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Linear and Accurate Quantitation of Proenkephalin-Derived Peptides by Isotopic Labeling with Internal Standards and Mass Spectrometry

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Abstract

Proenkephalin (PE) represents the precursor protein of the active peptide neurotransmitter enkephalin. Quantitative analysis of peptides and proteins is an objective of mass spectrometry-based studies of biological systems and will be important for studying the proteolytic conversion of proproteins to active enkephalin and neuropeptides. The goal of this study was to define and optimize quantitation of different amounts of tryptic peptides derived from PE using light H4- and heavy D4-succinic anhydride for isotopic labeling of peptides analyzed by LC-MS/MS tandem mass spectrometry. Comparisons were made between PE-derived peptides with and without internal standards. Importantly, incorporation of internal standards of known amounts of heavy isotopically-labeled tryptic peptides of PE provided linear calibration plots with accurate quantitation. In contrast, comparison of light- and heavy-isotopic labeled peptides without internal standards produced variable and inaccurate non-linear isotope ratio comparisons of PE-derived peptides. These results demonstrate that use of internal standards composed of defined amount(s) of standard peptides (PE-derived tryptic peptides) is necessary for high quality linear quantitation of peptides by isotopic labeling and tandem mass spectrometry.

Keywords

Peptide; proenkephalin; isotopic labeling; quantitation; linearity; mass spectrometry; proteomics

Introduction

Enkephalins and related neuropeptides function as endogenous peptide neurotransmitters involved in mediating analgesia and brain control of behavior [1–3]. Enkephalin neuropeptides are produced by proteolytic processing of the proenkephalin (PE) precursor [4–7]. Analyses of the conversion of PE into the smaller enkephalin neuropeptides will require quantitative assessment of its conversion to small enkephalin-related peptide products by chromatography and mass spectrometry.

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Isotopic labeling represents a primary approach used for peptide and protein quantitation [8–13]. Comparisons of the relative amounts of a protein in two different samples can be achieved by trypsin digestion followed by *in vitro* isotopic labeling of each of the different samples by heavy and light isotopic labels. The samples are combined in equal protein/protein ratios and subjected to LC-MS/MS mass spectrometry. The relative amounts of each peptide in the samples are indicated by ratios of heavy compared to light isotopically-labeled peptides. Alternatively, different samples (after trypsin digestion) can be compared by using isotopically-labeled internal peptide standards [14,15]; in this approach, the relative amounts of each peptide in the sample are compared to peptide internal standards prior to sample-to-sample comparisons. However, direct comparison of quantitation by isotopic-labeling with and without internal peptide standards has not yet been analyzed.

Therefore, to define optimized conditions for mass spectrometry quantitation of PE-derived peptides in this study, isotopic-labeling using internal peptide standards was compared to labeling without such standards. In essence, we are comparing a more absolute quantitation strategy versus a more relative quantitation strategy, the two predominant methods utilized for proteomic quantitation. Results demonstrated significantly increased consistency and accuracy for quantitation of PE-derived peptides using internal standards for isotopic ratio analysis. After labeling of PE tryptic digests with heavy (D4, 4 deuteriums) or light (H4, 4 hydrogens) succinic anhydride, the designated ratios of 1/10 to 10/1 were consistently and accurately measured by mass spectrometry. Use of heavy-labeled PE-derived peptides as internal standard yielded linear, one-to-one relationships among the different ratios of heavy and light isotope-labeled peptides. In contrast, without internal standards, considerable variability and inaccuracy in relative quantitation of isotope-labeled PE-derived tryptic peptides was observed. These results demonstrate that use of internal standards composed of defined amount(s) of heavy isotope-labeled PE-derived tryptic peptides provides high quality linear quantitation of peptides and PE protein by isotopic labeling.

Experimental Methods

Expression and purification of recombinant human proenkephalin (PE)

The expression vector pET19b was used to generate recombinant human proenkephalin (PE) in *E. coli* cells from the human PE cDNA [16]. The human PE cDNA (without the signal peptide sequence) incorporated NdeI and BamHI restriction sites at the 5' and 3' ends of PE, respectively, that was generated by RT-PCR of total RNA from human striatum (Stratagene, La Jolla, CA) using Taq DNA polymerase (Qiagen, Valencia, CA) and primers 5'-AAAAAC-ATATGGAATGCAGCCAGGATTGCGCGAC-3' and 5'-AAAAAGGATCCTTAAATCTC-ATAAATCCTCCGTATCTTTTCC-3' (Invitrogen, Carlsbad, CA). PCR reactions contained 1 ng cDNA as template and 0.3 μ M primers with 35 thermocycles each consisting of 60 seconds at 94°C, 60 sec at 45°C, and 70 sec at 72°C. Following digestion of the amplified PE DNA with restriction enzymes NdeI/BamHI, the digested PE DNA was ligated to the NdeI/BamHI digested plasmid expression vector pET19b using T4 DNA ligase (Invitrogen, Carlsbad, CA). The PE nucleotide sequence in the PE-pET19b vector was verified by DNA sequencing (Davis Sequencing, Inc., Davis, CA).

