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ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2009

Impact Factor: 5.64 · DOI: 10.1021/ac801654h · Source: PubMed

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Published in final edited form as:
Anal Chem. 2008 December 1; 80(23): 9298–9309.

Multiple isotopic labels for quantitative mass spectrometry

Cain Morano[1], **Xin Zhang**[1], and **Lloyd D. Fricker**[*]
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461

Abstract

Quantitative mass spectrometry is often performed using isotopically-labeled samples. While the 4-trimethylammoniumbutyryl (TMAB) labels have many advantages over other isotopic tags, only two forms have previously been synthesized (i.e. a heavy form containing 9 deuteriums and a light form without deuterium). In the present report, two additional forms containing 3 and 6 deuteriums have been synthesized and tested. These additional isotopic tags perform identically to the previously reported tags; peptides labeled with the new TMAB reagents co-elute from reverse phase HPLC columns with peptides labeled with the lighter and heavier TMAB reagents. Altogether, these 4 tags allow for multivariate analysis in a single liquid chromatography/mass spectrometry analysis, with each isotopically tagged peptide differing in mass by 3 Da per tag incorporated. The synthetic scheme is described in simple terms so that a biochemist without specific training in organic chemistry can perform the synthesis. The interpretation of tandem mass spectrometry data for the TMAB-labeled peptides is also described in more detail. The additional TMAB isotopic reagents described here, together with the additional description of the synthesis and analysis should allow these labels to be more widely used for proteomics and peptidomics analyses.

Introduction

A large number of bioactive peptides are known to play important physiological roles in a variety of organisms, ranging from yeast to humans [1–4]. Some peptides function in communication between cells (neuropeptides, peptide hormones, growth factors, and mating factors) while others function as toxins to capture prey or to repel predators (snake venoms, marine organism toxins, and antimicrobial peptides).

Developments in mass spectrometry (MS) in the last decade have led to a dramatic improvement in the detection of bioactive peptides [3;5–9]. Prior to mass spectrometry, peptides were primarily characterized using radioimmunoassays or related techniques [10]. While these techniques provided the ability to compare the immunoreactive levels of a peptide in a large number of different samples, there were three major drawbacks. First, the antisera often cross-reacted with longer or shorter forms of the peptide, and/or cross-reacted with post-translationally modified peptides (i.e. phosphorylated, acetylated, etc). Thus, it was difficult to be sure of the specific form being measured with the antibody-based assay. Secondly, it was very time consuming to raise antisera to a peptide, characterize the antisera, and set up a selective immunoassay to detect the peptide. Third, it was only possible to study known peptides. In contrast, mass spectrometry enables hundreds of peptides to be detected in a single experiment, with knowledge of the precise molecular form being measured, and can detect unknowns as well as previously identified peptides.

*Corresponding Author: Prof. Lloyd Fricker, Ph.D., Molecular Pharmacology, F248, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 fricker@aecom.yu.edu, Phone: 718-430-4225, Fax: 718-430-8922.
[1]authors contributed equally and should be considered co-first authors.

Some previous peptidomics studies have obtained an estimate of the relative levels of peptides in two or more samples by comparing the relative intensity among different LC/MS run of the peptides present in a complex sample [11–14]. While it may be possible to achieve some accuracy with this non-isotopic method, it is difficult to quantify small changes using this technique. A more accurate method to measure small changes in peptide levels involves stable isotopes [15–17]. In a typical experiment involving isotopes, peptides labeled with light isotopes (^{1}H, ^{12}C, ^{14}N, and/or ^{16}O) are combined with peptides labeled with heavy isotopes (^{2}H, ^{13}C, ^{15}N, and/or ^{18}O) prior to mass spectrometry (MS) and the relative peak intensity of the two forms provides an accurate indication of the relative levels of each peptide in the sample. For studies on cultured cells, as well as some lower organisms, differential isotopic labeling can be obtained by growing one sample in normal media while the other gets growth media supplemented with heavy isotopes [18;19]. However, for studies on mammals, the heavy isotopes are prohibitively expensive. Therefore, studies on mammalian peptides have generally used post-extraction labeling of the peptides with isotopic tags [15].

A number of isotopic tags have been developed for the quantification of proteins and peptides [15;20–26]. The Isotopically Coded Affinity Tag (ICAT) labels were developed for proteomics studies; this reagent reacts with the thiol group of Cys and also contains a biotin affinity tag to allow for purification of the labeled peptide [20]. However, because most peptides do not contain thiol groups, the ICAT labels will not work for peptidomics analysis. Instead, peptidomics studies have focused on isotopic tags that label primary and secondary amines which are contained on the N-terminus and on the side chain of internal Lys residues (unless acetylated or otherwise modified) [22]. Commercially available reagents that label amines and can be used for quantitative peptidomics include acetic anhydride, succinic anhydride, and iTRAQ reagents [15;20–22;22;27]. However, these suffer from various drawbacks. Although relatively inexpensive, the acetic and succinic anhydrides convert positively charged amines on a peptide into neutral sites (acetyl) or negative charges (succinyl), and for some peptides this results in a weak signal when analyzed by MS in positive ion-mode [22]. Furthermore, peptides labeled with the heavy forms of these anhydrides do not always co-elute on LC/MS with the light forms, and quantitation of these peptides is less accurate than for those isotopically tagged peptides that do co-elute [22]. The iTRAQ reagent solves these latter problems, but it is very expensive, difficult to synthesize, and only shows signals when peptides are subjected to collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) [21]. Because many peptides can be detected in MS spectra of complex mixtures but only one of these is analyzed by MS/MS at any given time, the iTRAQ reagent will miss those peptides not selected for MS/MS analysis [21].

The ideal isotopic label has several key properties. The label should react with amines efficiently and quantitively but also have a long shelf life, be inexpensive and either commercially available or easy to synthesize, and maintain the positive charge on the labeled amine. In addition, the isotopically-labeled peptides should co-elute on LC and be stable to MS. Finally, it would be ideal if the label came in more than just two forms (i.e. “heavy” and “light”) so that multiple comparisons could be performed within the same LC/MS run.

The TMAB labels were developed by Regnier and colleagues and contain a quaternary amine labeled with methyl groups which imparts a permanent positive charge on the peptide [23]. While the TMAB labels are close to ideal, there are several problems. First, the synthesis is fairly simple, but the description of the synthesis in the literature is difficult for a non-chemist to follow. Second, the isotopic tags often partially decompose to stable intermediates upon CID and MS/MS analysis and this complicates computer and manual interpretation of the data [22;28]. Finally, only heavy and light forms of these TMAB labels have been previously reported [23]. In the present study, we report the synthesis and evaluation of additional isotopic TMAB tags that allow 4 samples to be compared in the same LC/MS run. In addition, we describe the

synthesis in more detail, with instructions for a non-chemist and tips on how to get high yields. Finally, we describe the problems with data interpretation, providing guidelines for manual analysis. Although developed for quantitative peptidomics applications, these labels should be useful for proteomics studies too, much like the iTRAQ reagents but with several advantages.

Materials and Methods

Reagents

Gamma-aminobutyric acid (GABA), potassium bicarbonate, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide, methyl iodide (sold as iodomethane) and its d_1, d_2, and d_3 isotopes, dimethylsulfoxide (DMSO), sodium hydroxide, glycine, and hydroxylamine hydrochloride were purchased from Aldrich Chemical Company (Milwaukee, WI). Acetonitrile of HPLC grade, anhydrous solvents such as tetrahydrofuran (THF), acetonitrile, methanol, and acetone were obtained from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid (6N, sequanal grade, constant boiling), trifluoroacetic acid, and formic acid were purchased from Pierce (Rockford, IL). Water was purified with a Synergy® UV system (Millipore S.A.S., Molsheim, France).

Synthesis of TMAB labels: Overview

The overall reaction scheme is shown in Figure 1, and is described in detail below both in a standard protocol for chemists, as well as a less technical protocol intended for biochemists without formal training in organic chemistry. Additional notes on the synthesis are provided as supplemental information. All of the procedures involving organic solvents should be performed in a fume hood, and some of the reagents are especially toxic (methyl iodide and dicyclohexylcarbodiimide). Proper safety equipment should be used to handle these compounds and care should be taken to minimize exposure. The procedures described below are identical for all four isotopic tags; although the molecular weights and densities of the methyl iodide vary, the differences are so small as to be negligible. The use of CH_3I results in the production of D0-TMAB-NHS (Figure 2), which was previously referred to as H9-TMAB-NHS[22]. The use of methyl iodide with 3 deuterium atoms in place of the hydrogen atoms (Cd_3I) produces D9-TMAB-NHS, as previously described [22;23]. The two new labels are produced from CH_2dI and CHd_2I and result in the formation of D3-TMAB-NHS and D6-TMAB-NHS, respectively (Figure 2).

