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Stable Labeled Isotopes as Internal Standards: A Critical Review

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Abstract

Over the past few years, stable labeled isotopes (SILs) have played a critical role in bio-analysis, nearly replacing the use of structural analogues as internal standards. SILs are now the first choice of researchers/chemists when selecting an internal standard for day-to-day analysis to avoid process and analytical variation. However, although SILs are widely used in analytical labs today, they have challenging issues, such as the matrix effect, recovery, and ionization problems. The purpose of this review is to brief about both advantages and disadvantages of stable label internal standards from recent publications. Despite of having SILs in analytical methodology chemists observed the method issues and these were resolved later by replacing with structure analogs in their methodology.

Keywords: Liquid chromatography mass spectrometry (LC-MS/MS); Electrospray ionization (ESI); Atmospheric chemical ionization (APCI); Matrix effect and recovery

Introduction

SIL internal standards are used in a wide range of analyses, including of small and large molecules, quantification of metabolites, and the determination of the *in vivo* metabolism of certain molecules. For liquid chromatography mass spectrometry (LC-MS/MS) applications, there are two different types of internal standards that can be used, which include structural related compounds or analogues and isotopically labeled compounds such as deuterium (D), ^{13}C, or ^{15}N [1]. Commercially available SIL internal standards provide structural information, which enable researchers/chemists to better understand analyte fragmentation patterns in LC-MS/MS, and also metabolism during *in vivo* administration. Today, commercial sources can provide custom designed SIL internal standards, which are easy for researchers to use.

Applications of SIL Standards

The switch from analogue internal standards to SILs for LC-MS/MS analysis has proven to reduce variations in mass spectrometry results, such as ionization issues, and has also improved the accuracy and precision for the analysis of both small and large molecules. For example, Stokvis et al. [2] observed an improved performance in the assay of a novel anticancer drug, Kahalalide F, using an SIL internal standard. In another application of SIL standards, Freisleben et al. [3] described in their work the use of synthesized labeled vitamins of folic acid as internal standards in stable isotope dilution assays. Pawlosky & Flanagan [4] similarly contributed to this research area by developing a negative mode electrospray ionization (ESI) LC/MS method for the quantitative determination of folic acid in fortified foods with the aid of a stable labeled folic acid ($^{13}C_5$) internal standard, which helped diminish variation produced by the sample extraction procedure.

The use of stable labeled macromolecules, such as peptides and proteins, as internal standards for large bio-molecule assays is also becoming more widely available. These stable labeled macromolecules (e.g., peptides and oligonucleotides) are produced using labeled starting materials and automated synthesizers. In addition, internal standards of macromolecules and proteins can be produced using recombinant, fermentation, and semi-synthetic approaches, which incorporate ^{13}C and ^{15}N building blocks into the biosynthetic process. Ong & Mann [5] discussed in their review the characterization of complex protein mixtures using MS, and explain how the post-harvest incorporation of stable isotopes can be achieved through chemical or metabolic processes in living cells. Using these methods, peptides can be distinguished by the predictable mass difference between the native and isotopic versions of the biomolecule. This isotopic harvesting process helps to quantify and provide precise functional information about peptides using mass spectra. In another application of SIL standards in the study of biomolecules, Hsu et al. [6] described in their paper a strategy for labeling the N-terminus and the ε-amino group of lysine with a stable isotope using a reductive amination procedure in the presence of a formaldehyde reagent. In another work, Palermo et al. [7] demonstrated the practical application

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of SILs in their methodology using gas chromatography to profile 3-oxo-4-ene urinary steroids, using a series of D-labeled cortisone and hydroxycortisol internal standards.

Guo et al. [8] also demonstrated the use of SIL internal standards to study complex biomaterials, analyzing the metabolome by quantifying target metabolites with amino groups using LC-MS/MS. The authors were able achieved this by introducing stable isotope tags onto the amine groups of the metabolites by reductive amination in the presence of formaldehyde. This similar strategy was investigated in approximately 20 amino acids and 15 amines. Creek [9] also showed that novel metabolites and their pathways can be identified using SIL standards in metabolomics. Stable isotopes can also be used for bioavailability and bioequivalence studies. Being less toxic, D and ^{13}C are well suited for such research in humans, as well as for *in vivo* studies as a pharmacological tool. These isotopic drugs can be administrated concomitantly by different routes, including oral and parenteral, and in different forms, such as solid and solution dosages. Concomitant administration reduces the variability and also enables the use of a single assay. This process also minimizes drug exposure and discomfort for the volunteer. Using this single-dose administration method also makes it easier to compare two different routes or dosage forms. The technique is also well suited for "pulse" administration, and the kinetics from a single dose during multiple dose or chronic dosing regimens can be compared with single-dose kinetics [10]. In another study, Heck et al. [11] described a methodology to compare the bioavailability of two commercially available brands of imipramine hydrochloride relative to an SIL internal standard. In this work, each formulation was compared with an SIL drug that was consumed orally at the same time the tested formulation was ingested. In Kasuya et al. [12] publication, "Stable-isotope methodology for the bioavailability study of phenytoin during multiple-dosing regimens," the authors determined accurate clearance values of unlabeled phenytoin at a steady state condition in the plasma concentration by comparing the results from the plasma concentration of a small amount of intravenously administrated SIL phenytoin (DPH-d10), and analyzing the plasma samples using a highly sensitive and specific gas chromatography-mass spectrometry (GC-MS). In another study, Gilbert et al. [13] studied the bioavailability of the drug timolol in dogs using both oral and ophthalmic formulations. In this work, the authors quantified the amount of timolol in plasma and urine samples in the presence of the drug's SIL internal standard ($^{13}C_3$ and $^{2}H_9$) using LC with atmospheric-pressure chemical-ionization (APCI) tandem MS Bertilsson et al. [14] conducted an investigation of the autoinduction of carbamazepine metabolism in younger children (10 to 13 yrs old) using an SIL version of carbamazepine d4.

