Accurate mass filtering of ion chromatograms for metabolite identification using a unit mass resolution liquid chromatography/mass spectrometry system

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Identification of drug metabolites in biological matrices such as urine, plasma, bile, and feces presents a formidable challenge for bioanalytical scientists. Effective analysis of the metabolites by liquid chromatography/mass spectrometry (LC/MS) is often compromised by overwhelming interferences from the endogenous matrix ions. These ions are notoriously abundant and can appear throughout a typical LC/MS run to an extent such that the metabolite profile can be difficult to observe in the total ion current (TIC) chromatogram or a base peak chromatogram. A great deal of effort has been devoted to removal of the interferences physically prior to or during the LC/MS analysis. One approach is to clean up the *in vivo* samples by solid-phase extraction (SPE) or liquid-liquid extraction (LLE) before injection into the LC/MS system. These sample preparation methods appear to be simple and feasible, but they also pose a risk of losing metabolites, especially those present at low concentrations. Another approach is to use neutral loss (NL) or precursor ion (PI) scan modes with a triple-quadrupole instrument to selectively detect certain types of metabolites. For example, any glucuronide conjugation of a drug can be identified using a NL scan for 176 Da, corresponding to the mass of the glucuronide functional group. Both NL and PI scans can significantly filter out signals from the interfering endogenous ions to reveal the target metabolites and their profiles. However, this approach requires prior knowledge of characteristic fragmentations and is not universally applicable to other metabolites. Moreover, these two scan modes involve tandem mass spectrometry (MS/MS) experiments in which the abundances of minor metabolites may be too low to produce sufficient fragment ions from collisionally induced dissociation for reliable detection of the target metabolite types.

The endogenous interferences can also be removed or reduced by post-acquisition data processing. The most common utility used to search for the metabolites in complex mixtures is the extracted ion chromatogram (XIC); this approach does detect the metabolites with much improved selectivity compared with the TIC chromatogram, but many false positives can be produced when the metabolites are contained in complex sample matrices such as bile and feces. Based on the observation that the parent drug and its metabolites have similar mass defects, mass defect filtering has been used to successfully remove signals from matrix ions and to produce a metabolite profile similar to the trace obtained from a radioactivity detector. Currently, this filtering process can be performed only on the data acquired using high-resolution instruments such as quadrupole...
time-of-flight (qTOF) or Fourier transform mass spectrometers (FTMS), where the higher resolution allows for better separation of ions on-the-fly and the accompanying high mass accuracy enables the mass filtering in the post-processing mode. The filtering performance, however, depends on whether the endogenous ions or any of their significant isotopes have mass defects similar to those of the drug under investigation. Experienced users have also used isotope pattern analysis for the identification of chlorine- and bromine-containing metabolites, but this method is generally not applicable to metabolites that do not contain such distinctive isotope patterns.

Earlier work has shown that experimental mass accuracy follows the relationship given as Eqn. (1):

\[
\sigma \propto \frac{1}{R \sqrt{S}}
\]  

(1)

where \( R \) is the mass spectral resolving power and \( S \) is the ion signal level; this relationship assumes only ion counting noise. With internal mass calibration, a mass uncertainty of 10 mDa has been achieved using an enhanced resolution quadrupole instrument (FWHM = 0.1 Da) when operated in selected reaction monitoring (SRM) mode. The SRM operating mode obviously resulted in a high \( S \) value and partially contributed to the mass accuracy improvement. When comparing mass uncertainties achieved using an MS system that can operate in both low- and high-resolution modes, the resolving power at unit mass resolution (FWHM = 0.5 Da) for a small molecule of mass 500 Da is only 1000, resulting in a theoretical (Eqn. (1)) loss of mass accuracy by a factor of 5 compared to that obtained using a higher resolving power of 5000; this loss of mass accuracy resulting from low resolving power can, however, be partially or fully compensated by the higher signal available when operating at a lower resolution. It has been shown that it is possible to achieve a high mass accuracy of a few mDa or even 5 ppm using unit mass resolution systems in both infusion mode and on the chromatographic time scale. Nonetheless, the higher resolution available on qTOF systems still holds an intrinsic advantage over unit mass resolution systems in the physical rejection of ions with masses close to those of the metabolites of interest. On the other hand, there is significantly more information available from unit mass resolution data than what is currently exploited. A complete isotope profile that includes all isotopes of a metabolite ion, even measured under unit mass resolution, can provide the critical distinguishing power necessary to distinguish true metabolites and matrix ions.