(3-Carboxy-propyl)-trimethyl-ammonium chloride; common name: trimethyl-ammonium butyric acid chloride (TMAB Acid)

Chemist protocol

GABA (Gamma-aminobutyric acid) (2.23 g, 21.6 mmol) and potassium bicarbonate (10.0 g, 99.9 mmol) were combined in 250 mL of methanol under N_2 with stirring. After 15 min, 6.9 mL methyl iodide (approximately 16 g, 110 mmol) was added. The reaction was stirred for 66 h at ambient temperature. Then the solvent was removed by evaporation under reduced pressure at ~55ºC. The resulting white solid was washed four times with 50 mL of chloroform. The solid was then dissolved into 50 mL diluted concentrated HCl (3:1 water:acid), followed by removal of bulk water by evaporation under reduced pressure under moderate heat (~60–65ºC). The resultant orange residue was placed under high vacuum for further drying. After 2–3 h the amber residue was extracted four times with 60 mL acetone. The acetone was removed by evaporation under reduced pressure at ~55ºC, yielding an oily amber residue. To this, 150 mL of THF was added and then cooled to 0ºC for 0.5 h. The resulting solid was filtered and washed further with THF, then dried under vacuum. Overall, the reaction produced 2.02 g (11.1 mmol, 52% yield) of TMAB Acid.

Biologist protocol

1) Prepare reagents and equipment—Place 500 mL round bottom flask, glass 10 mL pipette, excess potassium bicarbonate, glass flask stopper, and Teflon stir bar in vacuum oven at approximately 50ºC overnight; after approximately 16 h, turn off heat and allow cooling to room temperature under vacuum. Place excess GABA in an Erlenmeyer flask inside a desiccator, then place under high vacuum at room temperature overnight. Methyl iodide and isotopes are liquids and should remain sealed, refrigerated, and under inert gas if possible.

2) Set up reaction—Weigh out 10.0 g potassium bicarbonate and 2.23 g GABA into flask. Add stir bar and fill flask slightly more than halfway with anhydrous methanol. If a nitrogen tank is available, blow some N_2 into the flask above the methanol and then stopper. Begin vigorous stirring on magnetic stir plate, but without splashing. After 15 min, add 6.9 mL methyl iodide to reaction vessel by glass pipette (caution – methyl iodide is toxic; wear gloves and perform this step in a fume hood). Stopper flask and let stir for 2 to 3 days (40 hours and 66 hours have produced similar yields.

3) Stop reaction—Remove stir bar from solution. Using a rotary evaporator, reduced pressure and mild heating, evaporate all methanol. Solvent distilled from reaction may contain residual methyl iodide; dispose of accordingly.

4) Wash and acidify product—In a fume hood, pulverize the white reaction product into a fine powder and add to a fritted funnel, attached to side-arm flask connected to a vacuum line. Wash the powder 4 times with 50 mL chloroform. Add powder to a 250 mL round bottom flask and remove remaining solvent under reduced pressure. Acidify the powder with 50 mL of a 3:1 mixture of water:concentrated HCl. Agitate flask or stir while adding acid slowly. Remove the acid/water on a rotary evaporator, with reduced pressure and heating to 60–65 ºC. Then place under high vacuum for further drying.

5) Extract product into acetone—Add 60 mL anhydrous acetone to product, agitate to suspend all solids then pour into fritted funnel/side-arm flask apparatus as before. Wash solid 3 more times with 60 mL acetone. Discard solid. Add acetone to round bottom flask, remove solvent by rotary evaporator as before, with mild heat (approx. 55ºC).

6) Clean and dry product—Slowly add 150 mL anhydrous THF to product while mixing (swirling flask by hand). It may be necessary to break up oily product with spatula to yield a solid. Stopper the flask and place flask in ice to allow further precipitation. This precipitate is the reaction product (TMAB Acid). Filter the precipitate in a glass funnel, wash several times with 50–100 mL of anhydrous THF, and then dry under vacuum for 16 h. After weighing, if you will not proceed with the second step (below), it is best to store under nitrogen in a desiccator that is cold and dark.

[3-(2,5-Dioxo-pyrrolidin-1-yloxycarbonyl)-propyl]-trimethyl-ammonium chloride; common name: trimethyl-ammonium butyric acid chloride *N*-hydroxy-succinimide ester (TMAB-NHS)

Chemist protocol

TMAB Acid (2.02 g, 11.1 mmol), the methylated amine, was dissolved into 250 mL of anhydrous acetonitrile and cooled to 0ºC. NHS (2.18 g, 19.0 mmol, 1.7 eq) was added with stirring, followed by dicyclohexylcarbodiimide (4.30 g, 20.9 mmol, 1.9 eq). The reaction was allowed to warm to ambient temperature over 16 h. The reaction was cooled to 0ºC again at 16 h, and the stirring terminated. The reaction was filtered, the solid discarded, and the

acetonitrile evaporated under reduced pressure. The compound was slurried with 125 mL THF and cooled to 0ºC for 1 h. The resulting white solid was filtered and dried under vacuum. This reaction typically yielded 2.72 g (9.74 mmol, 88% yield for the second step) of TMAB-NHS.

Biologist protocol

1) Prepare equipment—Dry a 500 mL round bottom flask, stopper, and stir bar in vacuum oven, as above. Set up flask above magnetic stirrer, as before, but include a tray on top of the magnetic stirrer that can hold ice (don't add ice yet – see below).

2) Start reaction—If you have higher or lower yields from step 1 (formation of the TMAB acid), scale the following reaction up or down as appropriate, being careful to maintain the same proportion of all reagents. Weigh 2.02 g of TMAB Acid and 2.18 g of NHS and add to the flask with stir bar. Add 250 mL of anhydrous acetonitrile, stopper the flask, and stir to dissolve the reagents at room temperature for approximately 15 mins. Then, add ice to the tray so that the acetonitrile solution cools while it is stirring. Weigh out 4.30 g of dicyclohexylcarbodiimide and dissolve in 43 mL of anhydrous acetonitrile at room temperature. Add the dicyclohexylcarbodiimide/acetonitrile solution to the flask, replace the stopper and stir for 16 h. During this time, the ice in the bath will melt and the reaction will warm to room temperature.

3) Remove precipitate and isolate product—Turn off the stirrer and place the flask in an ice bath to allow the dicyclohexylurea to precipitate. Filter off the precipitate using a fritted funnel and wash the precipitate with 100 mL acetonitrile. Discard the precipitate; the desired reaction product is in the acetonitrile. Place the acetonitrile solution into a round bottom flask and remove solvent on a rotary evaporator under reduced pressure, as before, yielding a brown oily residue.

4) Clean and dry product—Slowly add 125 mL of anhydrous THF to the residue while swirling by hand. Place flask in ice bath to allow a precipitate to form (usually 1–2 hours). It may be necessary to break up oily product with spatula to yield a solid. Once formed, isolate this solid with a glass filter, washing the residue with 50–100 mL of THF (wash until the compound is nearly white). Dry the precipitate under vacuum for 16h, then weigh and place in a storage vial. For long-term storage, it is best to gently blow nitrogen into the tube, then tightly seal and store in a cold and dark desiccator.

Labeling of mouse brain extract

Four mice (C57B6/J, 2–3 month old) were bred in the barrier facility of the Albert Einstein College of Medicine. Each mouse was sacrificed by decapitation and the head was immediately irradiated in a conventional microwave oven for 8 seconds to raise the brain temperature to 80°C, as described[29]. This microwave irradiation step is necessary to rapidly inactive proteases that can rapidly degrade cellular proteins within the postmortem processing; these protein degradation fragments overwhelm the signal from neuropeptides [29–31]. In addition, microwave irradiation inactivates proteases that degrade neuropeptides, and the level of recovered neuropeptides is several-fold higher in microwave-irradiated brain extracts than in non-microwaved tissue[29;31]. After cooling, the mouse brain was isolated, the olfactory bulb and cerebellum were removed, and the remainder of the brain was frozen in dry ice and stored at −70°C until peptide extraction.