The PE-pET19b vector was utilized to express PE in *E. coli*, induced with 1 mM IPTG for 3 hours at 37°C. Cells were collected by centrifugation at 4000 $\times$ g for 15 minutes at 4°C, and lysed by sonication in 100 mM Tris-Cl, pH 7.0. The cell homogenate was centrifuged (16,000 $\times$ g for 15 minutes at 4°C), and PE was localized primarily in pelleted inclusion bodies. The PE fraction was solubilized in 100 mM Tris-Cl, pH 7.0, 6 M urea and was purified by a Ni²⁺-affinity column, as described previously [16]. After elution from the affinity column in buffer containing 300 mM imidazole, PE was dialyzed against buffer with decreasing urea (5 M, 3 M, 2 M, 1 M, and 0.5 M) in 100 mM sodium phosphate, 20 mM Tris, pH 7.5, 5 mM glutathione

(GSH), and 0.5 mM oxidized glutathione (GSSG) (Calbiochem, San Diego, CA), with final dialysis in 50 mM Tris, pH 7.5, for storage of purified PE. The purity of the recombinant PE was confirmed by SDS-PAGE that showed a single band, as well as peptide sequencing by trypsin digestion and nano-LC-MS/MS tandem mass spectrometry.

Reduction/alkylation and trypsin digestion of recombinant proenkephalin (PE) and bovine serum albumin (BSA)

Reduction and alkylation of PE was performed by reduction with 2-tris-carboxyethyl phosphine hydrochloride (TCEP, Pierce, Rockford, IL) followed by cysteine alkylation with iodoacetamide (Sigma-Aldrich, St. Louis, MO). Briefly, 14 µg PE sample was lyophilized and redissolved in 16 µl 20% acetonitrile. After addition of 2 µl of 100 mM TCEP in 20% acetonitrile (28.7mg/ml), reduction proceeded at 55°C for 20 minutes. The reduced sample was cooled to room temperature and 2 µl of 100 mM iodoacetamide in 20% acetonitrile was added to alkylate free cysteine residues by incubation at room temperature for 30 minutes in the dark.

The reduced and alkylated PE sample was diluted with 60 µl 25 mM ammonium bicarbonate, pH 7.0, 1 mM CaCl₂. To this was added 20 µl of trypsin solution (20 ng/ml trypsin in 25 mM ammonium bicarbonate, pH 7.0, 1 mM CaCl₂, for a total of 400 ng trypsin (sequencing grade, Promega, Madison, WI), and the sample was incubated at 37°C for 18 hours. The final concentration was 0.14 µg/µl digested PE. A BSA control digest was performed using the same reduction/alkylation and digestion conditions.

Isotopic labeling of PE for analyses with and without internal standards for isotope ratio analysis

Isotope labeling of PE tryptic peptides was conducted for peptide quantitation by dividing a single digest into 3 identical aliquots. The first aliquot of PE tryptic digest were labeled with light succinic anhydride, the second with heavy succinic anhydride, and the third unlabeled aliquot was included as control (heavy and light succinic anhydride from (Sigma-Aldrich, St. Louis, MO). Stock solutions of light (H4) and heavy (D4) succinic anhydride were prepared at 50 mg/ml DMSO. Labeling was accomplished by 3 labeling cycles to each PE digest sample (33 µl) of 2 µl 2 M NaOH followed by addition of 2 µl succinic anhydride (heavy or light isotope-labeled) with incubation at room temperature for 20 minutes. Labeling was then forced to completion by 3 more additions of 4 µl of labeling reagent for 20 minutes/addition for a total of 6 labeling cycles (57µl total volume). No additional base was required as there was significant excess from the repeated cycles.

