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Isotope Dilution Assay in Peptide Quantification: The Challenge of Microheterogeneity of Internal Standard.

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Abstract

Isotope Dilution Analysis (IDA) allows quantitation of elements and different compounds in complex mixtures. The quantitation is based on a known amount of reference material (Internal Standard, IS) added to a sample that makes the result critically dependent on the value assigned to the standard. In the case of peptides, IS concentration is determined by nitrogen and amino acid analysis while purity is normally assessed by methods such as chromatography or electrophoresis that might not be able to detect many possible amino acid modifications, either naturally occurring or chemically induced. Microheterogeneity of the IS, if it isn't accounted for when assigning a reference value to the standard, results in highly overestimated values in target analyte quantitation. In this viewpoint article we illustrate the problem of internal standard microheterogeneity by analyzing synthetic human C-peptide labeled analogues.

Keywords

Isotope Dilution Assay; Reference material; Microheterogeneity; C-peptide

In IDA-MS, quantitative detection of an analyte signal is related to a heavy-isotope-labeled internal standard (IS) that is added at a known concentration [1,2]. In the case of peptides and other relatively low Mw substances, synthetic analogues containing stable heavy isotopes (e.g. ²H, ¹³C, ¹⁵N) can be easily derived [3,4] and used as an internal standards. The same physico-chemical properties of the analyte and its labeled analogue allow for isolation and purification prior to analysis. The identity of properties also provides the same signal intensity function for both analyte and standard, thus the analyte concentration can be calculated directly from the ratio of the signal responses. Due to very high sensitivity and specificity, IDAs currently are considered to be a good alternative to immunoassays in clinical chemistry.

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Conflict of interests

The authors declare no conflict of interest.

The internal standard serves as the “absolute standard” or “etalon”; thus manufacturers who provide researchers with the internal standards play the role no less important than does the International Bureau for Weights and Measures. Unfortunately, there are some reasons to believe that many current protocols are prone to considerable errors due to the fact that the assigned IS concentration is not correct, specifically in the analysis of peptides and other high molecular weight compounds. Although different purity requirements are normally associated with product quality (*e.g.*, analytical grade) or its intentional use (drugs or products for in vivo use only), manufacturers generally provide purity certifications with respect to the content of the specified analyte that are based on electrophoretic or chromatographic separations. In many cases these methods do not discriminate between tiny chemical or structural differences when it comes to relatively large molecules. For example, some post-synthetic modifications do not affect chromatographic or electrophoretic behavior significantly and thus remain undetectable with these analytical technologies. This microheterogeneity could be due to a number of factors, including unperfect synthesis, labelling and/or post-synthetic modifications.

As an illustration, consider the example of human C-peptide quantification. C-peptide is a marker of intact insulin secretory capacity that can differentiate true insulin deficiency characteristic of type 1 diabetes from the relative insulin insufficiency seen in type 2 diabetes [3,4]. Measurement of C-peptide secretion and clearance can also help to characterize dysfunctional insulin secretion in the prediabetic state [5-7]. The technique of isotope dilution assay (IDA) has been successfully employed by several different groups and the results appear to demonstrate excellent analytical metrics. After the work of Kippen and co-workers [8] who first used IDA for C-peptide determination using single quadrupole MS, other groups have reported IDA-tandem mass spectrometry procedures [9-11].

In the process of method development we analyzed the full scan data for the three synthetic C-peptide analogues, one laboratory-synthesized [11] and two commercially available. Deuterium labelled synthetic C-peptide +8, manufactured by Bachem (#1036214), as well as laboratory-synthesized +30 (labeled with ^{13}C and ^{15}N) can be used as standards in IDA; native synthetic analogue (unlabelled) produced by Sigma-Aldrich (#C862) can not be used for this purpose, but can be a useful tool for optimization of separation conditions and isolation procedures.

We found a high degree of heterogeneity exhibited by each of standards tested, while the purity specified by the manufacturers based on chromatographic separation was very high (>95% for Bachem C-peptide and >85% for Sigma). Our HPLC data also confirmed these high purity levels of synthetic C-peptide (Fig. 1A). The apparent contradiction can be easily explained by the fact the HPLC analysis used does not provide “fine-structural resolution” comparable to MS. On Figure 1B the spectrum for unlabeled synthetic C-peptide, Sigma-Aldrich, is shown. The predominant signal comes from the triple-charged ionized species; the two and four times protonized molecules are also easily detectable (in Fig. 1B, the 3+ and 4+ states are visible). The spectrum clearly shows a substantial degree of heterogeneity, while chromatographically the sample appears to be almost perfectly homogeneous.

With a full scan data it's easy to estimate the real purity of each standard. Since each preparation exhibits a number of modified forms, the real content of reference material can be estimated by calculating the ratio of the peak area corresponding to the isoform of interest (C-peptide without adducts) to the sum of all peak areas (unmodified plus all detectable modifications). In our particular example, the possibility of observing different ionization states together with a periodic pattern can assure the observed satellite peaks are indeed derivatives of synthetic peptide and not impurities. Figure 2 shows the composition of two different labeled synthetic C-peptides and the native C-peptide. For the C-peptide[²H₈-Val^{7,10}] standard, the real content of the ion 1013.0 serving as a reference point is ~ 20% of the total C-peptide (Fig.2A). The lab synthesized C-peptide[9,10], labeled with ¹³C and ¹⁵N, under the same LC-MS conditions shows a higher content of reference material, ~ 35% (Fig. 2B). Based on this finding, an appropriate correction factor $KC_{actual} = k C^*$ must be introduced to reflect properly the real content of the reference substance (here C* is the concentration specified by manufacturer). Thus for the first standard, +8 deuterium labeled produced by Bachem, it should be $k=0.20$. While for +30 lab synthesized ¹³C and ¹⁵N labeled analogue - $k=0.35$.

