurate mass filter of m/z 455.2904 of 2.0×10^{-8} μmol on column of verapamil sample, **(b)** zoomed base peak MS chromatogram with accurate mass filter of m/z 455.2904 of 2.0×10^{-5} μmol on column of verapamil sample, **(c)** high resolution full scan MS spectrum (scan # 2465) of 0.001 μM verapamil sample, **(d)** data-dependent CID MS² spectrum of precursor ion of m/z 255.9439 (scan # 2466) of 0.001 μM verapamil sample, **(e)** data-dependent CID MS³ spectrum of precursor ion of m/z 214.9 (scan # 2467) of 0.001 μM verapamil sample, **(f)** high resolution wide isolation width (m/z 250–850) HCD MS² spectrum (scan # 2468) of 0.001 μM verapamil sample, **(g)** data-dependent CID MS² spectrum of precursor ion of m/z 455.2902 (scan # 2469) of 0.001 μM verapamil sample, **(h)** data-dependent CID MS³ spectrum of precursor ion of m/z 303.2 (scan # 2470) of 0.001 μM verapamil sample, **(i)** data-dependent high resolution HCD MS² spectrum of precursor ion of m/z 255.9439 (scan # 2471) of 0.001 μM verapamil sample, **(j)** data-dependent high resolution HCD MS² spectrum of precursor ion of m/z 455.2902 (scan # 2472) of 0.001 μM verapamil sample

Figure 5. Typical MS^M workflow using hybrid linear ion trap Orbitrap mass spectrometer

verapamil with the help of the pre-built precursor ion list in the MS method (Figure 4g, h, and j).

Upon the optimization of the MS^M experimental method, a workflow was designed for typical in vitro and in vivo metabolite identification experiments (Figure 5). A given in

vitro or in vivo sample is analyzed on the hybrid linear ion trap Orbitrap mass spectrometer using the generic MS^M experiment method. Using the information-rich data set, the subsequent step in the design is a search for all metabolites of interest through chromatographic analysis using different accurate mass-based filters. The predictive accurate mass precursor ion filter (pAMPF) can be applied to the high resolution full scan MS data to confirm existence of expected metabolites if there is prior knowledge of the general metabolic pathways of the drug candidate of interest. The multiple mass defect filter (MMDF) is another accurate mass filter that can help elucidate potential components related to the parent compound. Applications of accurate mass product ion filters (AMPIF) to the wide isolation width HCD MS² data facilitate the search for metabolites, especially for unexpected products. The *m/z* values of precursor ions of identified components can be obtained through analysis of adjacent high resolution full scan MS data. Another complementary approach for detection of metabolites of interest is the application of an accurate mass neutral loss filter (AMNLF) on the high resolution full scan MS and wide isolation width HCD MS² data collected in the same experiment. Following completion of metabolite detection utilizing these accurate mass filters on the high

Figure 6. Extracted ion chromatograms (XICs) of verapamil rat hepatocytes sample with application of different accurate mass filters: (a) extracted ion chromatogram with application of high resolution full scan MS filter [*m/z* 80–850], (b) extracted ion chromatogram with application of high resolution full scan MS filter [*m/z* 80–850] and predicted accurate mass precursor ion filter (pAMPF), (c) extracted ion chromatogram with application of high resolution wide isolation width HCD MS² scan filter with center mass at *m/z* 550 and mass isolation range of *m/z* 250–850, (d) extracted ion chromatogram with application of high resolution wide isolation width HCD MS² scan filter with center mass at *m/z* 550, mass isolation range of *m/z* 250–850 and accurate mass product ion filter (AMPIF) of *m/z* 165.0910, a signature product ion of verapamil, (e) extracted ion chromatogram with application of high resolution isolation width HCD MS² scan filter with center mass at *m/z* 550, mass isolation range of *m/z* 250–850 and accurate mass product ion filter (AMPIF) of *m/z* 151.0754, a signature product ion of some verapamil metabolites with mono-demethylation occurred on the product ion of *m/z* 165.0910

resolution Orbitrap scans, the MSⁿ data, including HCD MS², CID MS², and MS³ spectra assists in the structural elucidation of all identified metabolites. Data-dependent MSⁿ scans can sometimes fail to be triggered on some components of interest, including unexpected species not included in the precursor ion list, low abundance species that co-elute with abundant metabolites, or those obscured under higher intensity background ions with close m/z values. A second targeted MSⁿ experiment is warranted for more definitive structural confirmation of these particular metabolites.