Many researchers have shown that SIL internal standards are the best choice to correct recovery, matrix effects, and variability in ionization during extraction and analysis of an analyte in a complex matrix using LC-MS/MS. For example, Häubl et al. [15] found that matrix effects were corrected during the ionization process of an unclean sample of mycotoxins in the MS source in the presence of a ^{13}C-labeled internal standard. In addition, the authors found these SIL standards also improved the accuracy and precision of the determination of the mycotoxin deoxynivalenol by LC-MS/MS and LC-MS. Sheppard & Henion [16] developed a quantitative method for determining the concentration of EDTA in human plasma and urine. In their method, the samples were prepared with the addition of a ^{13}C SIL internal standard, and extracted using an automated anion-exchange solid phase procedure. The authors then analyzed and quantified the extracted samples labeled with the isotopic internal standard using capillary electrophoresis/ion spray tandem Berg & Strand [17] demonstrated that the ion suppression effects of drugs in biological samples during analysis using LC-MS/MS can be significantly reduced using ^{13}C SIL internal standards. Berg et al. [18] also evaluated and determined the amount of amphetamines in biological samples using reverse phase ultra-high performance LC-MS/MS in the presence of isotopically labeled internal standards, including ^{13}C and D. SIL internal standards that use isotopes, such as ^{13}C, ^{15}N, and ^{18}O were expected to behave more closely to their respective unlabeled analytes, compared with the results for the more classically used D-labeled internal standards. Despite this hypothesis, the authors found that data from the samples which used ^{13}C-labeled internal standards was more promising for analytical purposes compared to the D-labeled standards. Fierens et al. [19] described a methodology for the quantitative LC-MS/MS analysis of urinary C-peptide in the presence of a D- and ^{14}C-labeled peptide as an SIL internal standard.

Limitations for SIL Internal Standards

Despite the many uses of SIL internal standards in analytical applications, many researchers and scientists still face the challenges of matrix effects/recovery and ionizations issues even when stable isotopes are used. In addition, some researchers have reported a slight change in the retention of the analyte with the use of SILs, which can lead to ion suppression. For example, during their study in the determination of carvedilol enantiomers by LC-MS/MS, Wang et al. [20] observed a matrix effect in two specific lots of human plasma in spite of using a D-labeled carvedilol internal standard. This observation was further verified after diluting the extracted sample with the mobile phase, post-column infusion followed by extracted plasma blank. From these experiments, the S-enantiomer of carvedilol and its respective deuterated internal standard was shown to cause a matrix effect by ion suppression in two different lots of human plasma. The authors also observed that the deuterated internal standard caused a slight change in the retention time of the analyte, which resulted in different ion suppression between the analyte and the SIL internal standard. The authors concluded that this slight change in the presence of the SIL was significant enough to change the area ratio and affect the accuracy of the method.

Liang et al. [21] extensively studied the phenomena of ion suppression in ESI, as well as ion enhancement in APCI while monitoring select-ions between the analytes and their respective labeled internal standards during the analysis of nine different drugs. The authors showed that ion suppression in ESI mode was

due to the co-elution of the labeled internal standards with the analyte. Additionally, other factors, such as the analyte's structure and concentration, matrix effects, and flow rate, which could cause ion suppression, were investigated apart from the ESI data. The authors observed that seven out of the nine analytes and their corresponding co-eluted labeled internal standards showed ion enhancement. This mutual ion suppression or enhancement between the analyte and the labeled internal standard may affect the sensitivity, linearity, accuracy, and reproducibility of quantitative analysis using LC/MS or LC-MS/MS. However, the authors concluded that their calibration curves were linear, and the response factor was constant with the addition of an appropriate concentration of the internal standard to the desired calibration sample ranges.

Remane et al. [22] extensively investigated ion suppression and enhancement effects of fourteen different SIL internal standards in the presence of their native analogues using APCI and ESI. Multi-analyte (different class of drugs) quantification, measured during a single run, is a common procedure followed in many clinical and forensic toxicology labs. The authors found that both ion suppression and enhancement were influenced by the concentration of the native analyte, in which ion suppression increased with the concentration of the analyte, particularly for ESI analysis. However, ion enhancement effects were observed in solutions prepared in methanol and analyzed using APCI, with one exception, which occurred when plasma extracts were used under these conditions. Eleven SIL internal standards showed relevant ion suppression under ESI mode, but only one analyte showed suppression effects when APCI was used. The authors concluded from this study that researchers should ensure that the selection of internal standards used in multi-analyte quantification in matrix samples should be free of ion suppression and enhancement effects to avoid incorrect quantification. If not, a different ionization technique should be considered.

Conclusion

SIL internal standards have distinct advantages and disadvantages; however these materials still play a vital role in analysis. SIL internal standards are the first choice for quantitative bioanalytical LC/MS assays, as they generally produce better results. Despite this, SIL internal standards may or may not necessarily be appropriate for all quantitative bioanalytical methodologies when using LC/MS. D-labeled internal standards can behave differently compared to their native analytes, sometimes displaying different retention times and recoveries. In addition, the use of SIL internal standards, being structurally and chemically identical to their corresponding analyte, may inadvertently cover up problems in the assay, such as stability, recovery, and ion suppression. While SIL internal standards are useful in many aspects of validation experiments for different analytical methods, as well as for regular analysis, at the same time these compounds are not always available or can be very expensive, in which case structural analogues can be useful alternative standards [23].

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