Each mouse brain was sonicated for 50 pulses at 1 pulse/sec in 1.0 mL ice-cold water using an ultrasonic processor (W-380, Ultrasonic Inc., Farmingdale, NY). The homogenate was incubated in a 70°C water bath for 20 min, cooled in an ice bath, and acidified with 120 μL of 0.1 M HCl to a final concentration of 10 mM HCl. The homogenate was centrifuged at 13,000

g for 30 min at 4°C and the supernatant (peptide extract) was transferred to a new low-retention tube. Aliquots (60 μL each) of the brain peptide extract from each of four mice were pooled, mixed well, and then split into four equal parts. Each part was neutralized to pH 8 by 75 mM borate buffer, and the final pH 9.5 was reached by addition of 1M NaOH before the labeling reaction. For the labeling reaction, 6.4 μL of 350 μg/μL D0-, D3-, D6-, or D9-TMAB-NHS dissolved in DMSO were added separately to four peptide extract sample tubes. After 10 min, an appropriate volume of 1.0 M NaOH was added to adjust the pH back to 9.5 and the tubes were also incubated for 10 min. The addition of TMAB and NaOH were repeated seven times, then the mixtures were incubated at room temperature for another 1 h, after which 10 μL of 2.5 M glycine was added into each labeling tube to quench the remaining TMAB reagents. After labeling, the four TMAB-labeled samples were combined and filtered through a Microcon® YM-10 unit (Millipore) to cut off proteins >10 kDa. To remove any labels from Tyr residues in the peptides, the pH of filtrate was adjusted to 9.0 and a total of 13.5 μL of 2.0 M hydroxylamine (in DMSO) was added in three aliquots of 4.5 μL each, with an interval of 10 min between each addition. After desalting with a PepClean™ C-18 spin column (Pierce), peptides were eluted out of the column with 80 μL of 70% acetonitrile in 0.1% trifluoroacetic acid in water, frozen, and concentrated to 20 μL in a vacuum centrifuge. Aliquots of the peptide samples were stored at −70°C until MS analysis.

LC/MS/MS

The peptide mixture was trapped and washed on a PepMap™ C18 trapping column (5 μm, 100Å, 300 μm i.d. × 5 mm, LC Packing, Marlton, NJ) using a Eldex MicroPro™ Syringe Pumping System with 5% solvent B for 20 min at a flow rate of 4 μL/min. Then the peptides were separated on a Grace Vydac MS C18 capillary column (3 μm, 100Å, 75 μm i.d. × 150 mm, Hesperia, CA) at 4 μL/min with a gradient elution 5% solvent B for 45 min, then to 35% solvent B in 40 min, to 95% solvent B in the following 20 min. Solvent A was 2% acetonitrile in 0.1% formic acid. Solvent B was 80% acetonitrile in 0.1% formic acid. Flow from the column was directed to an API Q-Star Pulsar-i™ quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The nanoelectrospray ion source was operated in the positive mode with a spray voltage of 2 kV. The information-dependent acquisition mode was used with a 1-s survey scan and 2-s MS/MS scan on the most intense ions in the MS survey scans. The collision energy for MS/MS (20–45 eV) was dynamically selected based on the *m/z* value and charge state of the ion selected.

Data analysis

The Mascot program (http://www.matrix-science/com) was used to aid in the interpretation of tandem mass spectra. In Mascot searches, fixed modifications were selected as GIST-Quat, GIST-Quat:2(H)3, GIST-Quat:2(H)6, or GIST-Quat:2(H)9 for D0-, D3-, D6-, or D9-TMAB labeled peptides at the N-terminus and Lys residues. (Note that this involved 4 Mascot searches, one for each isotopic tag, because in the “fixed modification” mode the different isotopes are mutually exclusive.) While Mascot searches identified a number of peptides, this program was not able to identify some MS/MS spectra that were of medium-high quality, primarily due to partial breakdown of the TMAB tags. Those MS/MS spectra that could not be identified by Mascot were interpreted manually. Also, manual re-interpretation of Mascot hits was done to verify the predicted peptide sequence. The criteria for considering a peptide identified from the MS/MS analysis were: (i) a parent mass within 40 ppm (preferably 20 ppm) of the theoretical mass; (ii) the observed number of TMAB tags on the peptide matched the predicted number of free amines available (i.e., Lys residue and N-terminus); (iii) the observed charge state(s) of the peptide was consistent with the expected number of positive charges; (iv) 80% or more of the major fragments observed in MS/MS matched predicted fragments (minimum five matches). Representative spectra of the peptides are included in the Supporting

Information accompanying this paper. Mascot search results are shown for some peptides, and manual interpretations are also provided for those peptides that were not found by Mascot.

The intensity of each TMAB-labeled isotopic peak was determined by measuring peak heights of the monoisotopic peak and the additional peaks containing 1 and 2 atoms of ^{13}C. As an alternative to peak height, it is possible to quantify by measuring peak area when the peaks are well separated, but this approach is more difficult to use when there is peak overlap and so for the present study, all measurements were made using peak height. When the peaks for the D0, D3, D6, and D9 forms of each peptide are well separated (Figure 3A), the quantitation was performed by simply measuring the peak heights. However, for those peptides with overlapping peaks it was necessary to calculate the theoretical peak intensity of the ^{13}C isotope-containing peaks (Figure 3B). This calculation was performed using the Protein Prosepector Program (http://prospector.ucsf.edu/). The baseline was then adjusted for the D3, D6, and D9 peaks based on this theoretical distribution, as shown in Figure 3B.

To measure the reproducibility between the four TMAB labeling reagents, the peak heights for each of the TMAB labels was compared to the average peak height of the other 3 peaks. For example, the following ratios were determined: the D0-TMAB peak versus the average of the D3, D6, and D9 peaks; the D3-TMAB peak versus the average of the D0, D6, and D9 peaks; the D6 peak versus the average of the D0, D3, and D9 peaks; and the D9 peak versus the average of the D0, D3, and D6 peaks. The standard deviation of all four ratio measurements for each peptide was determined using Excel.

Results

The synthetic scheme for the production of the TMAB labels (Figure 1) is a simple two step reaction that can be performed by non-chemists with relatively simple equipment that, except for the rotary evaporator, is standard in biochemistry laboratories. The first step involves the reaction of methyl iodide with 4-aminobutyric acid (also called gamma-amino butyric acid, or GABA). The yield of the reaction product is high if care is taken to avoid the introduction of water into the reaction by drying the reagents and using anhydrous solvents. Typical yields of the first reaction step are 60–70%. The reaction with regular methyl iodide (CH_3I) and fully deuterated methyl iodide (Cd_3I) has been previously described[22;23]. In the present study, the reaction with CH_2dI and CHd_2I was performed and found to proceed with the same overall yield as for the other compounds. The product of this first reaction was then reacted with dicyclohexylcarbodiimide and N-hydroxysuccinimide (NHS) to form the active ester (Figure 1). The yield of this second step was typically 80–90%, for an overall yield of 50–60%. The active NHS ester is stable for over one year when stored under anhydrous conditions.

The four isotopic tags differ in mass by 3 Da, and after reaction with the amine group of a peptide they increase the mass by 129 Da to 138 Da per label incorporated (Figure 2). To test the labels under typical experimental conditions, the peptide extract from several mouse brains was divided into equal parts and labeled with the four isotopic tags. After quenching, the reactions were pooled, treated with hydroxylamine to remove the tags from Tyr residues, and then desalted and analyzed by LC/MS. Representative data are shown in Figure 3. For all peptides identified in the present study, every free N-terminal amine and Lys side chain was labeled with the TMAB reagent, indicating that the labeling efficiency was close to 100% (Table 1). In most cases where multiple tags were incorporated into the peptide (i.e. when there were Lys residues), the 3 Da difference between the mass of each tag was easily resolved upon analysis of the data (Figure 3A). For those peptides that did not contain Lys residues and therefore incorporated only a single tag on the N-terminal amine, the separation of the individual peaks was less pronounced (Figure 3B). In these cases, determination of the peak heights attributed to the D3, D6, and D9 peaks required calculation of the distribution of ^{13}C

isotopes in the peptide and subtraction of their contributions to the overall intensity (Figure 3B).

For isotopic labels to be optimal for quantification applications, it is important that the labeled peptides co-elute on the HPLC columns used for LC/MS. Peptides labeled with all four of the isotopic labels tested in the present study co-elute from reverse phase columns (Figure 4). This is true both for peptides with a single isotopic tag (Figure 4, right panels) as well as those with multiple tags (Figure 4, left panels). In all cases, the elute time for each of the isotopic peaks was identical.

