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ACCURATE MASS:

The best solution for metabolite identification in discovery, development and clinical applications.

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Abstract

Definitions of common terms appropriate in the use of modern accurate mass applications is provided for those new to the field. A discussion of the reasons why accurate mass offers a significant advance over nominal mass systems is given with examples of how accurate mass data can differentiate between the analytes of interest and matrix. A review of how accurate mass data affords an increased ability to identify the metabolites observed through enhanced parent and fragment ions specificity is provided.

Introduction

In recent years, accurate mass based analyses have become an increasingly important approach for qualitative mass-spectrometric analyses (1, 2, 3, 4, 5, 6). The use of accurate mass applications has steadily replaced the use of nominal mass approaches (7, 8, 9, 10, 11) and has resulted in many scientists who are new to this instrumentation making greater use of the technologies now available.

The goal of this paper is to review appropriate definitions and describe the theoretical basis of why accurate mass approaches offer a significant advance over nominal mass approaches in the arena of qualitative analysis and, specifically, metabolite identification.

Definitions

- Average Mass: Calculated using the average atomic mass of each element weighted for its natural isotope abundance.
- Nominal Mass: The integer mass of the most naturally occurring stable isotope.
- Monoisotopic Mass: Calculated mass of an ion or molecule calculated esing the mass of the most abundant, naturally-occurring isotope.
- Exact Mass: Calculated mass of an ion or molecule containing a single, specified isotope of each atom.
- Accurate Mass: The measured mass of an ion to high mass accuracy.
- High Resolution Mass Spectrometry (HRMS): Describes a mass spectrometer technology that exhibits high mass resolving power.

The average mass is a value that is frequently used by medicinal chemists, but has no meaning in mass spectrometry. No molecule exists that is represented by the average mass, as the various isotopes present in a molecule result in generating an isotope pattern in the mass spectrum.

The monoisotopic mass and exact mass are often used interchangeably since the most abundant, naturally-occurring isotope is commonly used in calculating an exact mass.

Resolution

Unit mass resolution describes a mass spectrometer that can differentiate between adjacent masses such as m/z 50 and 51 or m/z 1000 and 1001. This term is appropriate when discussing resolution of a quadrupole or ion trap mass spectrometer.

The definition of resolving power that is appropriate for Quadrupole orthogonal acceleration Time of Flight (Q-TOF) and Orbitrap mass spectrometers is based on full width half maximum (FWHM); thus the resolving power is defined as the mass being analyzed divided by the peak width of the raw mass data at that mass. So a mass spectrometer that affords an ion at m/z 500 that has a peak width of 0.1 Da is operating at 500/0.1 = 5000 resolving power. A mass spectrometer exhibiting 5000 resolving power can differentiate between m/z 50.000 and 50.010 or m/z 1000.000 and 1000. 200.

Since mass resolution is a function of the mass of the ion being examined, when an instrument resolution is stated the mass at which that resolution was determined has to also be defined. In the example above, the mass spectrometer is exhibiting a resolution of 5000 at mass 500.

Mass Accuracy

Mass accuracy is a key parameter of mass spectrometer performance and determines the specificity of the mass measurement being made. The most commonly used definition of mass accuracy is parts per million (PPM), which is determined as the difference between an exact (or theoretical) and measured (accurate) mass. In Table I, the PPM error for 3 different measured masses that have the same absolute mass error (25 mDa) relative to their exact masses are reproduced. The PPM error, since it is a relative term, alters with the mass of the ion being examined. Traditionally, a mass error

of 5 PPM has been accepted by medicinal chemistry journals as sufficient to afford a definitive elemental composition. Because PPM mass accuracy is a relative term, the use of mDa as an absolute measurement of instrument performance is often used as a convenient day-to-day tool.

A mass spectrometer with high mass resolving power is generally considered an essential criterion for achieving good mass accuracy. In reality, high resolving power is used to eliminate chemical interferences and is not otherwise required for obtaining good mass accuracy. In fact, accurate mass measurements can be routinely achieved with even a quadrupole mass spectrometer operated at low mass resolving power if interferences are not a problem. Since the samples being analyzed are usually not pure standards but mixtures or chromatographic peaks from LC-MS analyses, interferences are a frequent occurrence and HRMS is required to obtain quality accurate mass data.

Why Accurate Mass?

The application of accurate mass technology offers several advantages in conducting metabolite identification studies when compared with the use of nominal mass instrumentation such as triple quadrupole or ion trap mass spectrometers.

A review of the 3 main goals in conducting a metabolite identification study is provided to create a framework for understanding these advantages. These goals are the same whether the samples are derived from in-vitro or in-vivo experiments.

The first goal is to differentiate between ions derived from the drug itself, drug-related metabolites and ions derived from the matrix. The second goal is to identify the biotransformation that has taken place, such as addition of an oxygen atom, loss of a methyl group, etc. The third goal is to define what site on the drug molecule has undergone the biotransformation. Accurate mass data enables all three of these goals to be achieved more quickly than when using nominal mass data.