Application of MS^M Workflow for In Vitro Metabolite Identification in Verapamil Rat Hepatocyte Samples

Samples from verapamil incubations with rat hepatocytes were analyzed using the MS^M experimental method and the hybrid linear ion trap Orbitrap mass spectrometer. Compared with the full mass high resolution scan (Figure 6a), the application of the predicted accurate mass precursor ion filter (*p*AMPF) with 3 ppm mass accuracy tolerance resulted in a significantly cleaner extracted ion chromatogram (XIC) (Figure 6b). Based on structural features of verapamil and knowledge of general metabolic pathways [20–23], 50 parent ions were included for the *p*AMPF filtering process, including multiple phase I and II biotransformation pathways. Given the high mass accuracy setting and stable instrument performance, all components identified in the XIC trace (Figure 6b), including those with relatively low ion intensity, are putative metabolites of verapamil [24–28]. While application of *p*AMPF filters to the high resolution full scan MS spectra offered a reliable and efficient way of identifying possible metabolites, the quality of the results is dependent on an accurate and comprehensive prediction of metabolic pathways. The search does not detect unusual metabolites generated through less common metabolic pathways since parent ion masses of these components are not included in the precursor ion list.

However, the wide isolation width HCD MS² scan (Figure 6c) contains fragment ions of all precursor ions that were isolated in the mass range of m/z 250–850. Application of the accurate mass product ion filter (AMPIF) offered a new means of metabolite fishing without a precursor ion list as all components that generate a targeted product ion will be detected. As an example, an extracted ion chromatogram of m/z 165.0910 (Figure 6d), a signature product ion of verapamil, revealed a number of components with significantly different relative abundances based on the observed signal response. Since all metabolites detected share the same product ion, and therefore the same substructure, at m/z 165.0910, it is reasonable to assume that metabolites that are formed by modifications on this substructure were not detected. As an interesting comparison, a different metabolite profile of verapamil was revealed in the extracted ion chromatogram generated with a product ion of m/z 151.0754,

mono-demethylation analog of product ion of m/z 165.0910 (Figure 6e). With the use of such AMPIF analysis and the data from an adjacent high resolution full MS scan, the masses of corresponding precursor ions associated with these characteristic product ions can be confirmed. Furthermore, the different metabolite profiles observed in Figure 6d and e suggest that the proper selection of the signature product ion is a critical step to efficient metabolite detection using AMPIF filter.

Application of MS^M Workflow for In Vivo Metabolite Identification in Verapamil Rat Bile Samples

Bile samples from verapamil-treated rats were analyzed using the MS^M experimental method, including a predicted precursor ion list incorporated into the data-dependent scan settings. Even with a more complex matrix (i.e., whole bile, Figure 7a), accurate mass-based filters were applied successfully to both high resolution full scan MS spectra and wide isolation width HCD MS² data to facilitate accurate

Figure 7. Extracted ion chromatograms (XICs) of verapamil rat bile sample with application of different accurate mass filters: **(a)** extracted ion chromatogram with application of high resolution full scan MS filter [m/z 80–850], **(b)** extracted ion chromatogram with application of high resolution full scan MS filter [m/z 80–850] and predicted accurate mass parent ion filter (*p*AMPF) of m/z 589.2754

Figure 8. Mass spectra of a tridesmethyl verapamil glucuronide in rat bile sample: **(a)** high resolution full scan MS spectrum [m/z 80–850], **(b)** data-dependent high resolution HCD MS² spectrum of m/z 589.2754, precursor ion of the glucuronide, **(c)** data-dependent CID MS² spectrum of m/z 589.3, precursor ion of the glucuronide, **(d)** data-dependent CID MS² spectrum of m/z 263.2, a product ion of the glucuronide

and efficient identification of in vivo verapamil metabolites. For example, an accurate mass precursor ion filter of m/z 589.2754 was applied to the high resolution full scan MS data to identify all possible tridesmethylated glucuronides in rat bile (Figure 7b). With a mass accuracy tolerance of ± 3 ppm, two related glucuronides were identified readily from the complex bile matrix, even though base peak ion intensities of both glucuronides were approximately 50- to 100-fold less than co-eluting background ions (Figure 7a). The high resolution full scan MS spectrum of the sample is shown in Figure 8a, where m/z 589.2753 is buried in a cluster of interference ions endogenous to bile. Without a pre-populated precursor ion list, such a high level of noise would pose a significant challenge to the collection of useful data-dependent MSⁿ information in the same experiment. At the mass accuracy tolerance of ± 10 ppm, a precursor ion list allowed generation of good quality data-dependent HCD MS² data (Figure 8b), as well as CID MS² and MS³ data collected with the linear ion trap. Structural elucidation of the glucuronide was achieved by analysis of the fragments from the major product ions detected in the high resolution HCD MS² scan (Figure 8b). For example, the product ion observed at m/z 413.2434, a fragment formed through loss of m/z 176.0317, is a neutral loss that is characteristic of glucuronides. The CID MS² and MS³ data (Figure 8c and d), which were collected in parallel with the high resolution scans, provided further confirmation of the proposed fragmentation pathways of the glucuronide