Another important feature of isotopic tags is that they allow for the conclusive identification of the peptide from MS/MS data. The TMAB labels undergo partial break-down upon CID that can complicate the spectra and lead to lower scores on Mascot and other search programs. However, manual interpretation of the spectra with knowledge of the typical break-down patterns can greatly increase the confidence in the assignments and/or eliminate false positives. For example, Figure 5A shows the MS/MS spectrum of a peptide identified by Mascot, with many matches to predicted *b* or *y* series ions. However, the two major ions in the spectrum (791.43 and 761.89) were unmatched by Mascot. This program only considers partial breakdown of the TMAB tags in the MS/MS mode, and not partial breakdown of the parent ion. In this example, the parent MH^{2+} ion (detected at 820.97) undergoes neutral loss of one and two trimethylamine groups (i.e. −59 and −118 Da for the D0 form of the TMAB labels) to produce the 2+ ions at 791.43 and 761.89, respectively (Figure 5A). All of the daughter ions have completely lost the trimethylamine groups, which is the state considered by Mascot and so this program correctly identified the peptide. However, other peptides not identified by Mascot showed beautiful sequence ladders that perfectly matched known peptides present in brain; for example, the proSAAS derived peptide shown in Figure 5B. In this example, the peptide fragments do not lose the trimethylamine group (68 Da for this D9-TMAB label) and therefore are not considered by the Mascot program. Because every major MS/MS fragment matches a predicted fragment from this proSAAS-derived peptide, and Mascot failed to identify a better match, it is highly likely that this assignment is correct. Additional notes on the interpretation of spectra and the complication of the partial breakdown of the TMAB labels is provided as supplemental information.

Altogether, 107 peptides were identified in the present study (Table 1). The Mascot results and/or manual interpretation are provided in a supplementary file. Of these 107 peptides, 72 are derived from secretory pathway proteins and the other 35 are fragments of cellular proteins. The secretory pathway protein fragments generally represent either known neuropeptides or other peptides that are predicted to be produced based on the presence of prohormone convertase cleavage sites flanking the peptide. Most of these secretory pathway protein fragments have been previously identified in peptidomics analyses. Some, but not all, of the cellular protein fragments have been previously reported in peptidomics studies. The majority of the observed peptides, both secretory pathway and cellular, represent unmodified peptides (except for the proteolytic cleavages). However, some show post-translational modifications beyond the proteolysis required for their production. These post-translational modifications include C-terminal amidation, N-terminal acetylation or pyroglutamylation, phosphorylation of Ser, oxidation of Met, and two additional modifications; one a +25 Da modification (cyanylation) on the N-terminal Cys of the ubiquinol-cytochrome C reductase hinge protein fragment of 1324.75 Da, and the other the loss of 36 Da (2 waters) from the proSAAS fragment of 1296.68 Da (Table 1).

For the vast majority of the peptides (>90%), the relative intensity of each isotopic peak was within 20% of the average intensity of the three other peaks (Table 1 and Figure 6). Only two peptides showed spectra with one of the peaks substantially different than the other three peaks;

the prodynorphin peptide of 1027.55 Da, and the ubiquinol cytochrome C reductase hinge protein fragment of 2450.15 Da (Table 1). The reason for this variability for these two peptides is not clear. The standard deviation of the relative peak intensities was within 0.20 for all but six of the 107 peptides detected in the present study. Thus, the 4 isotopic labels are able to provide quantitative data on the levels of a large number of peptides in a single analysis.

Discussion

Although the TMAB labels were developed several years ago by Regnier and colleagues [23], use of these labels has been limited[22;28;29;32]. This is presumably due to the lack of a commercial supplier for these compounds. However, the synthesis is very simple and can be done by a non-chemist with respectable yields. For example, the protocol described in the Methods section was performed by a biologist with no experience in synthetic organic chemistry and an overall yield of 45% was achieved. The TMAB-NHS labels are produced by convenient “dump-and-stir” reactions in two steps. The first step is the reductive alkylation of an amine by methyl iodide, and the second is a standard carbodiimide-catalyzed activation of a carboxylic acid to form a reactive ester (i.e. the NHS reagent that will subsequently react with amines on the peptides in the biological sample). To increase the yield of the synthetic reaction it is important to start with anhydrous reagents, solvents, and glassware, and to protect the solvents from ambient atmospheric conditions. Additional tips to improve the yield for the synthesis are included as supplementary information. This reaction is easily scaled up or down and the product will keep for years if protected properly.

Previously, an advantage of the iTRAQ reagents was their availability in 4 versions, allowing for multiple comparisons in a single LC/MS run. With the synthesis of the D3 and D6 isotopic forms of the TMAB labels, it is now possible to perform multivariate comparisons using the TMAB labels. In addition to the 4 TMAB labels described in the present manuscript, a fifth TMAB isotopic label is possible using $^{13}Cd_3I$ in the first step. This would result in a compound that is 3 Da heavier than the D9-TMAB reagent, extending the series by one additional label. Although the 3 Da difference between each TMAB isotopic label is not sufficient for the baseline separation of each isotopic form of some peptides that incorporate only a single isotopic tag (see Figure 3), it is possible to deconvolute the data by calculating the theoretical isotopic distribution of ^{13}C. This allows for the appropriate baseline to be subtracted from the D3, D6, and D9 peaks, providing more accurate quantification of the peak intensities.

As expected based on the previous results with the D0-TMAB (formerly called H9-TMAB) and the D9-TMAB reagents[22;23], all isotopic forms of the TMAB labeled peptides co-elute from reverse phase HPLC columns (see Figure 4). This is an important requirement for isotopic tags because the relative intensities of peptides are more accurate when comparing the same MS scans than when comparing peptides in distinct scans. This has been found to be a problem with many isotopic labeling reagents that use deuterium, and has led to the development of ^{13}C and/or ^{15}N-based labeling reagents[15;33]. These other isotopes do not affect elution from the reverse-phase HPLC columns and so the heavy and light forms co-elute. However, ^{13}C and ^{15}N-labeled compounds are much more expensive than deuterated compounds. The design of the TMAB reagents by Regnier et al[23] was based on the theory that the deuterium in the heavy form of the label would not interact with the reverse phase matrix due to the adjacent quaternary amine. This elegant idea combines the advantages of the deuterium labels (low cost, easy synthesis, and labels that differ by 3 Da or more) and eliminates the major limitation (labeled peptides that do not co-elute on HPLC). However, the quaternary amine causes the label to be unstable in many different MS applications (discussed below).

Although in theory each peptide labeled with the 4 isotopic TMAB reagents should appear in precise 1:1:1:1 ratios, this was only the case for some of the peptides and most showed a slight

deviation of one or more of the peaks. The data shown in Figure 3 are truly representative; for example, the peptide in Figure 3B (the chromogranin B-derived peptide LLDEGHYPVRESPIDTA) has peak heights that are among the most variable of all peptides found in the present study (standard deviation = 0.18; see Table 1). Most peptides showed less variability than this peptide. In this experiment, variability is not due to animal-to-animal variation because extracts from several animals were pooled prior to labeling. In those cases where one peak is higher than the others, it is possible that this is due to the co-elution of another peptide with similar m/z as one of the peaks. But, in cases where one peak is lower than the other 3, this is less likely (it would require 3 co-eluting peaks of precisely the same mass and charge). Still, most peptides showed a ratio that was very close to the theoretical 1:1:1:1 ratio, and those that didn't were still within the normal animal-to-animal variations seen with typical experiments using the D0 and D9 isotopic tags[34–37]. Thus, the small background variation observed with the labels in the present study will be negligible for most experiments. Furthermore, performing experiments with 3–4 replicates using different tag combinations for each replicate, will greatly reduce the potential error due to minor fluctuations in individual isotopic peaks.

In matrix assisted laser desorption ionization time-of-flight MS, the TMAB labels lose trimethylamine from a substantial fraction of the tagged peptides, thus reducing the level of the isotopically-labeled peptide and complicating the spectra with additional peaks. Ion trap mass spectrometers with electrospray ionization have not been successfully used with the TMAB-labeled peptides[16]. Best results have been observed with electrospray ionization quadrapole time-of-flight mass spectrometers (ESI Q-TOF MS). Comparable results were obtained with TMAB-labeled peptides analyzed on ESI Q-TOF MS instruments manufactured by several different companies (MDS Sciex/Applied Biosystems; Waters/MicroMass). All ESI Q-TOF MS instruments tested show the intact TMAB-labeled peptide when analyzed in MS mode, with virtually no breakdown of the label. However, after CID for MS/MS sequencing, the labeled peptides usually decompose and lose trimethylamine (which is neutral and therefore not detectable by MS). The positive charge is retained on the label attached to the peptide. Because the differential isotopes are usually lost from the molecule, the MS/MS results for each of the various isotopic forms are generally identical. However, for some peptides the breakdown of the TMAB label is not complete and the trimethylamine moiety remains attached to the peptide. This is especially a problem for peptides with multiple tags. When MS/MS results are obtained for multiple isotopic forms of a particular peptide, it is easy to spot these incompletely degraded TMAB-labeled fragments; they are the ones that differ in m/z between the spectra for each of the isotopic forms.