Determination of the linear range for measurements of heavy-labeled PE-derived peptides for internal standard experiments

The quantity of heavy-isotope labeled peptides used for internal standards was determined by selecting an amount in the linear range of mass spectrometric measurements of designated PE-derived peptides (peptides #2, 5, and 6 shown in Figure 1). Heavy isotope-labeled PE tryptic digests were subjected to analysis by mass spectrometric signal intensities at 41, 90, 150, 225, 300, 360 and 410 fmol total digested PE concentration. The designated peptides showed approximately linear measurements in this range of injected quantities of PE digests. The quantity of heavy-labeled PE-derived peptides selected for internal standard experiments was 270 fmol which is in the center of the linear range of the peptides measured (peptides #2, 5, and 6).

Preparation of heavy- and light-labeled PE-derived peptides at different ratios. For the internal standard experiment

(illustrated in Figure 3A), the heavy- and light-labeled PE digests were then combined using 1 μ l of heavy labeled PE digest as an internal standard in all samples combined with varying amounts of light-labeled PE digest. We used this quantity of heavy labeled PE digest as an internal standard after determining that it was in the linear range for quantitation by nano-LC-MS/MS. The amount of light-labeled PE digest added to the heavy labeled PE digest (1 μ l) varied from 0.1 μ l to 10 μ l in steps of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μ l added (as shown in Figure 3A). All samples were then diluted to 20 μ l with 0.1% formic acid. Injections of 2 μ l provided 270 fmole of heavy-labeled peptides, with a total amount of heavy and light labeled peptides of 2970 fmole (by total PE protein) for LC-MS/MS analysis.

For the experiment without internal standard (illustrated in Figure 3B), heavy- and light-labeled PE digests were combined in ratios of light/heavy of 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 that were made by addition of 1, 2, 2, 2, 4, 8, 10 μ l of light-labeled PE digest to 10, 8, 4, 2, 2, 2, and 1 μ l of heavy-labeled PE digest, respectively. This produced equal concentrations of protein in each sample. A 2 μ l aliquot of each sample was diluted with 8 μ l 1% formic acid in HPLC grade water. The final concentration of total PE-derived peptides was 470 fmole/ μ l and 1 μ l was injected for each LC-MS/MS analysis (470 fmole total injected PE-derived peptides).

LC-MS/MS tandem mass spectrometry for identification and analyses of isotopic peptide ratios

PE digests were loaded at 2 μ l for each LC-MS/MS analysis with internal standard. To maintain similar injection quantities between all experiments with and without internal standards, 1 μ l was used for each LC-MS/MS analysis in the experiments without internal standard. All nano-LC-MS/MS analyses were performed in triplicate on the XCT Ultra ion trap mass spectrometer coupled to the 1100 nano-HPLC system fitted with a HPLC-Chip injection system (Agilent Technologies, Santa Clara, CA). The LC separation was performed on an C18 analytical HPLC chip (Zorbax C18 Chip, 43 mm x 75 μ m, 40 nl trap, Agilent Technologies) and utilized solvent B (acetonitrile with 0.25% formic acid) in solvent A (water with 0.25% formic acid), and the gradient progressed from 3% to 45% solvent B in 10 minutes followed by an increase to 75% solvent B in 2 minutes. The mass spectrometer was set for data dependant scanning in MS/MS mode on the three most abundant ions present in the MS scan. The exclusion time was set to 0.1 minutes, isolation window set to 4 amu, and voltages set to -1850 V (capillary), -500 V (counterelectrode) and 1.30 V (fragmentation). Smart ion target was set to 500,000 to correct for accumulation of background ions. The maximum injection time was set to 100 ms. All other default settings were used and left unaltered in all experiments. Extracted ion intensities or peak areas from extracted ion chromatograms are used to define the relative amounts of heavy and light versions of a peptide [15].

PE-derived peptides were identified in their isotope-labeled form by Inspect [17] and used for quantitation if they met the following criteria: (1) observed in both heavy- and light-labeled forms, (2) exhibited complete labeling in all experiments as indicated by the lack of unlabeled peptides and presence of only succinic anhydride-labeled peptides, (3) demonstrated chromatographic coelution while having separation in mass ≥ 3 amu, (4) exhibited signal-to-noise $>10/1$ and no appreciable signal from similar mass peaks within 1 minute of the observed retention time and (5) were present in all experiments conducted without and with the internal standards. The bioinformatic search used no protease specificity against user-constructed databases consisting of the target proteins. Statistical criteria were set at $p \leq 0.10$ with the caveat that p values are not particularly useful in small database searches. However, this cut-off sufficiently ranked the best identifications. Peptides conforming to these criteria had their mass spectra averaged over their elution profile. The resulting averaged spectrum was Gaussian