A paradigm shift is possible in using accurate mass approaches because the data is rarely compromised with interferences and provides more information on the molecule being analyzed. Compared to nominal mass studies, the overall result is that conclusions are reached more rapidly, with a higher level of confidence in their accuracy.

Table I: Example of PPM and mDa error at different masses

EXACT MASS	MEASURED MASS	ERROR (mDa)	ERROR (PPM)
1000	1000.0025	2.5	2.5
500	500.0025	2.5	5
	250.0025	2.5	10

There are four main reasons that accurate mass data is superior to nominal mass data in conducting metabolite identification studies, and these are discussed below:

1. Analyte versus Matrix Specificity. How is it that having accurate mass data enables differentiation between the analytes of interest and matrix? In most instances the matrix components to be eliminated as false positives are derived from biological sources such as microsomes, hepatocytes, plasma or urine. Such components are predominantly made of carbon, hydrogen, nitrogen and oxygen (C, H, N and O).

Most xenobiotics that are being analyzed, though mainly derived of the same elements, often contain a higher level of unsaturation and occasionally incorporate halogens. Table II lists some basic parameters of common elements. Carbon does not contribute to the fractional mass (the numerals after the decimal point) of a molecule's mass, while both hydrogen and nitrogen increase and oxygen, halogens and sulphur decrease the fractional mass; thus the fractional mass of a molecule will change with the elements that constitute the structure of the compound.

Table II: List of common elements with their accurate masses

ELEMENT	NUCLIDE	NOMINAL MASS	EXACT MASS	MASS DEFECT
Carbon	C^{12}	12	12.0000	0.0000
Nitrogen	N^{14}	14	14.0031	0.0031
Hydrogen	H^{1}	1	1.0078	0.0078
Oxygen	O^{16}	16	15.9949	-0.0051
Chlorine	Cl^{35}	35	34.9689	-0.0311
Fluorine	F^{19}	19	18.9984	-0.0016
Sulphur	S^{32}	32	31.9721	-0.0279

For example, for each double bond introduced into a molecule, the fractional mass will decrease by – 0.0156 Da. Since many of the xenobiotics that are drug candidates are more highly aromatic than naturally occurring "interfereneces" such as peptides, the observed fractional mass is less. A stylized example of this is shown in Figure 1 where a tripeptide, Tyr-Gly-Gly, is compared with a possible drug compound of similar nominal mass. The mass difference of 32 mDa would be easily differentiated using a modern accurate mass instrument.

The difference in fractional mass enables elimination of false positive mass "hits" when dealing with biological matrices. Figure 2 reproduces two extracted ion chromatograms from the same datafile. The top trace uses a mass window of ± 0.5 Da and the bottom trace uses a mass window of ± 0.010 Da. The nominal mass top trace has multiple peaks or "hits" for potential metabolites, most of which are false, while the accurate mass bottom trace has only metabolite peaks.

2. Parent Ion Specificity. Accurate mass data facilitates differentiation of biotransformations that result in an identical nominal mass shift. For , when using a nominal mass instrument, it is impossible to differentiate between M+O-2H or M+CH2, as the mass shift (in both instances) is +14 Da. When the same comparison is made using accurate mass data, the difference is very clear, as M+O-2H (M + 13.9793) or M+CH2 (M + 14.0156) result in a mass shift that differs by 36 mDa.

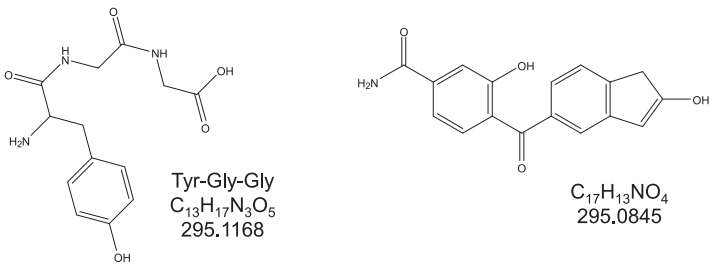
3. Fragment ion Specificity. An essential step in conducting a parent drug and, thus, determine the part of the molecule that has undergone biotransformation. The use of accurate mass data in this step can prevent errors in localization of the biotransformation, since nominal mass data cannot differentiate between different substructures in the molecule that afford the same nominal mass fragment ions.

Figure 3 shows a molecule that would afford fragment ions with similar nominal mass and require an accurate mass analysis to be correctly interpreted. Incorrectly interpreting the parent drug MS/MS spectrum would result in incorrect localization of the biotransformation. An incorrect localization of the site of metabolism could lead to medicinal chemisty decisions made to address a metabolic instability that would alter the wrong site on

Figure 1:

EXAMPLE OF A TRIPEPTIDE

and a possible "xenobiotic" with similar nominal mass

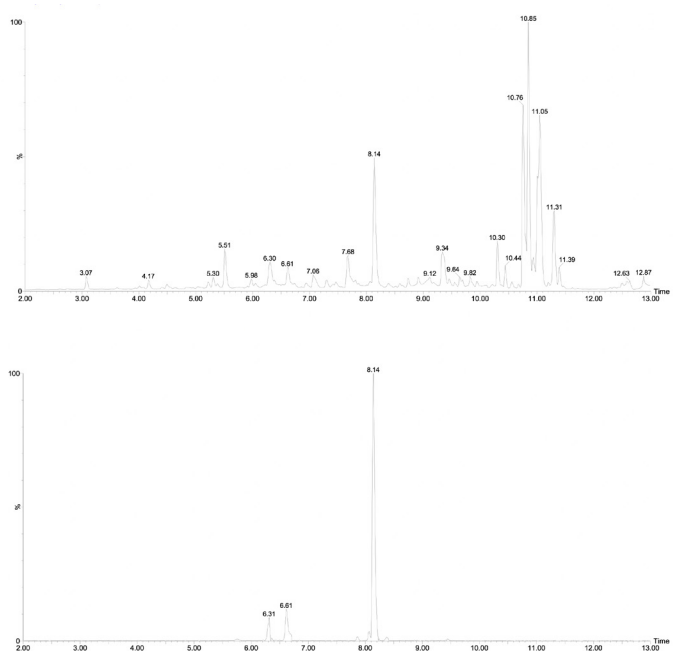


295.1168 – 295.0845 = 0.0323 • •32.3 mDa

Figure 2: Two extracted ion chromatograms

TWO EXTRACTED ION CHROMATOGRAMS FROM THE SAME DATA FILE

The top trace is generated using a 1 DA wide window; the bottom trace is from using a 10 mDa wide window.



the molecule.

4. Analytical Sensitivity. Nominal mass instruments use MS/MS methods to obtain specificity, such as precursor ion scans and constant neutral loss scans for metabolite identification. These MS/MS approaches are less sensitive than full-scan experiments, simply as a consequence of transmission efficiency in such MS/MS instrument modes. The application of accurate mass using full-scan experiments is more sensitive than nominal mass MS/MS methods because equal, or superior, specificity is obtained without the resultant loss of ion current.

Metabolite identification plays an important role throughout the drug discovery, preclinical and clinical stages of small molecule development (see Figure 4).

In drug discovery, metabolite identification can be used to define the sites on a molecule that are subject to metabolism to assess the potential for a structural scaffold to form reactive metabolites and to compare the metabolites formed across various species, including humans.

In preclinical drug development, CYP screening studies will determine which metabolites are generated by which CYP isoforms and in vivo studies, typically in rats, are conducted to determine the routes of excretion of a drug and its metabolites. Later in preclinical development, animal mass balance studies illuminate the routes of excretion and their relative quantitative importance.

The clinical development role of metabolite identification is generally two-fold. First, a metabolites in safety testing (MIST) assessment determines that the circulating metabolites observed in humans are also present in the IND-enabling toxicity species, thus demonstrating that the toxicity of the human metabolites has been tested. Second, it defines the structures of the metabolites observed in human, plasma, urine and feces and the relative quantitative importance of the observed routes of excretion.

Figure 3: Potential Drug Molecule

FIGURE OF POTENTIAL DRUG MOLECULE

that affords multiple fragment ions with the same nominal but different exact masses

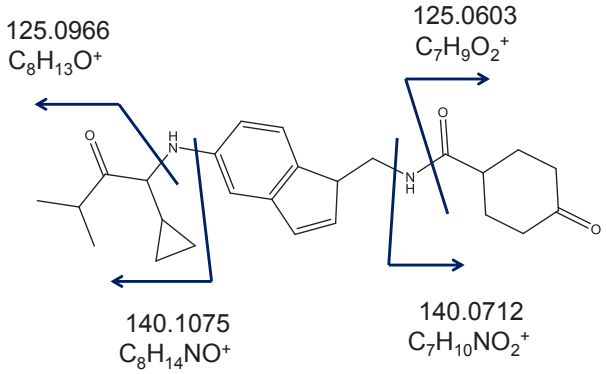
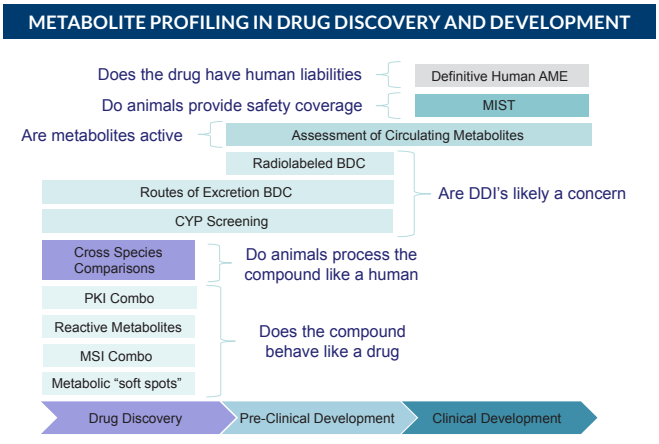


Figure 4: Metabolite Profiling in Drug Discovery and Development

SCHEMATIC OF DIFFERENT METABOLITE IDENTIFICATION EXPERIMENTS

performed throughout the drug discovery, pre-clinical and clinical development stages



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