Due to the partial breakdown of the TMAB labels in CID and MS/MS, Mascot scores are often lower than those considered to be acceptable for conclusive identification of a peptide. However, manual reinterpretation of these spectra is relatively straightforward, and if the majority of the peaks can be assigned (taking into account the partial breakdown of the TMAB labels, as well as other possible ions such as *a* ions, loss of water or ammonia from *b* or *y* ions, and internal fragments) then it is highly likely that the identification is correct.

The majority of the peptides identified in the present study are previously identified secretory pathway peptides. Many result from cleavages at known processing sites, often pairs of basic amino acids, by an endopeptidase followed by a carboxypeptidase. Some of the peptides detected in the present study have undergone additional post-translational modifications, including phosphorylation of Ser, oxidation of Met, and amidation of the C-terminus (by oxidation of a Gly on the C-terminus of the processing intermediate). One of the peptides, a 1296.68 Da fragment of the peptide named little SAAS, appears in a form 36 Da lighter than the theoretical peptide. The loss of 36 occurs on the C-terminal 5 amino acids (sequence VETST) based on analysis of the *b* and *y* ions. This peptide presumably has undergone loss of

2 water molecules; a peptide 18 Da lighter than the theoretical peptide was detected in some LC/MS analyses, and although this other peptide has not been sequenced, it likely reflects the SAAS-derived peptide with the loss of one water molecule. In addition to the peptides derived from secretory pathway proteins, a number of peptides derived from cellular proteins (i.e. cytoplasmic, nuclear, lysosomal, mitochondrial) were detected in the present study. Some of these peptides have been reported in previous peptidomics studies. Interestingly, one of these peptides, a fragment of ubiquinol cytochrome C reductase contains a +25 Da modification on the N-terminal Cys residue; this corresponds to the mass difference of a cyano group.

Conclusions

The major finding of the present study is that 4 isotopic forms of the TMAB labels can be readily synthesized, and that the labeled peptides co-elute from reverse phase HPLC columns and show the expected 1:1:1:1 ratio. The development of 4 labels allows for a large variety of studies that are either not possible, or are less efficient than studies with only 2 isotopic forms of the labels. For example, with 4 labels it is possible to include duplicates of control and treated groups in same study, thus providing a reflection on the variation between duplicates in a single LC/MS run. Alternatively, it is possible to compare several different treatment groups in the same experiment. Although the applications described in this study involve peptidomics analyses, these TMAB labels should be applicable to proteomic studies much like the iTRAQ reagents. But, because the TMAB labels do not require MS/MS for the quantitation, it is possible to obtain quantitative data on any peptide detected in the MS mode. Thus, these TMAB labels should have broad applications for a variety of projects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants DA-04494 and DK-51271 to L.D.F. Thanks to Iryna Berezniuk for providing the mouse brain extract used in the present study, and to Dries Cardoene for testing the ‘biochemist’ version of the synthetic scheme and offering comments. Mass spectrometry was performed in the Laboratory for Macromolecular Analysis and Proteomics of the Albert Einstein College of Medicine, Dr. Ruth Angeletti, director.

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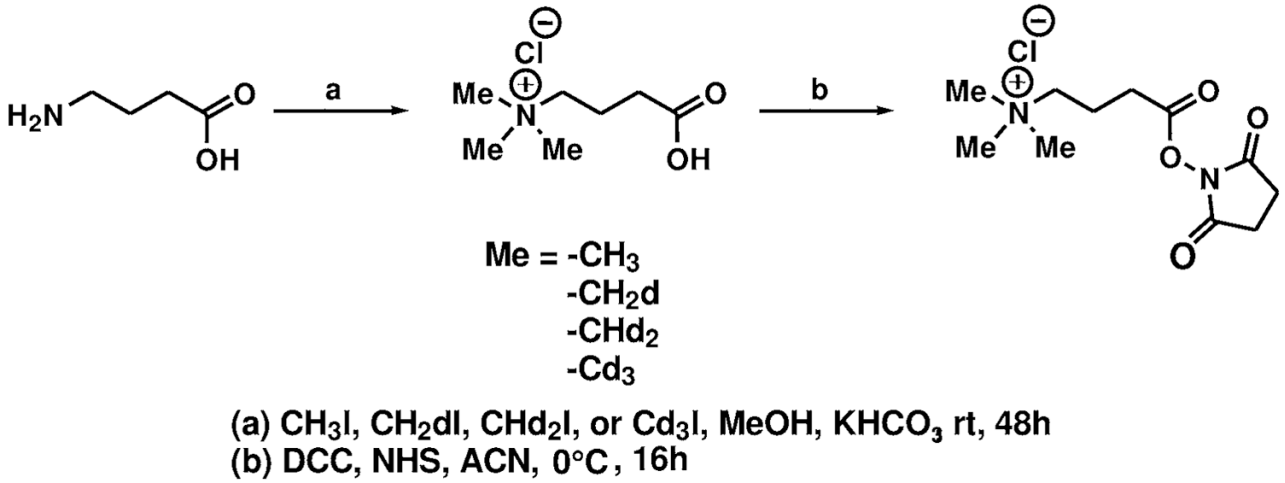


Figure 1.
Synthetic scheme of TMAB labels. Step (a) is the alkylation of gamma-amino butyric acid (GABA) using methyl iodide or one of its deuterated isotopes. This generates a trimethylammonium iodide salt, which is washed with chloroform, and then converted to the chloride salt by acidification with HCl. Step (b) is the activation of the acid by formation of the *N*-hydroxysuccinimide (NHS) ester using dicyclohexylcarbodiimide (DCC).

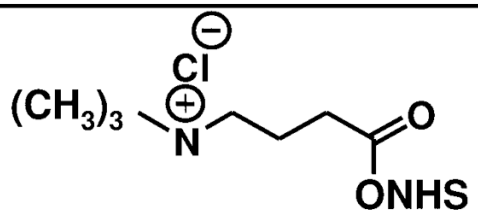
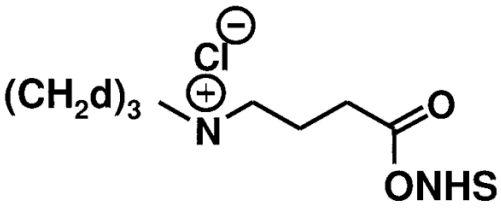
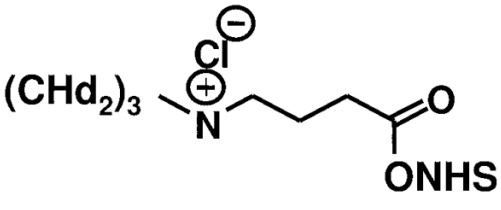
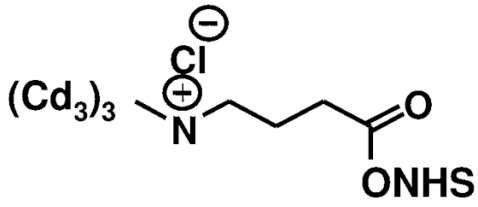
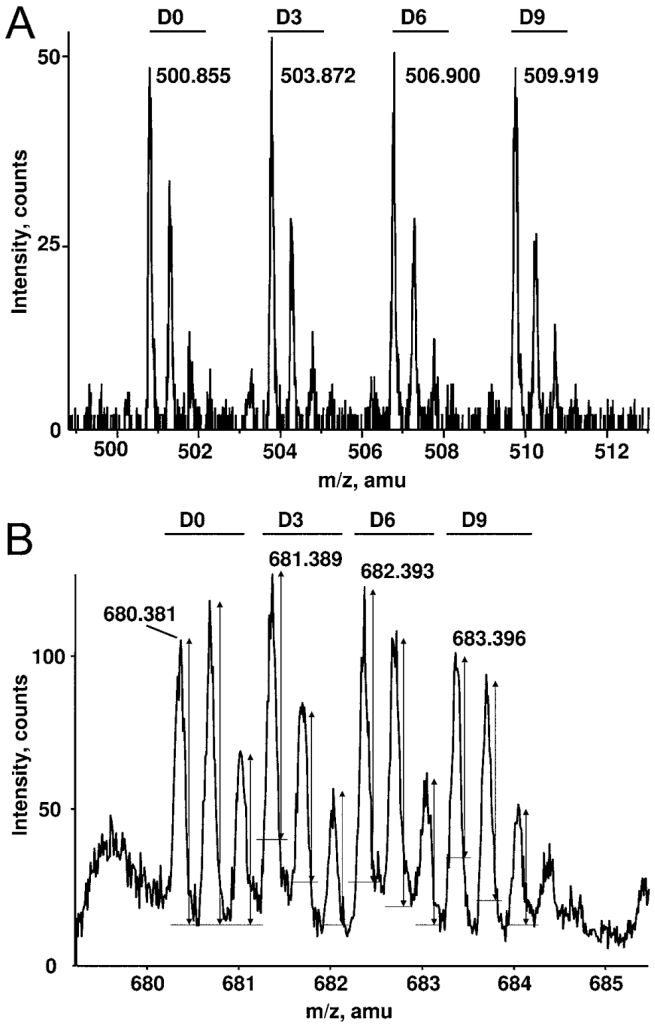
Name	Net Mass	Structure
D0-TMAB -NHS	129	
D3-TMAB -NHS	132	
D6-TMAB -NHS	135	
D9-TMAB -NHS	138	