smoothed at 1.0 point and peak intensities for heavy and light labeled versions were recorded. For all experiments, with and without internal standards, the peak intensities (from peptide chromatograms) for heavy- and light-labeled peptides were directly utilized for ratio calculations (light-peptide signal intensity/heavy-peptide signal intensity). Curve fitting and data analysis was performed using Excel Microsoft program. The linear relationship between peptide ion intensity and peptide quantity was assessed directly by linear fitting of the data using least-squares analysis. Quality of fit was evaluated by manual validation and analysis of R^2 (R-squared or coefficient of determination) where at perfect linear correlation $R^2 = 1.0$ and at no correlation $R^2 = 0.0$. For these experiments, we considered $R^2 \geq 0.99$ to be an accurate correlation between the linear regression equation and the observed data [18]. Percent standard deviation (% RSD, also known as coefficient of variance [19]) was calculated by dividing the average observed standard deviation by the average of the observed isotopic ratio.

Results

PE-derived peptides used for isotopic quantitation analyses were expected to meet several criteria. These criteria indicate that peptides should exhibit (1) unambiguous identification, (2) complete labeling in all labeling reactions (indicated by the presence of succinic anhydride labeled peptides and absence of unlabeled peptides), (3) equivalent chromatographic and mass spectrometric behavior with either isotopic label, (4) high signal-to-noise ($>10/1$) with low interference with background signals (no appreciable signals at that mass within 1 minute of observed retention time), and (5) presence in all experiments. Six PE-derived tryptic peptides met these criteria; these PE-derived peptides were all tryptic and were either singly- or doubly-labeled. Their sequences within the PE precursor are illustrated in Figure 1.

The appropriate quantity of heavy-labeled PE-derived peptides for use as internal standards was then experimentally selected from the linear range of detecting heavy isotope-labeled PE-derived peptides. The linear range for selected peptides (peptides #2, 5, 6) was determined by evaluating the relative signal intensities of different amounts of injected heavy-labeled PE-derived peptides corresponding to 41, 90, 150, 225, 300, 360 and 410 femtomoles of total PE-derived peptides. The data (Figure 2, and data provided in Supplemental Table A) show excellent measurements of peptides #2, 5, and 6 derived from PE (peptides illustrated in Figure 1). In these experiments, the Agilent XCT Ultra exhibited excellent reproducibility and linearity depending on the peptide analyzed. Analysis of these data indicated that the linearity observed was sufficient for internal standard concentrations across the range of PE-derived peptide quantities evaluated. Therefore, 270 fmol was selected from the linear range as the amount to be utilized for heavy-labeled PE peptides for internal standard experiments.

PE-derived peptides were quantitated by ratios of light- and heavy-isotopic labeling in experiments with internal standards and without (Figure 3). Experiments with internal standards (considered as an absolute quantitation strategy) utilized a constant amount of heavy isotope-labeled PE tryptic peptides combined with different amounts of light isotope-labeled PE tryptic peptides to obtain light/heavy isotope ratios of 1/10 to 10/1 (Figure 3A). All experiments were performed in triplicate.

Using this strategy for heavy-labeled PE tryptic peptides as internal standards, measurements of the ratios for light- and heavy-isotope labeled peptides accurately represented the known ratios (plotted in Figure 4, with data shown in Supplemental Table B). The calibration plots showed linear relationships for measurements of light/heavy isotope ratios of 1/10, 1/5, 1/2, 1/1, 2/1, 5/1, and 10/1. Moreover, the slopes of the calibration plots showed an average of 1.03 with R^2 value of 0.995 (Table 1) for the six PE-derived tryptic peptides. This slope value indicated the one-to-one relationship for accurate measurements of the known ratios of light- and heavy-isotope labeled PE-derived peptides.

In contrast, without use of internal standards (considered as a relative quantitation strategy) during combination of different ratios of light and heavy isotope-labeled peptides (shown in Figure 3B), resultant plots of the theoretical light/heavy isotopic ratios compared to the observed ratios showed lack of one-to-one relationships that were nonlinear (plotted in Figure 5, and data shown in Supplemental Table C). The lack of the one-to-one relationship was shown by the average slope of 0.644 for the calibration plot (Table 2), rather than a slope near 1.0. Furthermore, correlation of observed and known ratios of light and heavy isotopically labeled peptides was poor (average R^2 of 0.948 for six peptides, Table 2) compared to the internal standard experiment (average R^2 of 0.995, Table 1). In fact, the calibration plots showed curves for isotopic quantitation conducted without internal standards (Figure 5), which contrast with the linear plots obtained with internal standards (Figure 4). These data show that the measurements of light compared to heavy isotopically-labeled peptides without internal standards were variable. Importantly, use of internal standards provided accurate and linear quantitative measurements of isotopically-labeled peptides.

