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Application Note

ISO-012

ISOTEC® Stable Isotopes

Labeled standards

Comparison of Deuterium, ^{13}C, and ^{15}N Isotopic Labels in Mass Spec Standards

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Introduction

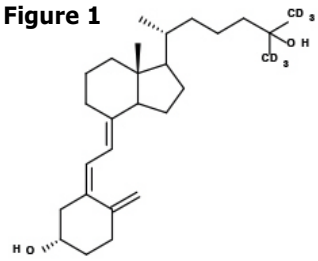
Clinical applications of LC-MS/MS have risen steadily over the last ten years. MS-based methods to quantify key biological components have been based on the improved sensitivity, specificity, and throughput of the MS-based analytical method. Stable isotope-labeled internal standards are important features of these assays, helping to optimize the accuracy of the method.

However, the first generation of stable isotope labeled internal standards had the drawback of relying on deuterated compounds, which were readily available and relatively inexpensive. Procedures and Standard Operating Procedures (SOPs) were established and clinical assays performed with these first generation deuterated standards.

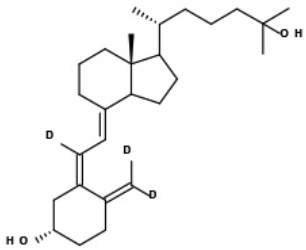
A second generation of labeled internal standards is readily available, characterized by ^{13}C and ^{15}N labeling or, in a few cases where there is no viable alternative, deuterium placed into stable, non-exchangeable positions.

The Vitamin D assay shows the progress made in the synthesis of labeled internal standards. This assay was among the first to switch from RIA (radioimmunoassay) to LC-MS/MS and is now among the most widely performed LC-MS assays.[1] The first available labeled internal standard was 25-hydroxyvitamin D_3-[26,26,26,27,27,27-d_6], which was then followed by 25 hydroxyvitamin D_3-[6,19,19-d_3] (see **Figure 1**).

Figure 1



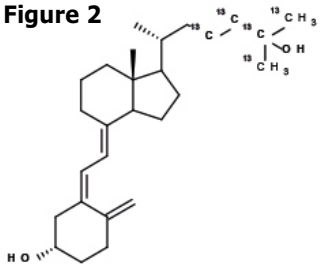
25-hydroxyvitamin d3-[26,26,26,27,27,27-d_6]



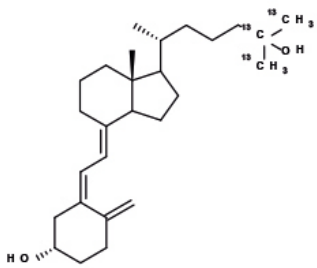
25-hydroxyvitamin d3-[6,19,19-d_3]

More recently, two ^{13}C labeled forms of 25-hydroxyvitamin D_3 have become available (see **Figure 2**).

Figure 2



25-hydroxyvitamin d3-[23,24,25,26,27-$^{13}C_5$]



25-hydroxyvitamin d3-[25,26,27-$^{13}C_3$]

In light of the availability of a second generation of labeled internal standards, the question arises as to which is “the best” internal standard for LC-MS/MS assays?

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Isotope Label Choice

Deuterium Label

The use of deuterium labels can lead to challenges during analysis. First and foremost is the possibility of deuterium loss from the standard due to exchange with protons. This can occur when the deuterated compound is in solution or in the mass spec instrument and will be detailed in a case study with aldosterone.

Loss of deuterium compromises the accuracy of results obtained with the labeled internal standard and has two scenarios. First, the deuterated internal standard may lose all of its deuterium, thus giving rise to a "false positive," where the internal standard appears to be the unlabeled analyte. However, this should be obvious to the analyst since the internal standard signal would not be detected.

Second, and far more consequential, is in instances when the internal standard loses 15–20% of deuterium over the course of the assay. The internal standard is used to standardize the concentration of the analyte, and if this internal standard signal is not reproducible sample to sample, its instability will lead to reporting of erroneously high analyte concentrations.