Figure 2.
Chemical structures of the TMAB labels. The net masses, in Daltons, are of the carbon chain, the carbonyl oxygen, and the trimethyl ammonium group, minus the N-hydroxy succinimide group and the chloride ion (i.e. the mass difference in a peptide after incorporating one of these compounds on a free N-terminal amine).



Sequence: LLDEGHYPVRESPIDTA
Elemental Compositon: C84 H131 N22 O29

Isotope Number	mass	%Total	% Maximum
0	1911.94468	33.11	95.08
1	1912.94762	34.82	100.00
2	1913.95039	20.09	57.70
3	1914.95307	8.29	23.81
4	1915.95569	2.72	7.81
5	1916.95826	0.75	2.15

Figure 3.

MS spectra of peptides labeled with the isotopic TMAB tags and analyzed by LC/MS. A: Example of a peptide that displays baseline resolution between each isotopic tag. The peptide was identified by MS/MS analysis as neuromedin N (KIPYIL) which incorporated 2 isotopic tags. The indicated monoisotopic m/z values represent the 2+ ion. B: Example of a peptide with relatively smaller mass differences between isotopic peaks and which therefore shows substantial peak overlap. This peptide was identified by MS/MS analysis as a chromogranin B-derived peptide corresponding to residues 438–454 (LLDEGHYPVRESPIDTA). The indicated monoisotopic m/z values represent the 3+ ion that incorporated 1 isotopic tag (on the N-terminus) and protons on the His and Arg residues. To determine the relative abundance of

each isotopic form, it is necessary to subtract the contribution from the ^{13}C-containing peptides of the lower m/z values. For this, Protein Prospector was used to determine the natural isotope distribution of this peptide and the baseline of each peak was then adjusted to account for the contribution from the ^{13}C-containing isotope of the lower m/z peak, as shown in panel B.

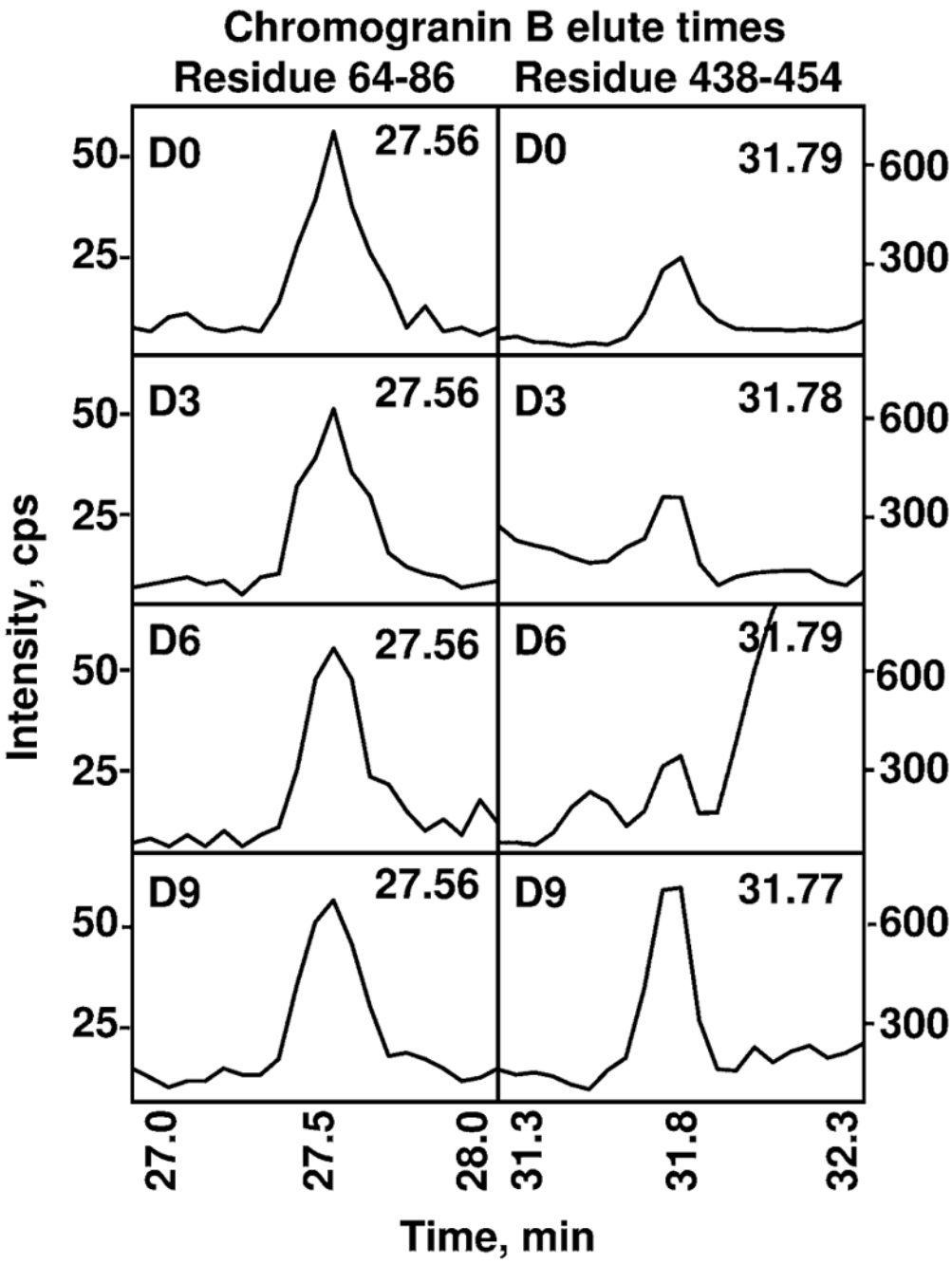


Figure 4.

The 4 isotopically-labeled forms of each peptide co-elute from a reverse phase LC column. Left panels represent chromogranin B (64–86) and the right panels represent chromogranin B (438–454). The relative intensity for m/z values representing each isotopic form are plotted versus elute time in minutes.

