Metabolite Identification Using a Unit Mass Resolution Liquid Chromatography/Mass Spectrometry with Accurate Formula Identification and Mass Defect Filtering

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Overview

Purpose

- *In vitro* metabolite identification using a unit mass resolution liquid chromatography/mass spectrometry with accurate formula identification, accurate mass profile extract ion chromatogram and mass defect filtering

Methods

- Rat liver microsomal incubation
- Liquid chromatography/Tandem mass spectrometry
- Convert the nominal mass data to the accurate mass data using a post acquisition computer program

Results

- Post acquisition processing obtained mass accuracy in a range of 3-10 ppm and spectral accuracy greater than 99.0% using a single reference ion for phase I metabolites.
- The accurate mass profile extract ion chromatogram (AMPXIC) obtained through accurate mass measurement and isotope profile-mode filtering allowed elimination of the false-positive peak observed using conventional XIC.
- Application of MDF technique requires accurate mass measurements in an entire mass range for all ions including parent drugs, metabolites and endogenous interferences, which could not be achieved using a single reference ion for calibration.

Introduction

Early metabolite identification provides essential information to chemists in synthesizing metabolically stable compounds and identifying pharmacologically active or toxic metabolites. Tandem quadrupole LC/MS/MS systems are extensively used for metabolite profile, but only provide nominal mass data. Newly developed mass spectrometry technology is available to convert nominal mass data to accurate mass data for accurate formula determination of metabolites. Further, this technology allows performing an Accurate Mass Profile Extract Ion Chromatogram or a Mass Defect Filtering to eliminate false-positive peaks or endogenous interferences on a unit mass spectrometer. These approaches substantially improve the throughout of data processing for metabolite profiling. We described here this approach for *in vitro* metabolite identification using a tandem quadrupole LC/MS and a post acquisition computer program.

Experimental

Microsomal incubation and sample preparation:

Incubations of Verapamil, Loperamide and Buspirone were performed with rat liver microsomes at a substrate concentration of 10 μM in the presence of NADPH at 37 °C. The incubations were quenched with acetonitrile. The incubation mixtures were centrifuged and the supernatants were transfer to HPLC vials for LC/MS analysis .

LC/MS analysis:

All LC/MS analyses were carried out using a Waters Quatro Micro mass spectrometer coupled with an Agilent HP1100 HPLC. The mass spectra were collected at a continuous scan mode at a scan rate of 700 amu/sec.

Post acquisition data processing:

A post acquisition computer program MassWorks™ was used to convert the nominal mass data to the accurate mass data using a single reference ion in a LC/MS run. The Accurate Mass Profile Extract Ion Chromatogram (AMPXIC) or Mass Defect Filtering (MDF) program was then employed to eliminate false-positive peaks or endogenous interferences in the samples.

Results and Discussion

Accurate Formula Identification

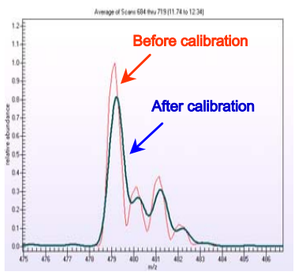
Table 1. Parameters to generate plausible formulas based on accurate mass converted from nominal mass

Charge	1
Mass Tolerance (mDa)	5
Electron State	Even
Double Bond Equivalent Minimum	-3
Double Bond Equivalent Minimum	100
Profile Mass Start (Da)	0
Profile Mass End (Da)	4.5

Element	Minimum	Maximum
C	0	50
H	0	100
O	0	50
N	0	31
Cl	0	20

- Elemental table for metabolites could be derived from the formulas of the parent compounds. To demonstrate the ruggedness of the post acquisition computer program, the elemental table was given in a wide range

Figure 1. Expanded view of a metabolite peak before and after calibration



Results and Discussion

Accurate Formula Identification

Table 2. Accuracy mass measurement and spectral accuracy of metabolites of Buspirone, Loperamide and Verapamil using the molecular ion of the parent compound as a single reference ion in the same LC/MS run