A further complication in the use of deuterated compounds is HPLC co-elution problems. Deuterium is less lipophilic than hydrogen, and this can result in the deuterated standard not co-eluting with the unlabeled compound.[3,4]

Carbon-13 Label

Labeling with ^{13}C has none of the disadvantages of deuterium labeling. Carbon-13 can be obtained in high isotopic purity (99.5%) for introduction into the labeled standard, while specifications for deuterium are typically only 98%. Carbon-13 is chemically stable; there can be no loss of ^{13}C via exchange with ^{12}C. This gives a very clean, sharp molecular ion of the final labeled standard. Furthermore, there are no co-elution problems in HPLC between the labeled and unlabeled versions of the same compound.

The only minor disadvantage is that more elaborate syntheses are usually required to introduce ^{13}C, and as a result, the final labeled standard tends to be more expensive that the deuterated analog. This is not always the case, and quite often, the difference in price between deuterated and ^{13}C labeled analogs is not significant. For new assays, the higher cost of the internal standard is frequently offset by the reduced time spent on initial method development and qualification.

Other Labels

Nitrogen-15 offers the same advantages as ^{13}C. Its use is more limited since there are fewer labeled starting materials for ^{15}N synthesis and fewer options in positioning the nitrogen.

Though oxygen-18 (^{18}O) is also available and offers a molecular weight gain of +2 amu per atom, it, unfortunately, is also easily exchangeable. The ^{18}O in accessible functional groups (acids, esters, or ketones) is readily exchangeable, so ^{18}O is rarely used.

Case Study in Solution Stability – Aldosterone

Background

Aldosterone is a mineralocorticoid steroid hormone that plays a key role in the regulation of blood pressure. Aldosterone tends to promote sodium and water retention and lower potassium concentration in plasma. Regulation of aldosterone is a key feature in several antihypertensive medications, such as Lisinopril.

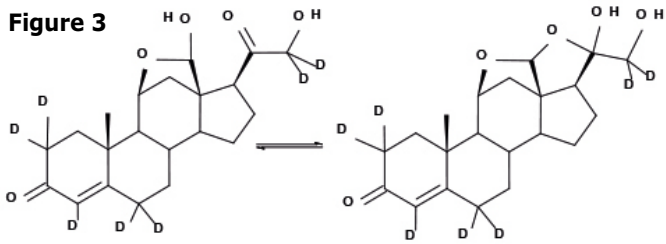
Primary aldosteronism (hyperaldosteronism) is a result of an increased level of aldosterone that exacerbates retention of sodium and loss of potassium. Hyperaldosteronism increases water retention and blood volume, resulting in an increase of blood pressure. The opposite condition, hypo-aldosteronism, can also exist and is characterized by decreased levels of aldosterone.

Measurement of aldosterone in blood is therefore of prime importance when managing hypertension. The concentration of aldosterone can now be analyzed by LC-MS/MS.[5] This method avoids the drawbacks of the more traditional measurement of steroids by immunoassays, such as a lack of specificity that can result in overestimation of the serum concentration of the steroid analyte, aldosterone.

The Chemistry

Deuterated aldosterone, available since 2004, exists in solution in equilibrium between two forms in roughly a 60:40 ratio (see **Figure 3**).