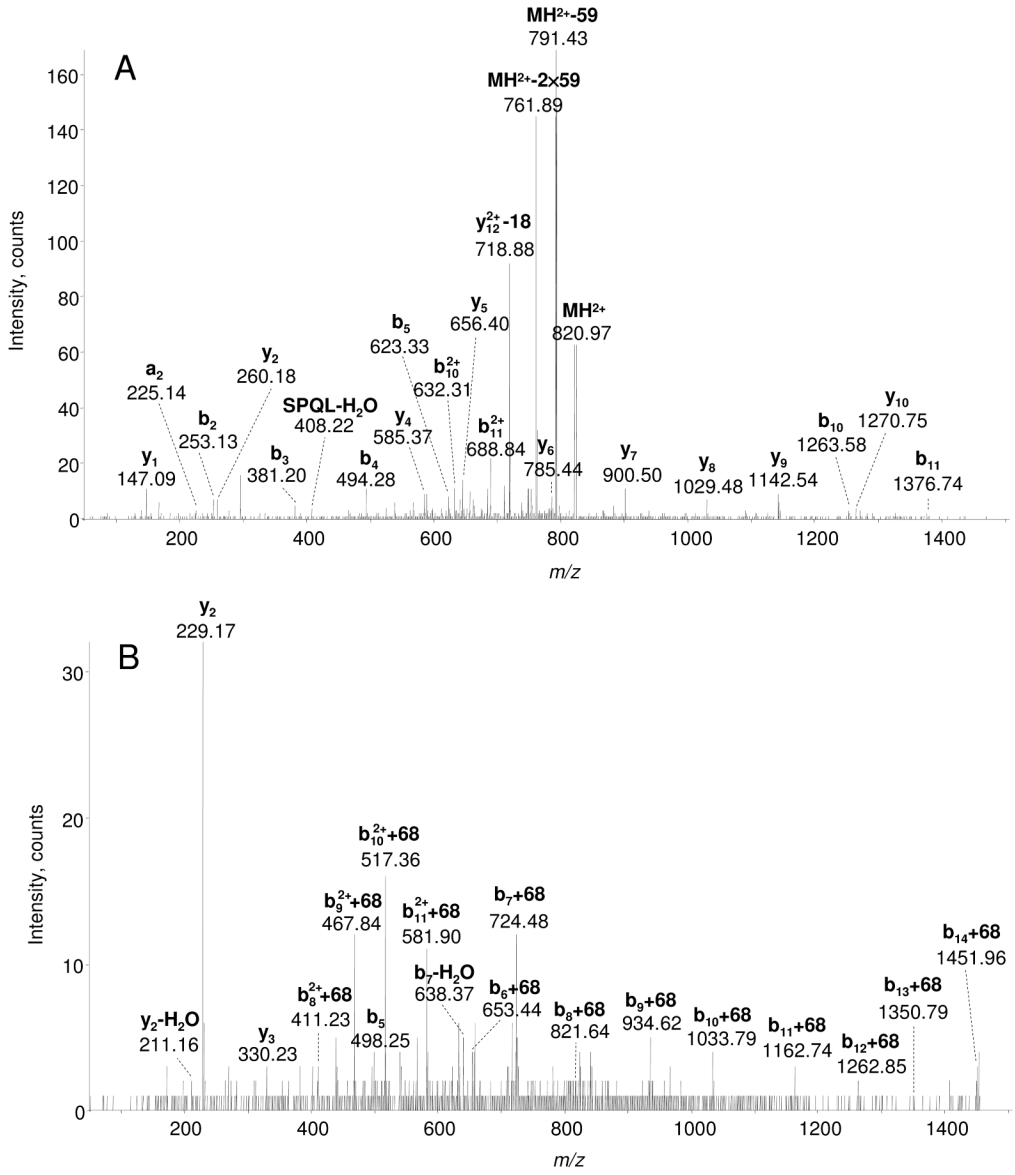


Figure 5.

Representative MS/MS spectra. (A) MS/MS of the proenkephalin-derived peptide SPQLEDEAKELQ, which incorporated two D0-TMAB tags. Note the complete loss of trimethylamine from the observed *a*, *b*, and *y* series ions. The spectrum also shows an MH^{2+} peak for the intact peptide, and stronger peaks corresponding to the loss of 59 and 118 Da (i.e. 2×59); these represent neutral loss of one and two trimethylamines from the parent ion. (B) MS/MS of D9-TMAB-labeled proSAAS-derived peptide SLSAASAPLVETSTPL. For this peptide, many of the trimethylamine groups are not lost in the collision-induced-dissociation, so most of the *b* series ions are indicated as +68 (for D9-TMAB-labeled peptide). In this example, partial breakdown of tags leads to inconclusive results upon Mascot database searches, so manual interpretation is required.

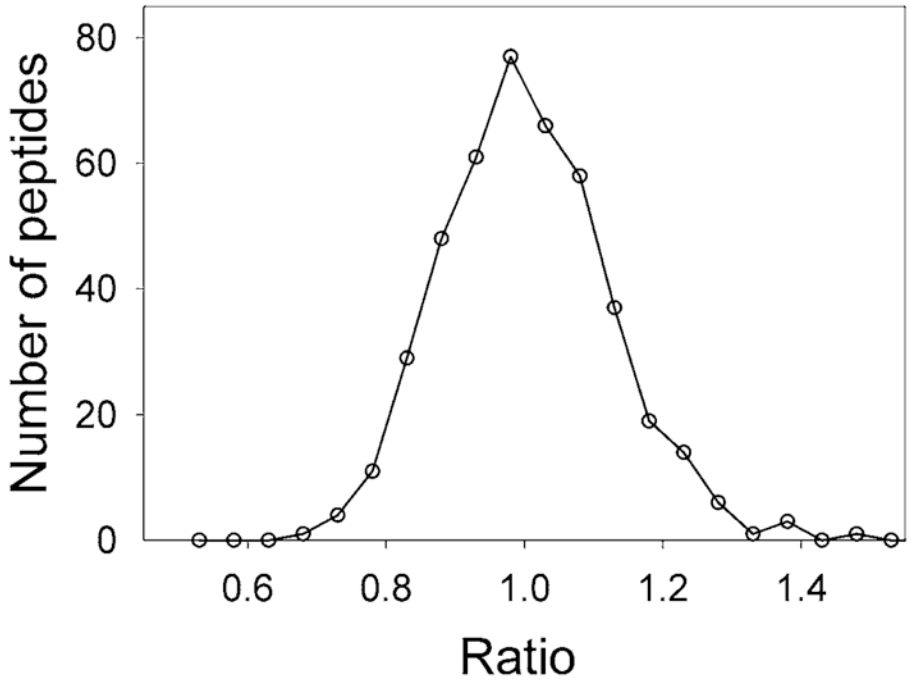


Figure 6.

Distribution of peak ratios of the 107 peptides detected in the analysis of mouse brain. The peak-to-peak ratios of each peptide fragment were measured and the individual peak intensity for each isotopically-labeled form was compared to the average peak intensity of the other 3 isotopically-labeled forms of the peptide (see Table 1; columns 8–11). Then, the number of peptides for each ratio was plotted versus the observed ratio. The theoretical ratio is 1.00, and nearly all of the isotopically-labeled forms of each peptide showed ratios within 20% of this theoretical ratio.

Table 1

Peptides identified in analysis of whole mouse brain with four isotopic TMAB labels.