At the same time, native C-peptide (endogenous) exhibits little heterogeneity (Fig 2C). Obtaining full scan data in this case is much more laborious compared to analyzing synthetic peptide due to the necessity of isolating a sufficient amount of concentrated sample, since C-peptide concentration in plasma or urine is very low (normal physiological range for C-peptide blood plasma is 0.5-10 ng/mL and urine concentration is approximately one order in magnitude higher [3,4], while zero concentration indicates a total lack of insulin production). However, we have developed the techniques for C-peptide purification that can provide sufficiently concentrated product [13,14].

The slope for the calibration curve representing “¹³C ¹⁵N” standard is approximately two times higher compared to the curve for the deuterium labeled standard (Fig 2D); this ratio is in a good agreement with the actual content of the reference isoform in the two standards. Results of uncorrected C-peptide quantitation will therefore be twice as high with the IS which is less rich in content of the “isoform of reference”. Even more important is that, without correction accurate values are not obtained with either standard. It has to be emphasized that microheterogeneity of the IS results in error regardless of whether SIM or MRM is used.

Although protein microheterogeneity (as well as heterogeneity of many other natural compounds) seems to be quite evident for many researchers in the area, the question of possible microheterogeneity of internal standards has not, to our knowledge, been previously explored. In our example, all three of the examined solid phase synthesized peptides (one unlabeled and two labeled) showed a high degree of microheterogeneity. Curiously, the microheterogeneity of endogenous C-peptide is much less pronounced. Here our data were also confirmed by Oran *et.al.* who investigated the naturally occurring heterogeneity of human C-peptide in their work [15]. They found several C-peptide isoforms, but the relative concentrations of these newly discovered C-peptide variants, which the authors hypothesized to be truncated forms - lacking one, two, or three N-terminal amino acids - C-peptides

(2-31), (3-31) and (4-31) - appear to be minor with the concentration of the “largest among minor components” being just around 5 percent. Also, another C-peptide variant, des{27-31}, has been reported earlier [16] as a minor component. While it has been pointed out recently that in complex mammalian systems, multiple proteins can serve as precursors of a single peptide and consequently, the result of protein quantitation is essentially dependent on peptide chosen and consequently may be significantly under- or overestimated [17], the described phenomenon is not connected with the complexity of the system under investigation, but with the imperfection of the tool used for quantitation. Fortunately, the problem can be easily addressed by analysing full scan data. Accounting for microheterogeneity of the IS by determining the real content of isoform of reference provides more accurate measurements.

Mass spectrometry is sought to be one of the powerful tools for detailed characterization of all isoforms of many natural compounds of interest. However, when performing quantitation it is important to take into account microheterogeneity of the internal standard. There are a number of possible reasons for this phenomenon, including errors in the process of synthesis or amino acid modifications during processing, or modifications due to storage or further manipulation. Perhaps, in some cases it may be possible to produce a highly homogeneous IS, but in other cases it might be technically impossible, or too costly. In this case the full scan data can provide a good idea about the real IS concentration in the total IS preparation, which can allow for the introduction of a correction factor that would facilitate accurate quantitation.

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Figure1.

ESI MS spectrum (positive mode) of the solid phase synthesized native C-peptide analogue (1-31) sequence (Sigma-Aldrich, unlabeled). A: The total ion count for this commercial synthetic C-peptide provides an illusion of a highly-pure-state and homogeneous preparation. Chromatographic (reversed phased) separation was performed on C18 Fenomenex column in a linear gradient of acetonitrile. HPLC (Prominence-Shimadzu) was coupled to MS, API-4000 (Sciex).B: Full scan data reveals high degree of heterogeneity of synthetic peptide. The predominate state for C-peptide is 3+ ; the most abundant ion that is typically used for quantitation in IDA has its M/z around 1008 (1007.7). On the left of the mass spectrogram, is the group of ions corresponding to C-peptide with a 4+ ionization state.

Figure 2.

Heterogeneity of two different human C-peptide analogues, isotope-labeled. ESI MS spectra in the range M/Z 900 –1200. A: The solid phase synthesized peptide, deuterium labeled [$^2\text{H}_8$ -Val 7,10], produced by Bachem. B: C-peptide analogue, containing 14 residues labeled with ^{13}C and ^{15}N (H3N-EA*EDL*QV*GQV*EL*G*G*G*PG**A*GSL*QPL*A*L*EGSLQ-COOH). C: Native C-peptide, purified and concentrated. D: Calibration curves for two isotopically labeled C-peptide standards. The lower curve represents the deuterium labeled standard, [$^2\text{H}_8$ -Val 7,10]. To generate the curves, two preparations were taken at the same concentrations (1 ng/mL) according to producers' specifications and the same series of progressively increasing volumes were injected.