Discussion

The objective of this study was to develop optimized strategies for quantitation of PE-derived peptides using light and heavy isotopic labeling with internal standards or without (Figures 3). These experiments with and without internal standards correspond to methods related to absolute and relative quantitation strategies, respectively. Results demonstrated linear and accurate quantitation of peptides using heavy labeled-peptides as internal standards. This findings was achieved by trypsin digestion of recombinant PE, labeling of peptides with heavy D4- or light H4-succinic anhydride, and peptide mixtures of light/heavy isotope-labeled peptides at different ratios were generated in the range of 1/10 to 10/1. Quantitative analysis by LC-MS/MS was performed using triplicate measurements for each peptide (peptides #1–6, derived from PE, Figure 1) at each of the different isotopic ratios. The quantity of heavy-labeled peptides used for internal standard was selected from the linear range of detected measurements. The constant amount of heavy-labeled PE digest was mixed with different amounts of light-labeled PE digest to achieve ratios of 1/10 to 10/1 for light/heavy isotope-labeled peptides. Significantly, linear quantitation was achieved using a constant amount of heavy D4-labeled PE-derived tryptic peptides as an internal standard (Figure 4). Calibration with internal standards provided consistency and accuracy for quantitation of peptides with linear correlation and one-to-one slopes for known and experimental values. These results demonstrate that use of internal standards composed of defined amount(s) of PE-derived tryptic heavy isotope-labeled peptides provides high quality linear quantitation of PE-derived peptides using isotopic ratio measurements.

In contrast, the data from isotopic ratio measurements for PE-derived peptides performed without internal standards demonstrated non-linearity and poor accuracy (Figure 5). Data from this study, obtained in LC-MS/MS, indicates that isotopic ratios may present systematic errors that are specific to particular peptides and areas of measurements; for example, the data show excellent 1/1 ratios, but significant errors at 4/1 and 10/1. Plots of these data demonstrate greatest variability in the highest ratio regions, as would be expected. Errors and variability in measurements for lower abundance ions would magnify errors when divided into much larger measurements for more abundant ions. Additionally, considerable variation in measured ratios was observed between peptides and could produce significant variation in calculated protein/protein ratios. Therefore, the calculated protein differences would be dependent on which peptides were observed and used for the calculations. While isotopic ratio experiments without internal standards may provide effective differential analysis between samples, it apparently may not reflect actual relative quantitative abundances.

Quantitation must occur within the physiological range of measurements for PE-derived peptides estimated in tissue samples. Therefore, it is important that the standard plots of different ratios of light/heavy isotopically-labeled PE-derived peptides were conducted in the physiological range of 300–800 fmol PE-derived peptides that corresponds to the estimated levels of such peptides in tissue samples of brain and related tissues (20). The physiological range is present in the mid-portion of the standard plots in experiments with internal standard (fig. 4). These standard plots show that the amounts of total protein used for analyzing ratios of 1/5 to 2/1 (light/heavy) in experiments with internal standards (fig. 4) correspond to approximately 300–800 fmol PE-derived peptides found tissue samples (21). Also, in experiments without internal standard (fig. 5), the amount of protein used in the experiments corresponds to a typical amount of PE-derived peptides in tissue samples (in the area of 470 fmol). Overall, the main point of these experiments is that quantitation can be obtained for physiological levels of PE-derived peptides.

It is noted that a few of the observed ratios differed from theoretical ratios of light and heavy isotope-labeled PE-derived peptides illustrated by data in Figure 4 compared to Figure 5. For example, peptide #1 shows an experimentally observed value of 12 for the theoretical ratio of 10.0 for light/heavy in Figure 4 (with internal standard), but its observed value is 6 for the theoretical ratio of 10.0 for light/heavy in Figure 5 (without internal standard). Such differences in isotope ratios in experiments with internal standard compared to without may explained by the total amounts of PE digests (light and heavy isotope-labeled mixture) injected into the mass spectrometer. The experiments with internal standard analyzed 2970 fmol of PE digest in each mass spectrometry experiment, but experiments without standard used 470 fmol digested PE (light and heavy isotope-labeled) injected into the mass spectrometer. These differences in total amount of PE-derived peptides injected can account for the differences in observed ratios of light:heavy peptides such as, for example, for peptide #1 (at known ratio of 10.0). The higher amount, 2970 fmol, was necessary for experiments to maintain the constant amount of internal standard, consisting of heavy isotope-labeled PE-derived peptides. However, the amount of sample, 470 fmol, in the experiments without internal standard is apparently outside the linear range of the mass spectrometer instrument (by scanning across a mass range). Nonetheless, the important aspect of the standard plots (figure 4 and figure 5) is that the majority of the points in the mid-domain of the plot correspond to physiological levels of PE-derived peptides that would be measured from tissue samples. Clearly, the internal standard experiment provides more accurate determinations of ratios of light:heavy isotope-labeled peptides.

