



Benefits of ^{13}C vs. D Standards in Clinical Mass Spectrometry Measurements

Andrew J. Percy, PhD

Cambridge Isotope Laboratories, Tewksbury, MA USA

Richard Tyburski

ASI Chemicals LLC, Cheyney, PA USA

The capabilities of mass spectrometry (MS) have made this analytical technique an invaluable tool in clinical-based developments and applications. As with any clinical test, accurate and precise results are paramount toward correct diagnosis and treatments. In MS testing, reliable results are best achieved by the inclusion of stable isotope-labeled standards. The utility of such standards has been demonstrated in clinical and translational research, with their benefits including the ability to help compensate for matrix effects and ion suppression.¹ For optimum results, the standards should be added as early in the analytical workflow as possible, such that they can effectively normalize the variations that may arise throughout the experimental stages. The nature of labeled standards is a critical element of a method and is predicated on its availability/cost as well as the study design and research aims. Important to recognize in the standard selection process is the isotope differences (e.g., between ^{13}C and D) and the potential impact this may have in the pre-analytical (e.g., storage and handling) and analytical (e.g., sample preparation and processing) phases. As standard selection is not always a straightforward procedure, this article compares the commonly used ^{13}C and D isotopes from production to analysis in an effort to edify the challenges and guide future selections.

Standards labeled with ^{13}C (and/or ^{15}N) have demonstrated broad research utility over the past couple of decades. This stems partly from the chemical stability of its isotope. Its stability ensures that the isotope remains intact irrespective of the experimental methodology employed (e.g., multidimensional LC or derivatization-based GC prior to MS/MS). In other words, the ^{13}C (and ^{15}N) isotope remains positioned at its point of synthesis throughout all stages of an analytical workflow (includes extraction, derivatization, separation, and analysis in metabolomics). This provides flexibility to the end user as there is no limitation on the choice of sample/solution preparation nor the mode of MS/MS analysis. Since ^{13}C (and/or ^{15}N) standards have exceptional isotope stability, as compared to their deuterated counterparts, these can be inserted

at an early stage of sample preparation. Of additional benefit is that this type of labeled compound co-elutes with its corresponding unlabeled (i.e., native or endogenous) analyte during chromatographic separation. This co-eluting result is optimal in correcting for both ion suppression and matrix effects. Further to the benefits, ^{13}C (and/or ^{15}N) standards are absent from isotope scrambling or loss during ionization and collisional activation in the mass spectrometer. Owing to these collective merits, ^{13}C (and/or ^{15}N) standards have incurred great value in preclinical and clinical MS applications (from qualification to absolute quantification).

Despite the benefits of ^{13}C (and ^{15}N) labeling, the production of such standards could entail complex and laborious synthesis. While carefully selected structural analogues (with ^{13}C and/or ^{15}N) may instead be used in cases where it is cost or time prohibitive to obtain or synthesize the required standard, deuterated standards are an alternate option to consider. These are comparatively straightforward to prepare, but invoke a number of potential issues at the pre-analytical and analytical phases. The first pertains to the isotope stability. If the D-label is placed at an exchangeable position (i.e., at acidic and polar groups), it could be susceptible to an isotope effect during storage and later in analysis. In this effect, the location of deuterium may scramble or undergo an exchange reaction with protium in solution or in the gas phase. Another situation to consider is deuterium loss on specific compounds from enzymatic reactions (e.g., deuterium abstraction from fatty acids due to fatty acid desaturation).² The impact of these collective effects could be significant and is best illustrated by a hypothetical example. In a complete exchange scenario, for instance, the labeled signal at the mass spectrometer would be unmeasurable, while the unlabeled signal (i.e., M+0) would be elevated. This would provide an invalid view of a patient's biochemistry and a false impression of the assay's fitness. A result that would clearly contribute to "imprecision medicine" in laboratory diagnostics. While this deleterious impact could be overcome by selecting alternate MRM

Continued ►

transitions (i.e., at sites verified to have label due to consistent scrambling), a preferred approach would be to incorporate deuterium at chemically inert, nonexchangeable positions. Doing so would aid its stability, but the integrity of the deuterated standards would still need to be validated at all phases of the analytical workflow (from reconstitution through extraction to MS analysis). Complicating these assessments is the difference in physicochemical properties between deuterium and hydrogen. The difference causes deuterated standards to typically exhibit an altered chromatographic retention from its native analogues.³ This elution impact is most pronounced in LC separations, but may also occur in GC separations. The shift could complicate the accuracy/reproducibility of identification and quantification in complex biosample analysis, such as human plasma or urine. Only if the stability and effectiveness of deuterated standards are first demonstrated can its subsequent use in large-scale analysis be considered acceptable for critical decision making studies (e.g., newborn screening, therapeutic drug monitoring, vitamin D deficiency).

To summarize, there are an array of factors to consider in designing experiments and implementing methods. Important amongst them is the type of labeled standard. As described

above, ¹³C (and ¹⁵N) standards provide excellent isotope stability and analytical reliability. This means that the position of label is not impacted by the pre-analytical and analytical processes. Since this type of standard has equivalent physicochemical properties as its unlabeled counterpart, we consider these to be ideal toward the accurate and reproducible quantitation of small or large molecules. Deuterated standards, in contrast, may exhibit isotope instability and an exchange or scrambling effect during storage and the experimental phases. These effects are magnified if the D-label is incorporated at exchangeable positions. Even if deuterium is placed at nonexchangeable positions, development time must be allotted for stability testing (e.g., at storage, in autosampler) and method evaluation (e.g., for mobile phase impact, preferable MRM transitions).⁴ That said, if validations have been performed and other options (e.g., ¹³C standards or surrogates) are absent, then this route could be suitable long-term. Overall, although Cambridge Isotope Laboratories, Inc. (CIL) offer a multitude of variably labeled standards (encompasses vitamins, steroids, and fatty acids/lipids, amongst others), our recommendation is toward a ¹³C (and/or ¹⁵N) variant, when possible, for accurate/reproducible quantification in clinical MS-based analyses.

References

1. George, R.; Haywood, A.; Khan, S.; et al. **2018**. Enhancement and suppression of ionization in drug analysis using HPLC-MS/MS in support of therapeutic drug monitoring: a review of current knowledge of its minimization and assessment. *Ther Drug Monit*, 40(1), 1-8.
2. Triebel, A.; Wenk, M.R. **2018**. Analytical Considerations of Stable Isotope Labelling in Lipidomics. *Biomolecules*, 8(4), pii: E151.
3. Guo K1, Ji C, Li L. **2007**. Stable-isotope dimethylation labeling combined with LC-ESI MS for quantification of amine-containing metabolites in biological samples. *Anal Chem*, 79(22), 8631-8638.
4. Honour, J.W. **2011**. Development and validation of a quantitative assay based on tandem mass spectrometry. *Ann Clin Biochem*, 48(Pt 2), 97-111.