This paper presents a new method that combines accurate mass ion chromatograms with profile mode mass spectral filtering to successfully extract metabolite-related information from complex LC/MS data acquired using a unit mass resolution triple-quadrupole mass spectrometer. Data obtained for verapamil metabolites buried in strong signals from the ions of a bile matrix demonstrate that this new approach removes the background ions and generates simplified ion chromatograms virtually free of false-positive peaks.

**EXPERIMENTAL**

**Reagents**

HPLC-grade water and acetonitrile (ACN) were purchased from EM Science (Gibbstown, NJ, USA). Verapamil, erythromycin, promethazine, reserpine, Tyr-Tyr-Tyr, terfenadine, buspirone, loperamide hydrochloride, potassium dihydrogen phosphate, magnesium chloride, and NADPH were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate (Bio-Chemika grade) was purchased from Fluka (Ronkonkoma, NY, USA). Male Sprague-Dawley (SD) rat liver microsomes were obtained from XenoTech, LLC (Lenexa, KS, USA).

**Instrumentation**

The HPLC system consisted of Shimadzu (Columbia, MD, USA) LC-10ADvp pumps and a SIL-HTA autosampler. An Advanced MAC-MOD Analytical (Chadds Ford, PA, USA) ACE 3 C18 HPLC column (18, 150 × 4.6 mm, 3 µm) was used. The mobile phases were A (0.4% formic acid in water) and B (ACN). A 68-min gradient from 0% B to 50% B was employed for the separation of verapamil metabolites at a flow rate of 0.7 mL/min during 31 min ramping. The HPLC eluent passed through a divert valve and was then introduced directly into the source of the mass spectrometer. An API 4000 Q TRAP (ABI-Sciex, Toronto, Canada) mass spectrometer was employed for MS analysis. All data were acquired in profile mode. The instrument was operated in positive electrospray ionization (ESI) mode, using the first quadrupole scanning from \( m/z \) 100–800. The Ionspray voltage was set to 5000 V. An ion source turbo gas temperature of 500°C was used, with curtain gas back-pressure set to 20 psi and the declustering and entrance potentials at 50 and 20 V, respectively.

**Sample preparation**

All incubations were conducted in 0.1 M potassium phosphate buffer (pH 7.4), with substrates (10 µM), magnesium chloride (4 mM), rat liver microsomes (1 mg/mL microsomal protein) and NADPH (1 mM). The verapamil was pre-incubated for 5 min at 37°C, and reactions were initiated by the addition of NADPH. A 1 mL volume of the incubate was removed after 1 h of incubation. The reactions were quenched with 2 mL of ice-chilled acetonitrile. Microsomal proteins were pelleted by centrifugation at 14000 rpm (10000 g) for 10 min at 4°C. The supernatants were transferred into clean tubes and evaporated to dryness under a gentle stream of nitrogen at room temperature. The residues were reconstituted with 250 µL of 50% methanol in water. Aliquots of incubated verapamil were spiked with blank SD rat bile or urine matrix to mimic a real in vivo sample.

A calibration standard mixture, containing erythromycin, promethazine, reserpine, Tyr-Tyr-Tyr, terfenadine, buspirone, and loperamide hydrochloride, was made in an ACN/water mixture (1:1) containing 0.1% formic acid.

**Data acquisition and processing**

The procedure for data acquisition and processing is summarized in Scheme 1. To achieve high mass accuracy using the Q TRAP instrument, acquisition of data for the
standard mixture is required for the ‘highly accurate mass spectral calibration algorithm’ (HAMSCA). This was accomplished by loop injection of the standard mixture with the mobile phases A and B at 1:1 ratio and the flow rate at 0.7 mL/min. These loop injection conditions were similar to those used for LC/MS analysis of verapamil metabolites.

After the loop injections, a sequence of samples that included a blank, a verapamil incubate, and the verapamil incubates spiked with a bile or urine matrix, was analyzed by LC/MS.