Use of internal standards for comparison of peptide ratios is a valuable approach for comparison of peptide quantities in two different biological samples. It is remarkable that the average of the peptide measurements (slopes) for PE-derived peptides were statistically indistinguishable from unity. Data from other studies indicate that while slopes from other protein isotope ratio measurements using internal controls may be linear, the “slopes themselves may deviate significantly from unity” [15]. The ability to detect isotope ratios with one to one correspondence to the theoretical ratios will be advantageous for applications to differential measurements of peptides in biological samples.

Several recent studies have compared multiple methods to achieve protein quantitation in complex biological matrices. Such studies have examined quantitation of protein and peptide components by 2D-GE, LC-MALDI, and LC-MS/MS ion abundance and spectral counting methods [22–24]. The general finding of these studies is that the different methods provide different quantitative evaluations of similar biological samples. While there is general agreement among methods, no one method provides a comprehensive view of protein abundances. These reports and our data may provide insight into a recent study by ABRF that focused on protein quantitation of a standard protein mixture by various laboratories [25]. The results of this study indicated high variability in quantitative measurements using isotopic

labeling methods. The data obtained in these studies suggests that significant variability in protein abundances can result from specifically which peptides were observed and quantitated, especially if no internal standard was used. This observation is critical because (1) only a subset of peptides will be observed in any LC-MS/MS analysis of a complex protein mixture and (2) different experiments could observe substantially different peptide subsets.

We emphasize that our experiments have been developed and optimized for our objectives for analyses of PE-derived peptides using an Agilent ion trap mass spectrometer. Other mass spectrometer designs may have inherently different qualitative and quantitative capabilities [26]. Therefore, it is important to employ the appropriate experimental design, instrumentation, analysis methods and controls to obtain reliable data. This is especially important in applying the optimized peptide quantitation for understanding regulation of tissue levels of enkephalin and related neuropeptides.

In summary, isotopic ratio analyses examined in this study shows that use of internal standards provide accurate quantitation of PE-derived peptides over a broad range of isotope ratios. In contrast, measuring relative ratios by a direct isotope-labeling strategy without internal standards provides poorer comparison of differential peptide levels, with lower accuracy in measurements of isotopic ratios. The data presented here demonstrates that inclusion of the internal control in the experimental design provides increased consistency and accuracy for peptide quantitation. Thus, the use of internal standards provides highly improved linear correlations for mass spectrometry-based measurements of relative peptide quantities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PE-derived tryptic peptides utilized for isotopic ratio analyses

Tryptic peptides derived from PE used for isotope ratio analyses are illustrated by the colored bars under the proenkephalin primary sequence that indicate the active enkephalin neuropeptides (highlighted in gray). Peptide #1, 2, 4, and 5 are illustrated by green bars. The positions of peptide sequence #3 (purple bars) and peptide #6 (blue bars) occur at several places within the primary sequence of PE. The peptides studied met the following properties required of peptides utilized for isotopic ratio analyses (see methods): (1) unambiguous identification, (2) complete labeling in all labeling reactions, (3) equivalent chromatographic and mass spectrometric behavior with either isotopic label, (4) high signal-to-noise with low interference with background signals and (5) presence in all experiments (for $n \geq 3$).

Figure 2. Linear ranges for measuring heavy isotope-labeled peptides: selection for internal standard experiments

The quantity of heavy-labeled PE peptides used for internal standard was determined experimentally by evaluating the linearity of several peptides present in the authentic digest. These peptides correspond to peptides 2, 5 and 6 shown in Figure 1. Injection quantities of 41, 90, 150, 225, 300, 360 and 410 fmol of heavy isotope-labeled peptides were used to establish the linear range of peptide detection by mass spectrometry. The linear plots of amounts of heavy isotope-labeled peptides illustrate mean peptide measurements (signal intensity) from triplicate determinations (data shown in Supplemental Table A); standard deviations of each point in the three plots showed low coefficient of variance values (Supplemental Table A).