metabolite. Without performing an extra experiment, HCD and CID MS/MS data collected within the same LC-MS run facilitated the structural characterization of this low abundant tridesmethylated glucuronide.

Conclusions

A workflow termed MS^M was developed which incorporates wide isolation width HCD MS² scans. The data from the wide isolation width HCD MS² scans provided additional search options that were not previously available, such as AMPIF and AMNLF. Unlike similar search functions based on data-dependent MS² scans [14], the current approach is based on the wide isolation width HCD MS² scan collected through a data-independent acquisition, which is conceptually similar to classic product ion and neutral loss scan modes on a triple-quadrupole mass spectrometer. While the triple quadrupole-based product ion and neutral loss scan modes offer impressive selectivity and sensitivity, such analysis often requires multiple experiments. The post-acquisition approaches used in the presented workflow allows comparable searches using different specified neutral loss and product ions masses with high resolution accurate mass data from a single injection. The combination of high resolution mass analysis, independent of the parent compound, along with multiple post-acquisition accurate mass-based filters provided a powerful, yet flexible solution to accommodate different sample types and different user

preferences. By utilizing the data-dependent scan functions and a pre-built accurate mass precursor ion list for all predicted metabolites, meaningful MSⁿ data of metabolites of interest could be efficiently obtained within the same LC-MS run. The method was particularly useful for samples in complex and analytically challenging matrices such as bile.

Overall, with multiple scan types built into one experiment method, along with flexible post-acquisition data analysis options, the MS^M workflow offered a unique way to achieve fast and reliable identification of common and uncommon metabolites in different kinds of in vitro and in vivo samples. The performance of this workflow could be further enhanced with more streamlined data analysis software and the next generation hybrid linear ion trap Orbitrap mass spectrometer, which has even faster scan rates.

Acknowledgments

The authors thank Dr. Bradley K. Wong of Amgen Inc. for very constructive discussion and careful review of the manuscript.