All the data acquired using the Q TRAP were converted into a MATLAB (MathWorks, Natick, MA, USA) data format by an Analyst script ‘Wiff to Matlab’. The converted data files were then processed by the software MSIntegrity™ (Cerno Bioscience, Monmouth Junction, NJ, USA). MSIntegrity™ features three major functions: mass spectra calibration, accurate mass and isotope profile filtering, and compound identification by accurate mass. Depending on the stability and the nature of a given instrument, the calibration can be performed as external calibration alone, internal calibration alone, or a combination of the external and internal calibrations. In this study, the profile mode mass spectrum of the calibration standard mixture was used to create an external calibration that was subsequently applied to every individual scan in the LC/MS data sets. Following the calibration, a list of possible biotransformations is created and loaded into MSIntegrity™ to perform the analysis with default values of 10 mDa for accurate mass filtering and 15% relative error for isotope profile filtering. The observed potential metabolite peaks in the resulting ion chromatogram, called the ‘accurate mass and profile extracted ion chromatogram’ (AMPXIC), were further confirmed by accurate mass measurements in the mass spectral dimension.

RESULTS AND DISCUSSION

Verapamil is a calcium channel blocker commonly used to treat hypertension and coronary artery disease. The in vitro metabolism of verapamil incubated with human or rat liver microsomes has been studied extensively, and results in several metabolites. The major metabolites are demethylated verapamil arising from loss of a methyl group from four possible O-methyl groups and one N-methyl group (see the molecular structure of verapamil in Fig. 2(c)). Verapamil has often been used as a model system to validate LC/MS method development for metabolite identification applications. Its well-characterized metabolites are thus used here as examples to demonstrate the effectiveness of this new approach for metabolite identification.

Mass spectrometer calibration

As a result of limitations imposed by current calibration technology and mass spectral peak analysis algorithms, quadrupole-based instruments do not offer adequate accurate mass capabilities which are increasingly popular as an additional confirmation tool for metabolite structural analysis. It is one of the major objectives in this study to achieve high accuracy mass measurement capability with these instruments of only unit mass resolving power.

The keys to high mass accuracy using such a low-resolving power MS system are comprehensive calibration and robust peak mass assignment (also called centroiding). Conventional MS calibration for quadrupole instruments concentrates only on correction of the mass errors, and does not address the issues of baseline and isotope interferences, noise filtering, and peak shape compensation. All of these issues,
along with the lack of a reliable centroiding algorithm, contribute to poor mass accuracy on these systems where peak width (FWHM) can be as wide as 0.7 Da. The new calibration approach used here focuses on the calibration of both mass scale and peak shapes with the added benefits of noise filtering. As a result of this comprehensive calibration procedure, the mass spectral peaks become well-defined symmetrical mathematical functions amenable to highly accurate peak mass measurement and peak area integration.

Figure 1 illustrates the processes and the results of the external calibration. The calibration standards consist of six small drug molecules with monoisotopic MH ions at m/z 152.071, 285.143, 386.256, 472.322, 609.281, and 716.459, as shown in the raw spectrum (Fig. 1(a)). An external calibration can be developed following the HAMSCA process from this calibration mixture. When this external calibration is applied to a weak peak in the mass spectrum shown in Fig. 1(a), both mass axis adjustment and peak shape compensation can be achieved along with improved signal-to-noise ratio (S/N, Fig. 1(b)). The mass calibration residuals for standard ions are all within 1 mDa (Fig. 1(c)). It is important to point out that the mass calibration residuals shown in Fig. 1(c) are only a measure of goodness of fit during calibration. To assess the true mass accuracy achievable, the calibration thus built needs to be applied to each mass spectral scan in an LC/MS run and the m/z values of ions other than the calibration ions calculated and compared to their theoretical values. The external calibration, created as described above, covered a sufficient m/z range from 156–716 and permitted high mass accuracy measurements for the verapamil metabolites on the LC/MS time scale. These measurements are demonstrated for representative metabolites of verapamil in Table 1. Although there was a time gap of about 6 h between the data acquisition of the calibration standards and that of the metabolites, the external calibration appears to have held as a result of the good instrument stability. Regardless of their m/z values, the accurate masses for all these metabolites were within 11 mDa of the theoretical values. The use of the external calibration for accurate mass measurements offers a number of advantages over the internal calibration. First, the approach has multiple calibration standards to account for all possible metabolites over the calibrated m/z range. Second, external calibration does not introduce ion suppression or interferences. Most importantly, this approach does not require dual ESI sprayers or the use of post-column addition of internal standards through a syringe pump, which greatly simplifies experimental procedures.