Based on the linear range of detection of heavy isotope-labeled peptides, 270 fmol of heavy-labeled PE peptides was selected for internal standard experiments.

A. With Internal Standard

PE Digest:		H4/D4 Ratio
H4-Labeled μ l	D4-Labeled μ l	
0.1	1.0	1/10
0.2	1.0	1/5
0.5	1.0	1/2
1.0	1.0	1/1
2.0	1.0	2/1
5.0	1.0	5/1
10.0	1.0	10/1

B. Without Internal Standard

PE Digest:		H4/D4 Ratio
H4-Labeled μ l	D4-Labeled μ l	
1.0	10.0	1/10
2.0	8.0	1/4
2.0	4.0	1/2
2.0	2.0	1/1
4.0	2.0	2/1
8.0	4.0	4/1
10.0	1.0	10/1

Figure 3. Analyses of different ratios of heavy- and light-isotope labeled proenkephalin-derived tryptic peptides by LC-MS/MS with internal standard and without standard

Recombinant purified human proenkephalin (PE), reduced and alkylated, was subjected to trypsin digestion. Separate samples of the PE digest was isotopically labeled with light (H4)-succinic anhydride and heavy (D4)-succinic anhydride. The light- and heavy-isotope labeled PE digest samples were combined in different ratios and subjected to nano-LC-MS/MS tandem mass spectrometry for quantitative analyses of relative amounts of PE-derived tryptic peptides. Analyses of different light to heavy isotope ratios of PE-derived peptides were conducted with internal standards (A) and without internal standards (B).

A. Analyses of ratios of light/heavy isotope-labeled PE tryptic peptides with internal standards. The light- and heavy-isotope PE-derived peptides were combined at ratios from 1/10 to 10/1 by combining different amounts of the light-labeled PE digest with a constant amount of heavy-labeled PE digest as internal standard. The samples with different isotope ratios were then subjected to LC-MS/MS for identification of peptides and quantitative analyses of their relative ratios. Relative quantitation was obtained by averaged ion intensity comparison between heavy- and light-labeled peptide chromatograms.

The amount of the constant heavy-labeled PE digest (as internal standard) in each sample injected into the mass spectrometer was 270 fmol. The light/heavy ratios of 1/5, 1/2, 1/1, and 2/1, and 5/1 correspond to total PE-derived peptide amounts of 324, 405, 540, 810, and 1,620 fmol that correspond to physiological amounts of such peptides in samples obtained from brain and related tissues [20,21].

B. Analyses of different ratios of light/heavy isotope-labeled PE tryptic peptides without internal standards. Light- and heavy-isotope labeled PE peptides were combined at ratios from 1/10 to 10/1 by combining different amounts of both the light- and heavy-isotope labeled peptides to maintain a constant total protein concentration. These mixtures of different isotope ratios of peptides were subjected to LC-MS/MS for peptide identification and quantitative analyses of relative ratios of peptides. Each injection of PE-derived peptides (at different ratios of light/heavy isotope labeling) into the mass spectrometer consisted of 470 fmol, representing an amount within the range of physiological levels of PE-derived peptides in tissue samples [20,21]. Relative quantitation was obtained by averaged ion intensity comparison between heavy- and light-labeled peptide chromatograms.

Figure 4. Quantitation of peptides illustrated by observed compared to theoretical ratios of PE-derived peptides analyzed by LC-MS/MS with internal standards in isotopic ratio measurements Plots of the known ratio versus the observed light/heavy isotopic ratios for peptides #1–6 derived from human proenkephalin are shown from analyses with internal standard calibration. Plots of observed and theoretical isotopic ratios are illustrated for each peptide #1–6 shown in panels a–f, respectively. Data points in each plot are illustrated as the mean of triplicate determinations; means and respective standard deviations are provided in Supplemental Table B. Standard deviations were less than the size of the symbols in these plots. These data demonstrate linear slopes for observed and theoretical light:heavy isotope ratios of the indicated peptides.