Figure 3

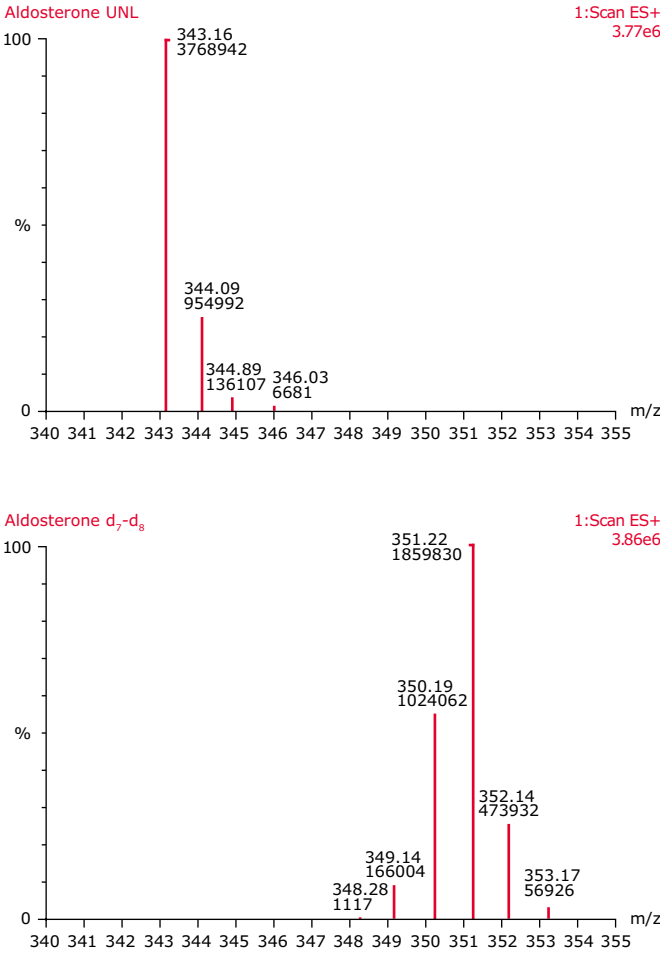


The mass spectra of the unlabeled and labeled standards are shown in Figures 4a and 4b. The top spectrum shows unlabeled aldosterone with the M+H molecular ion seen at m/z 343.16. The lower spectrum shows the deuterated aldosterone standard. This exhibits an M+H molecular ion at m/z 351.22, which corresponds to a D8 isotopomer (60.7%). However, as is typical for a deuterium labeled compound, several other isotopomers are present. There is a significant amount of M+7 (m/z 350.19, 32.6%) and M+6 (m/z 349.14, 5.5%).

The actual incorporation of deuterium in this standard is 7.5 D/molecule with M+8 being the major isotopomer. Additionally, the intensity of the M+8 molecular ion peak is significantly less than the unlabeled molecular ion peak even at identical concentrations in solution.

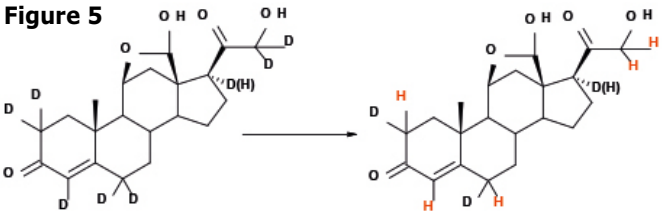
Figure 4a and 4b

Mass spectra of aldosterone (top) and aldosterone-d_7 (bottom).



As is typical with steroids of this type, the deuterium labels are in base-sensitive active positions. Exposure of the deuterated aldosterone to basic conditions in solution leads to the exchange of deuterium to the surrounding protic solvent. The result is a reduction in the intensity of the M+8 molecular ion peak. Loss of deuterium can be rapid or slow; the rate of exchange is dependent upon the molecule in question and the pH of the solvent (see **Figure 5**).

Figure 5



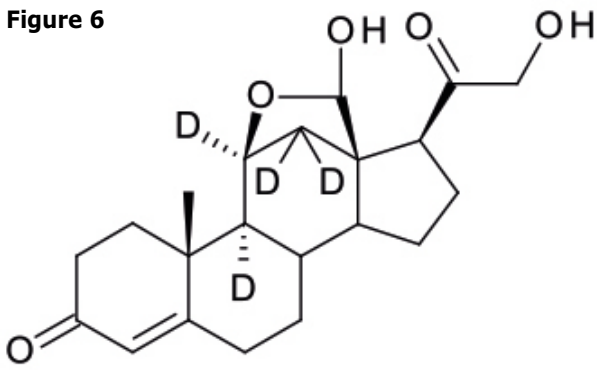
The Analytical Solution

When used as an LC-MS/MS internal standard, the possibility of deuterium loss mandates the analyst verify the integrity of the internal standard. Is the deuterium retained or lost once it is in the final medium for analysis? Is the deuterium stable over the length of time for which it is intended to be used? Those two critical questions often go unanswered and can lead to the reporting of erroneous results.

There are two approaches to avoid this problem. One is using a ^{13}C labeled form of aldosterone and the other is to place the deuterium in non-exchangeable positions. The preferred approach is to use ^{13}C, since there is no chance of ^{13}C loss and hence, no need for a validation study on the integrity of the label. The challenge of this approach is the difficulty in synthesizing ^{13}C-labeled materials.