Table 1. Accurate mass measurements of verapamil metabolites

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Exact mass (Da)</th>
<th>Retention time (min)</th>
<th>Measured mass (Da)</th>
<th>Mass errors (mDa)</th>
</tr>
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<tbody>
<tr>
<td>Parent drug</td>
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<td>33.200</td>
<td>455.285</td>
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<td>Demethylated</td>
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<td>6.500</td>
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<td>Standard mass errors (mDa)</td>
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Verapamil in bile, external calibrated

<table>
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<th>Metabolites</th>
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<th>Retention time (min)</th>
<th>Measured mass (Da)</th>
<th>Mass errors (mDa)</th>
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</table>

Verapamil in urine, external calibrated
Accurate mass and profile extracted ion chromatogram (AMPXIC)

While recent developments in mass spectrometry technologies have provided highly automated data acquisition procedures for high-throughput LC/MS analysis, the lack of sophisticated data-handling tools has made data processing a bottleneck in the overall analytical process. This is especially true when searching for metabolites in LC/MS data sets for in vivo samples, because the metabolite peaks are often barely visible in the TIC chromatogram as a result of the presence of background ions that dominate the mass spectra. Identification of individual metabolites is thus time-consuming and labor-intensive. With the present accurate mass and isotope profile filtering approach, the data processing can be fully automated to significantly remove the matrix ions and result in an AMPXIC that is much more selective than a conventional XIC. Unlike the isotope pattern analysis method applicable only for chlorine- and bromine-containing metabolites, this new approach is universally applicable to a diverse range of metabolite structures.

The drastic reduction in matrix ion signals by this approach is demonstrated by LC/MS analysis of a verapamil in vitro incubation spiked with a bile matrix as a model for in vivo samples. As expected, while the TIC chromatogram for the LC/MS separation (Fig. 2(a)) shows intractable signals arising from abundant matrix ions, the XIC created using the usual ±0.5 Da mass window (from m/z 444.8–445.8, Fig. 2(b)) includes the parent drug (verapamil, m/z 455.291) at a retention time of 33 min. Unfortunately, many other peaks are also observed in this same XIC. This poor selectivity in the XIC is largely the result of the conventional mass accuracy available on such a unit mass resolution system, and the fact that it does not exploit the information in the isotope profile. When the new filtering approach was applied to the same data set, however, it rejected almost all the false positives and resulted in an AMPXIC (Fig. 2(c)) that is much cleaner than the conventional XIC. This significant improvement in selectivity is first attributed to the filtration by accurate mass measurements. Two peaks (group b2, Fig. 2(b)), at retention times of approximately 13 and 14.5 min in the original XIC for the parent drug, were filtered out since their m/z values, 455.164 and 455.166, respectively, were more than 120 mDa away from the m/z value of the MH+ ion of verapamil at 455.291. The minor peaks, appearing in the regions labeled as groups b1, b3, and b4 in Fig. 2(b), were also eliminated as a result of their large mass differences from the selected value (that of the parent drug). Similarly, the AMPXIC identified all five demethylated metabolites and one dealkylated metabolite (data not shown), with virtually no false positives and very little if any baseline variation in the AMPXIC (cf. Fig. 3(b) with Fig. 3(a)). Nearly all of the interference peaks in the corresponding XIC (Fig. 3(a)) disappeared as a result of the accurate mass and profile filtering. For example, at retention times of 15.6 and 17.3 min (Fig. 3(a)), two well-defined peaks corresponding to m/z values of 441.198 and 441.196, respectively, were filtered out completely.

The effect of accurate mass filtering can be further exemplified by the profile for the parent drug in its AMPXIC (Fig. 4(b)). The peak appears to be split into two separated peaks, reflecting the varying mass accuracy from different scans across the parent drug LC peak. As the peak eluted from the column into the mass spectrometer, the signal for verapamil first rose, then saturated the detector, and finally declined. It is well known that detector saturation leads to poor mass accuracy. Evidently, both the rising and the declining portions of the chromatographic peak gave very accurate m/z values at 455.287 and 455.296, respectively, leading to the enhanced AMPXIC signal. The saturated peak top resulted in an assigned m/z of 455.384, almost 100 mDa off the correct value for verapamil (455.291), leading to a depressed AMPXIC signal and resulting in two apparent split peaks for the same MH+ ion.