Figure 5. Poor quantitation of peptides illustrated by observed compared to theoretical isotopic ratios for PE-derived peptides analyzed without internal standards

Plots of the known light/heavy isotopic ratios versus the observed ratio for tryptic peptides derived from PE are shown. Plots of observed and theoretical isotopic ratios are illustrated for each peptide #1–6 shown in panels a–f, respectively. Data points in each plots are illustrated as the mean of triplicate determinations; means and respective standard deviations are provided in Supplemental Table C. Standard deviations were less than the size of the symbols in these plots. These data demonstrate that quantitation in isotopic labeling experiments without internal standards generates variable and non-linear response slopes. This observation is not dependent on protein identity as several peptides from bovine serum albumin also exhibit these

non-linear relationships for light/heavy isotopic ratios assessed without internal standards (Bark et al., unpublished data).

Linear Equation Fitting Demonstrates Linear One-To-One Slope Quantitation of PE-Derived Peptides with Internal Standard Calibration

TABLE 1

Peptides		Linear Equation Parameters				
		Position	Sequence	# Labels	$y = mx + b$ (m, b)	R^2 % RSD
1		119-134	DAEEDDSLANSDDLK	2	1.242, -0.281	0.993 5.6%
		135-143	ELLETGDNR	1	0.867, 0.167	0.987 5.2%
		161-167				
3		185-191	RYGGFMR	1	0.943, 0.068	0.998 3.8%
		236-242				
4		171-180	RSPQLEDEAK	2	1.205, -0.229	0.998 5.5%
		172-180	SPQLEDEAK	2	0.902, 0.121	0.996 5.4%
6		162-167				
		186-191	YGGFMR	1	0.920, -0.099	1.000 4.5%
		237-242				
Average					1.030, -0.042	0.995 5.0%

Linear equation fitting for tryptic peptides derived from proenkephalin (PE) was conducted for data obtained from internal standard calibration experiments (Fig. 3A). The amino acid numbers corresponding to tryptic peptides derived from PE are listed as peptides 1-6. The amino acid sequences of each peptide are also shown. The number of succinic anhydride labels for each peptide is indicated. The PE digest was separated into two equal samples; one sample was labeled with light (4-H) and the other was labeled with heavy (4-D) succinic anhydride. The light-labeled peptide mixture was combined as 1/10 to 10-fold the amount of a constant quantity of heavy isotope-labeled peptides (Fig. 3A) and analyzed by LC-MS/MS. The experimentally measured light/heavy isotopic ratios (y-axis) were compared to known isotopic ratios (x-axis). Data was fitted by linear least squares analysis and reported as m,b pairs fitting the linear equation $y = mx + b$. R^2 was used as an estimate of the accuracy of the least-squares linear fit to the experimental data. For these experiments, $R^2 \geq 0.99$ was considered to be an accurate correlation between the regression equation and the observed data [18]. % RSD is the percent standard deviation (also known as coefficient of variance [19]) compared to the average of experimentally observed ratios for the triplicate experiments.

Linear Equation Fitting Demonstrates Poor Quantitation of PE-Derived Peptides in Isotopic Ratio Analyses without Internal Standards

TABLE 2

Peptides		Linear Equation Parameters				
		Position	Sequence	# Labels	$y = mx + b$ (m, b)	R^2 %RSD
1		119–134	DAEEDDSLANSDDLK	2	0.5931, 0.4508	0.941 10.8%
		135–143	ELLETDGDN	1	0.7164, 0.2909	0.989 7.5%
2		161–167				
		185–191	RYGGFMR	1	0.2042, 0.4778	0.785 13.0%
3		236–242				
		171–180	RSPQLEDEAK	2	0.7791, 0.3519	0.986 12.5%
4		172–180	SPQLEDEAK	2	0.8253, 0.16	0.992 7.2%
		162–167				
5		186–191	YGGFMR	1	0.7431, 0.154	0.992 8.7%
		237–242				
		Average			0.644, 0.314	0.948 10.0%

Linear equation fitting was investigated for quantitation of tryptic peptides analyzed in isotope ratio analyses without internal standards. The amino acid numbers and peptide sequences of tryptic peptides derived from PE are shown for peptides 1–6. The number of succinic anhydride labels for each peptide is indicated. The experimentally measured light/heavy isotopic ratios (y-axis) were compared to known isotopic ratios (x-axis), for mixtures of heavy- and light-isotope labeled peptides of 1/10 to 10/1. Data was fitted by linear least squares analysis and reported as m,b pairs fitting the linear equation $y = mx + b$. R^2 was used as an estimate of the accuracy of the least-squares linear fit to the experimental data. For these experiments, $R^2 \geq 0.99$ was considered to be an accurate correlation between the regression equation and the observed data [18]. Percent RSD is the percent standard deviation (also known as coefficient of variance [19]) compared to the average of experimentally observed ratios for the triplicate experiments.