References

- Lin, J.H., Lu, A.Y.H.: Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol. Rev.* **49**, 403–449 (1997)
- Fura, A.: Role of pharmacologically active metabolites in drug discovery and development. *Drug Discov. Today* **11**, 133–142 (2006)
- Guengerich, F.P.: Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J.* **8**, E101–E111 (2006)
- Yan, Z., Caldwell, G.W.: Metabolism profiling, and cytochrome P450 inhibition and induction in drug discovery. *Curr. Top. Med. Chem.* **1**, 403–425 (2001)
- Guidance for Industry, Safety Testing of Drug Metabolites. Center for Drug Evaluation and Research, Food and Drug Administration (2008)
- Makarov, A.: Electrostatic axially harmonic orbital trapping: A high-performance technique of mass analysis. *Anal. Chem.* **72**, 1156–1162 (2000)
- Makarov, A., Denisov, E., Kholomeev, A., Balschun, W., Lange, O., Strupat, K., Horning, S.: Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. *Anal. Chem.* **78**, 2113–2120 (2006)
- Perry, R.H., Cooks, R.G., Noll, R.J.: Orbitrap mass spectrometry: Instrumentation, ion motion and applications. *Mass Spectrom. Rev.* **27**, 661–699 (2008)
- Lim, H.-K., Chen, J., Sensenhauser, C., Cook, K., Subrahmanyam, V.: Metabolite identification by data-dependent accurate mass spectrometric analysis at resolving power of 60,000 in external calibration mode using an LTQ/orbitrap. *Rapid Commun. Mass Spectrom.* **21**, 1821–1831 (2007)
- Peterman, S.M., Duczak Jr., N., Kalgutkar, A.S., Lame, M.E., Soglia, J. R.: Application of a linear ion trap/orbitrap mass spectrometer in metabolite characterization studies: Examination of the human liver microsomal metabolism of the nontricyclic antidepressant nefazodone using data-dependent accurate mass measurements. *J. Am. Soc. Mass Spectrom.* **17**, 363–375 (2006)
- Zhang, H., Zhang, D., Ray, K.: A software filter to remove interference ions from drug metabolites in accurate mass liquid chromatography/mass spectrometric analyses. *J. Mass Spectrom.* **38**, 1110–1112 (2003)
- Bateman, K.P., Castro-Perez, J., Wrona, M., Shockcor, J.P., Yu, K., Oballa, R., Nicoll-Griffith, D.A.: MS^E with mass defect filtering for in vitro and in vivo metabolite identification. *Rapid Commun. Mass Spectrom.* **21**, 1485–1496 (2007)
- Zhu, M., Ma, L., Zhang, H., Humphreys, W.G.: Detection and structural characterization of glutathione-trapped reactive metabolites using liquid chromatography-high-resolution mass spectrometry and mass defect filtering. *Anal. Chem.* **79**, 8333–8341 (2007)
- Ruan, Q., Peterman, S., Szewc, M., Ma, L., Cui, D., Humphreys, W.G., Zhu, M.: An integrated method for metabolite detection and identification using a linear ion trap/orbitrap mass spectrometer and multiple data process techniques: Application to indinavir metabolite detection. *J. Mass Spectrom.* **43**, 251–261 (2008)
- Olsen, J.V., Macek, B., Lange, O., Makarov, A., Horning, S., Mann, M.: Higher-energy C-trap dissociation for peptide modification analysis. *Nat. Methods* **4**, 709–712 (2007)
- Zhang, Y., Ficarro, S.B., Li, S., Marto, J.A.: Optimized orbitrap HCD for quantitation analysis of phosphopeptides. *J. Am. Soc. Mass Spectrom.* **20**, 1425–1434 (2009)
- Bateman, K.P., Kellmann, M.K., Muenster, H., Papp, R., Taylor, L.: Quantitative-qualitative data acquisition using a benchtop orbitrap mass spectrometer. *J. Am. Soc. Mass Spectrom.* **20**, 1441–1450 (2009)
- Louris, J.N., Cooks, R.G., Syka, J.E.P., Kelley, P.E., Stafford Jr., G.C., Todd, J.F.J.: Instrumentation, applications, and energy deposition in quadrupole ion trap mass spectrometry. *Anal. Chem.* **59**, 1677–1685 (1987)
- Schwartz, J. C., Syka, J. P., Quarmby, S. T.: Improving the Fundamentals of MSⁿ on 2D Ion Traps: New Ion Activation and Isolation Techniques. Proceedings of the 53rd Annual Conference on Mass Spectrometry and Allied Topics; San Antonio, TX (2005)
- Guengerich, F.P.: Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **14**, 611–650 (2001)
- Levsen, K., Schiebel, H.-M., Behnke, B., D tzer, R., Dreher, W., Elend, M., Thiele, H.: Structure elucidation of phase II metabolites by tandem mass spectrometry: An overview. *J. Chromatogr. A* **1067**, 55–72 (2005)
- Gibson, G. G., Skett, P.: Introduction to Drug Metabolism, 2nd ed. Stanley Thorne Publishing Ltd., Cheltenham, p. 1–34: (1996)
- Parkinson, A.: Biotransformation of Xenobiotics in Casarett and Doull's Toxicology: The Basic Science of Poisons, 5th edn. McGraw-Hill, New York (1996)
- Walles, M., Thum, T., Levsen, K., Borlak, J.: Metabolism of verapamil: 24 new phase I and phase II metabolites identified in cell cultures of rat hepatocytes by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **798**, 265–274 (2003)
- Borlak, J., Blickwede, M., Hansen, T., Koch, W., Walles, M., Levsen, K.: Metabolism of verapamil in cultures of rat alveolar epithelial cells and pharmacokinetics after administration by intravenous and inhalation routes. *Drug Metab. Dispos.* **33**, 1108–1114 (2005)
- Nelson, W.L., Olsen, L.D.: Regiochemistry and enantioselectivity in the oxidative N-dealkylation of verapamil. *Drug Metab. Dispos.* **16**, 834–841 (1988)
- Nelson, W.L., Olsen, L.D., Beitner, D.B., Pallow Jr., R.J.: Regiochemistry and substrate stereoselectivity of O-demethylation of verapamil in the presence of the microsomal fraction from rat and human liver. *Drug Metab. Dispos.* **16**, 184–188 (1988)
- Eichelbaum, M., Ende, M., Remberg, G., Schomerus, M., Dengler, H.J.: The metabolism of DL-[¹⁴C] verapamil in man. *Drug Metab. Dispos.* **7**, 145–148 (1979)