Secondly, isotope profile filtering also plays an important role in the selectivity enhancement, as illustrated by the peak at retention time of 47.2 min in Fig. 2(b). Although the peak was eluted much later than the parent drug verapamil, and therefore not likely to be a metabolite derived from verapamil, the peak will be used as an example to show the effective use of isotope profile filtering when the peak has a reasonably accurate mass assignment and would be expected to be retained based on accurate mass filtering alone. A striking difference can be seen between the conventional XIC and the AMPXIC in the time window from 45 to 50 min (Figs. 2(b) and 2(c)). Although it was the second most abundant peak in the XIC, the peak completely disappeared in the AMPXIC due exclusively to isotope profile filtering. The peak had an accurate m/z assignment of 455.300 (Fig. 5(a)), 10 mDa less than the known m/z value (455.291) of the MH+ ion of the parent drug (verapamil). On comparing the mass spectra of rejected (Fig. 5(a)) and retained (Fig. 5(b)) peaks with the theoretically calculated isotope profile of verapamil (Fig. 5(c)), it is obvious that the mass spectral peak at m/z 455.300 from a 13C peak of an unknown component at m/z 454.297 in Fig. 5(a) was removed because its isotope profile deviates significantly from that of verapamil. On the other hand, the retained mass spectrum (Fig. 5(b)) had both good mass assignment and a peak profile consistent with that of verapamil.
Figure 6 demonstrates another example of profile-mode spectral filtering when the monoisotopic mass of a peak matches accurately with that of a suspected metabolite ion. In the XIC for demethylated verapamil from 30 to 30.5 min, a small peak (Fig. 3(a)) was observed to have an accurate \( m/z \) assignment of 441.279, only 4 mDa from the calculated \( m/z \) of 441.275 for demethylated verapamil; thus this peak would have been retained based on accurate mass filtering alone. However, this peak was successfully rejected by profile filtering and did not appear in the AMPXIC. Comparison with the theoretically calculated spectral profile (Fig. 6(c)) shows that the measured spectrum (Fig. 6(a)) evidently has significantly more abundant \( M^+ \) and especially \( M^+2 \) peaks, and is therefore rejected. Further confirmation based on MS/MS experimentation is underway in the next phase of this project. In contrast, the mass spectrum retained in the AMPXIC (Fig. 6(b)) has not only an accurate mass assignment, but also a spectral profile consistent with that theoretically predicted for demethylated verapamil. This double-filtering effect, by both the accurate mass and profile matching, eliminated all spurious peaks and retained the truly drug-related chromatographic peaks in the AMPXIC.

When operated at the usual unit mass resolution, mass spectrometers such as triple quadrupoles are more likely subject to interferences than higher resolution mass spectrometers, such as qTOF and FTMS. The presence of isobaric interferences could compromise the mass accuracy measurement and lead to possible false negatives. Many of these isobaric interferences, however, can be identified and mathematically resolved due to the comprehensive calibration performed. This is the subject of a separate publication under preparation. When there is significant overlap of isotope profiles between an ion of interest and other interferences, the AMPXIC will tend to filter out the ion due to the high selectivity. When interferences of this nature are severe enough to cause false negatives, MS/MS analysis will be needed for further confirmation. Also, in spite of the intrinsic...
noise filtering capability built into the HAMSCA calibration, there may be cases where the S/N is not enough to provide reliable identification, requiring the use of different sample preparation, separation, or detection techniques.

CONCLUSIONS

The combination of accurate mass and profile mode filtering represents an innovative approach to facilitate data processing for the quick and reliable identification of metabolites in a complex mixture. The approach is established on the solid foundation of a unique calibration procedure that corrects for both mass error and mass spectral peak shape variations, and achieves random noise filtering all in the same process. This approach makes it possible to achieve accurate mass measurements for the metabolites in the bile matrix on a chromatographic scale using a quadrupole instrument with unit mass resolution. With this added capability for accurate mass measurements on top of its versatile scanning functions, the widely accessible triple-quadrupole instruments can be made more useful for metabolite identification and other qualitative analyses. Given molecular formulae of expected drug metabolites, AMPXICs obtained through accurate mass assignment and profile-mode filtering have far superior selectivity compared with conventional XICs, resulting in much simplified ion chromatograms similar to those obtained from a radioactivity detector with radio-labeling of the suspected metabolite. While an AMPXIC can pinpoint all key metabolite peaks with virtually no false positives, the possibility of false negatives from severe interferences, requiring the use of MS/MS for further confirmation, does exist. The applicability of this method can be expanded to other structure identification applications such as detection of degradation products and impurities, confirmation of protein modifications, and quick identification of trace levels of hazardous chemical or biological compounds. It has the potential to provide accurate mass measurements of MS/MS fragments for metabolites or other structural elucidation applications, a subject under active research. Future research would include embedding this approach into the MS system to perform highly targeted data-dependent MS/MS for applications ranging from metabolite identification to protein sequencing.

REFERENCES