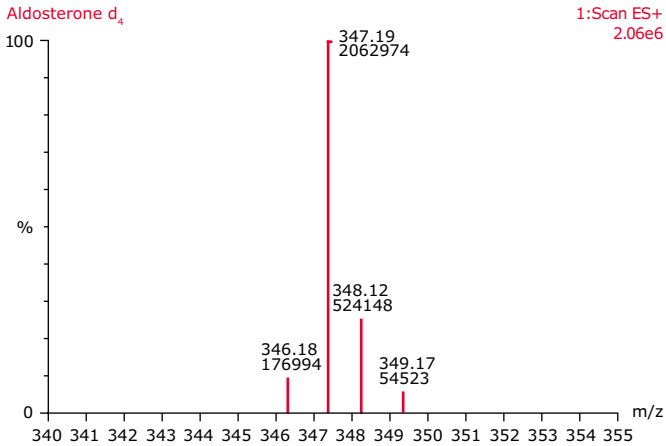
Instead, a second generation deuterated aldosterone internal standard that places the deuterium in completely non-exchangeable positions has been developed (**Figure 6**).

Figure 6



The mass spectrum of aldosterone-d_4 is shown in **Figure 7**. The molecular ion of the aldosterone-d_4 is much cleaner than aldosterone-d_7 and consists of 96.9% d_4 and 3.1% d_3. This indicates the ion selected for monitoring in the LC-MS/MS assay will be 96.9% as intense as the unlabeled aldosterone molecular ion. This is a vast improvement over the use of aldosterone-d_7 as the internal standard.

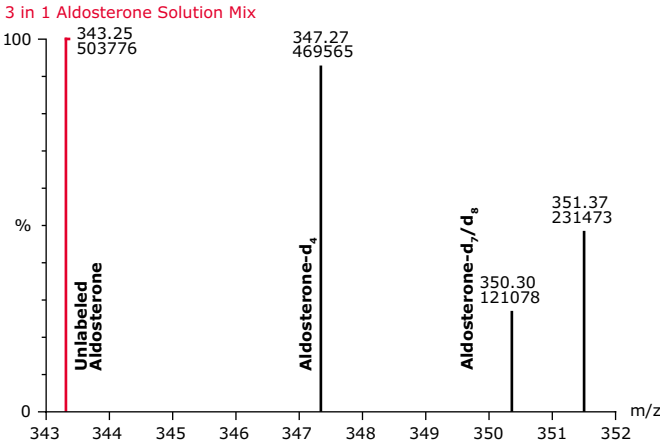
Figure 7



The significantly improved mass spec performance of the d_4 standard is clearly seen when the molecular ions of aldosterone, aldosterone-d_4, and aldosterone-d_7 are superimposed (see **Figure 8**). Two ions are shown for the d_7 standard since both the d_7 and d_8 ion could be chosen. Clearly the d_4 standard is the internal standard of choice.

Figure 8

Mass spectrum of aldosterone, aldosterone-d_4 and aldosterone-d_7. All are at identical concentrations.



Validation of Deuterium Solution Stability

The Aldosterone Case Study reinforces the need to validate any deuterated standard for use in an LC-MS/MS clinical assay, with particular attention paid to the following questions:

- Is the deuterated standard stable in the final matrix (Plasma, Buffer, Aqueous solution, Organic solution) for the length of time and under the conditions required for use?
- Is the deuterated standard stable in the HPLC mobile phase?
- Has a time course stability validation been carried out? (Stability at t = 0 does not mean stability at t = 60 minutes or …)

The answers are dependent upon the actual conditions and techniques used. Deuterium-containing standards can be used successfully in many instances when validation takes these potential issues into consideration.

Many deuterium-labeled standards are designed to minimize exchange, and ^{13}C and ^{15}N labeled standards are more readily available. The Stable Isotope team is ready to assist with product selection.

References

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3. R. Bawejaa, *Journal of Liquid Chromatography*, 9(12), 2609 (**1986**).
4. F. Masters et al., *Analytical Chemistry*, 60(19), 2131 (**1988**).
5. Michael Jarvis, AB SCIEX, Concord, Ontario, Canada, Ultra-Sensitive Analysis of Aldosterone in Serum Using the AB SCIEX Triple Quad™ 6500 LC/MS/MS System. www.absciex.com/documents/downloads/literature/6500_aldosterone.pdf.

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