Metabolites	RT (min)	Formula	Theoretical mass (Da)	Measured mass (Da)	Mass error (ppm)	Spectral Accuracy	Ranking Formula
Buspirone							
Di-hydroxylated	4.94	$C_{21}H_{32}O_4N_5$	418.2454	418.2418	-8.67	99.7	1
Hydroxylated	5.22	$C_{21}H_{32}O_3N_5$	402.2505	402.2536	7.67	99.3	1
Hydroxylated	5.41	$C_{21}H_{32}O_3N_5$	402.2505	402.2534	7.17	99.6	1
Di-hydroxylated	5.54	$C_{21}H_{32}O_4N_5$	418.2454	418.2415	-9.39	99.5	2
Loperamide							
Demethyl, hydroxyl	11.72	$C_{28}H_{32}O_3N_2Cl$	479.2101	479.2086	-3.22	99.7	1
Demethyl, hydroxyl	12.41	$C_{28}H_{32}O_2N_2Cl$	463.2152	463.2200	10.30	99.8	1
Verapamil							
Hydroxylated	6.90	$C_{27}H_{39}O_5N_2$	471.2859	471.2814	-7.50	99.1	2
Di-demethylated	7.60	$C_{25}H_{35}O_4N_2$	427.2597	427.2626	6.83	99.6	1
Demethylated	7.79	$C_{26}H_{37}O_4N_2$	441.2753	441.2777	5.36	99.3	1
Hydroxylated	8.02	$C_{27}H_{39}O_5N_2$	471.2859	471.2818	-8.60	99.3	2
Demethylated	8.21	$C_{26}H_{37}O_4N_2$	441.2753	441.2755	0.38	99.6	1

- The post acquisition processing obtained mass accuracy in a range of 3-10 ppm and spectral accuracy greater than 99.0% for most metabolites of these three compounds.
- The correct formulas of the majority of metabolites were the first hit on formula ranking with a wide range of elemental table. For metabolite identification, a marrow elemental table based on the predicted metabolites can be used, which will further improve the ranking for correct formulas.

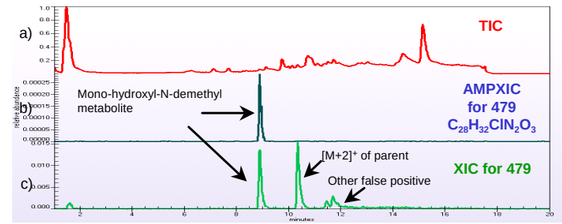
Table 3. Accurate mass measurement and spectral accuracy of dealkyl metabolites of Verapamil using the molecular ion of Verapamil as a reference ion in the same LC/MS run

Metabolites	RT (min)	Formula	Theoretical mass (Da)	Measured mass (Da)	Mass error (ppm)	Spectral Accuracy	Ranking Formula
Dealkylated	4.93	$C_{11}H_{18}O_2N$	196.1338	196.2200	439.53	98.8	1
Demethyl-dealkylated	6.25	$C_{16}H_{25}O_2N_2$	277.1916	277.2115	71.78	99.1	1
Demethyl-dealkylated	6.71	$C_{16}H_{25}O_2N_2$	277.1916	277.2024	38.95	98.0	1

- The molecular ions of the N-dealkyl and N-dealkyl-demethyl metabolites of Verapamil are m/z 196 and 277, respectively, which are more than 39 % lower than the calibration mass at m/z 455 (the molecular ion of Verapamil), resulting in low mass accuracy. However, the double-filtering effects of accurate mass and isotope profile allowed achieving high spectral accuracy for these N-dealkyl and N-dealkyl-demethyl metabolites. As a result, the correct formulas of these metabolites were the first hit.

The Accurate Mass Profile Extract Ion Chromatogram (AMPXIC)

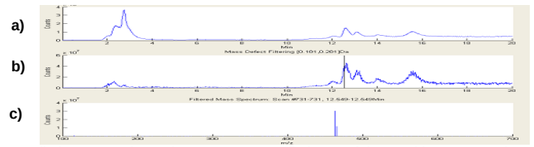
Figure 2. a) TIC chromatogram for Loperamide incubation mixture, b) AMPXIC for m/z 479 $C_{28}H_{32}ClN_2O_3$, c) XIC for 479



- The accurate mass profile extract ion chromatogram (AMPXIC) obtained through accurate mass measurement and profile-mode filtering allowed elimination of the false-positive peak observed using the conventional XIC for mono-hydroxyl-N-demethyl metabolites of Loperamide.

Mass Defect Filtering (MDF)

Figure 3. a) TIC chromatogram for Loperamide incubation mixture, b) MDF for potential metabolites, c) filtered mass spectrum of a metabolite



- Preliminary study using MDF to eliminate false-positive or endogenous interferences in the ion chromatogram of the incubation mixture of Loperamide was unsuccessful, due to the poor mass accuracy for the endogenous interferences, which had molecular ions far lower or higher than the reference ion.
- Using multiple reference ions in a wide mass range for external calibration could improve the mass accuracy of the interference ions.

Conclusions

- The double-filtering effects of accurate mass and isotope profile achieved high spectral accuracy for all metabolites studied using the molecular ion of parent drugs as a single reference ion to convert the nominal mass to the accurate mass.
- The accurate mass profile extract ion chromatogram (AMPXIC) obtained through accurate mass measurement and isotope profile-mode filtering eliminated the false-positive peaks observed using conventional XIC in the metabolite profile.
- Application of MDF technique requires accurate mass measurements in an entire mass range for all ions including parent drugs, metabolites and endogenous interferences, which could not be achieved using a single reference ion for calibration.