Protein	Sequence	Obs M	Thr M	Diff ppm	z	#T	D0/others	D3/others	D6/others	D9/others	Stdev
Actin (beta or gamma)	ALPHAILRL	1002.63	1002.64	−9	3	1	1.07	0.88	1.00	1.06	0.08
Cathepsin D	YTVFDRDNNRVGFANAVV	2056.05	2056.01	19	3	1	1.07	0.98	1.03	0.93	0.05
Cerebellin 1 precursor	GSAKVAFSAIRSTN	1407.75	1407.75	3	3	2	1.00	1.00	0.91	1.10	0.07
Cerebellin 1 precursor	SGSAKVAFSAIRSTN	1494.78	1494.78	3	3	2	1.10	0.91	1.19	0.82	0.15
Cerebellin 1 precursor	GSAKVAFSAIRSTNH	1544.78	1544.81	−15	4	2	0.93	0.95	1.03	1.09	0.06
Cerebellin 1 precursor	SGSAKVAFSAIRSTNH	1631.86	1631.84	14	4	2	1.04	0.76	1.15	1.06	0.15
Cerebellin 2 precursor	GSAKVAFSATRSTN	1395.71	1395.71	0	3	2	1.10	0.96	0.86	1.10	0.10
Cerebellin 4 precursor	SKVAFSAVRSTN	1265.67	1265.67	−3	3	2	1.09	0.97	0.84	1.11	0.11
Cerebellin 4 precursor	ANSKVAFSAVRSTN	1450.76	1450.75	4	3	2	1.15	0.93	1.00	0.94	0.09
Cerebellin 4 precursor	AANSKVAFSAVRSTN	1521.82	1521.79	17	3	2	1.16	0.91	1.05	0.90	0.11
Chromogranin A	AYGFRDPGPQL	1219.62	1219.60	16	2	1	0.99	0.96		1.05	0.03
Chromogranin A	WSRMDQLAKELTAE	1676.84	1676.82	11	3	2	0.83	1.04	0.95	1.20	0.13
Chromogranin A	LEGEDDPDRSMKLSF	1737.81	1737.79	13	3	2	0.86	1.13	0.87	1.16	0.14
Chromogranin A	LEGEDDPDRSMKLSFRT	1994.95	1994.94	9	4	2	1.06	0.96	1.00	0.98	0.04
Chromogranin B	LLDEGHYPV	1041.51	1041.52	−7	2	1	1.02	0.91	1.01	1.07	0.06
Chromogranin B	SFARAPQLDL	1116.59	1116.59	−3	2	1	1.06	0.94	0.90	1.10	0.08
Chromogranin B	QYDGVAELDQLLHY	1662.82	1662.79	16	2	1	1.06	0.89	0.99	1.06	0.07
Chromogranin B	LLDEGHYPVRESPIDT	1839.97	1839.90	19	3	1	1.18	0.79	1.04	1.01	0.14
Chromogranin B	LLDEGHYPVRESPIDTA	1910.97	1910.93	18	3	1	1.27	0.87	1.09	0.81	0.18
Chromogranin B	PSPKESKEADVATVRLGE	1912.00	1911.99	8	4	3	1.00	0.79	1.02	1.21	0.15
Chromogranin B	GLQYRGRGSEEDRAPRPR	2099.08	2099.07	2	5,6	1	1.05	0.85	0.94	1.19	0.13
Chromogranin B	GLQYRGRG-phosphoSEEDRAPRPR	2179.05	2179.07	−12	5	1	1.01	1.03	0.92	1.03	0.05
Chromogranin B	SGKEVKGEEKGENQNSKFEVRLL	2604.37	2604.35	6	5,6	5	0.96	0.91	0.86	1.31	0.18
Clathrin light chain A	SVLISLKQAPLVH	1403.85	1403.85	−3	3	2	0.98	0.98	1.02	1.02	0.02
Elongation factor 1 beta 2	GFGDLKTPAGLQVL	1414.76	1414.78	−13	2	2	1.02	0.88	1.15	0.96	0.10
Fibrinogen, alpha	TDTEDKGEFLSEGGGVR	1795.84	1795.82	8	3	2	1.12	0.96	0.90	1.03	0.08
Hemoglobin alpha	VLSGEDKSNIKA	1259.67	1259.67	−2	3	3	0.88	0.88	1.13	1.13	0.13
Macrophage migration inhibitory factor	AQATGKPAQYIAVHVVPDQL	2105.14	2105.13	8	3	2	1.02	0.88	1.27	0.86	0.16
Mast cell protease -3 or -4	IIGGVESRPHSRPY	1566.83	1566.83	0	4	1	1.08	0.87	1.08	0.97	0.09
Myelin basic protein	Ac-ASQKRPSQRSKYLA	1660.90	1660.91	−3	4	2	0.87	1.10	0.97	1.06	0.09
Myelin basic protein	Ac-ASQKRPSQRSKYLAT	1761.95	1761.96	−4	4	2	0.84	0.93	1.10	1.15	0.13
Myelin basic protein	Ac-ASQKRPSQRSKYLATA	1833.00	1833.00	4	4	2	1.03	0.91	0.85	1.24	0.15
Myelin basic protein	Ac-ASQKRPSQRSKYLATAS	1920.01	1920.03	−9	4	2	1.25	0.90	0.80	1.08	0.17
Peptidylprolyl isomerase A	EDENFILKH	1143.56	1143.56	4	3	2	0.91	0.90	0.98	1.22	0.13
Peptidylprolyl isomerase A	KTEWLDGKHVVF	1457.74	1457.77	−18	4	3	1.08	0.87	0.98	1.08	0.09
Peptidylprolyl isomerase A	ADKVPKTAENFRAL	1558.85	1558.85	1	4	3	1.13	1.00	0.88	0.99	0.09
Peptidylprolyl isomerase A	EDENFILKHTGPGILSM	1899.95	1899.95	1	3	2	1.04	0.85	1.00	1.12	0.10
Peptidylprolyl isomerase A	ELFADKVPKTAENFRAL	1948.05	1948.04	4	4	3	0.99	0.86	1.05	1.11	0.09
Peroxiredoxin V	APIKVGDAIPSVEVF	1540.84	1540.85	−9	2	2	0.90	1.36	0.82	0.96	0.21
Phosphatidylethanolamine-binding protein 1	DDYVPKLYEQLSGK	1653.78	1653.83	−25	3	3	1.06	0.93	0.96	1.05	0.06
Phosphatidylethanolamine-binding protein 1	AGVTVDELGKVLTPTQV	1725.97	1725.95	11	3	2	1.07	0.96	1.06	0.92	0.06
Procholecystokinin	AVLRTDGEPRARLGA	1580.88	1580.88	3	4	1	0.77	1.26	1.01		0.20
Procholecystokinin	AVLRTDGEPRARLGAL	1693.96	1693.97	−9	4	1	0.97	1.09	1.08	0.87	0.09
Procholecystokinin	AVLRTDGEPRARLGALL	1807.07	1807.04	17	4	1	0.83	1.12	0.94	1.12	0.12
Procholecystokinin	AVLRTDGEPRARLGALLA	1878.08	1878.08	2	4	1	1.11	0.91	0.92	1.07	0.09
Procholecystokinin	APSGRMSVLKNLQSLDPSHRISD	2507.33	2507.29	14	5	2	1.12	0.86	1.15	0.89	0.13
Procholecystokinin	KAPSGRMSVLKNLQSLDPSHRISD	2635.41	2635.39	8	6	3	1.20	0.82	1.01	1.00	0.14
Procorticotropin releasing factor	GAEDALGGHQGALERE	1608.79	1608.75	8	3	1	1.01	0.87	1.01	1.12	0.09
Prodynorphin	YGGFLRRI	980.56	980.56	3	3	1	1.08	0.82	0.86	1.27	0.18

Protein	Sequence	Obs M	Thr M	Diff ppmz	#T	D0/ others	D3/ others	D6/ others	D9/ others	Stdev
Prodynorphin	PKLKWDNQ	1027.54	1027.55	−53	3	0.77	0.95	0.86	1.49	0.28
Prodynorphin	YGGFLRKYPK	1227.67	1227.68	−84	3	1.00	0.95	0.81	1.27	0.17
Proenkephalin	YGGFL	555.27	555.27	−21	1	1.04	0.95	0.89	1.12	0.09
Proenkephalin	YGGFM	573.24	573.23	221	1	0.97	0.92	1.00	1.11	0.07
Proenkephalin	YGGFMRF	876.39	876.40	02	1	1.10	0.95	0.91	1.05	0.08
Proenkephalin	YGGF-Mox-RF	892.40	892.40	−22	1	1.08	0.87	0.93	1.14	0.11
Proenkephalin	YGGFMRSL	929.44	929.45	−82	1	1.18	1.02	0.89	0.92	0.11
Proenkephalin	SPQLEDEAKEL	1257.63	1257.61	−42	2	0.86	0.86	1.11	1.20	0.15
Proenkephalin	SPQLEDEAKELQ	1385.67	1385.67	42,3	2	1.02	0.93	0.93	1.12	0.08
Proenkephalin	VGRPEWWMDYQ	1465.67	1465.65	172	1	1.19	0.99	0.88	0.96	0.11
Proenkephalin	MDELYPMEPEEEANGGEILA	2235.95	2235.96	−32	1	1.10	0.92	1.00	0.98	0.07
Proenkephalin	FAESLPSDEEGENYSKEVPEIE	2497.14	2497.10	183	2	1.10	0.90	0.95	1.05	0.08
Proenkephalin	FAESLP-phosphoSDEEGENYSKEVPEIE	2577.10	2577.06	173	2	1.03	0.98	0.99	1.00	0.02
Prohormone convertase 2	SLQSILRKN	1057.62	1057.62	−73	2	0.97	0.84	1.00	1.21	0.13
Prohormone convertase 2	IKMALQQEGFD	1278.63	1278.64	−32	2	1.18	1.10	0.76	0.99	0.16
Promelanin concentrating hormone	EIGDEENSAKFPI-amide	1446.71	1446.68	183	2	1.06	0.87	0.92	1.16	0.12
Proneurotensin	KIPYIL	745.47	745.47	−72	2	1.16	0.93	0.94	0.97	0.09
Proneurotensin	pELYENKPRRPYIL	1671.92	1671.91	73	1	1.04	0.91	0.92	1.13	0.09
Proopiomelanocortin	Ac-SYSMEHFRWGKPV-amide	1663.80	1663.79	53	1	1.08	0.73	1.01	1.21	0.18
Proopiomelanocortin	AEEEAVWGDGSPEPSPRE-amide	1939.88	1939.86	123	1	0.98	0.85	0.96	1.23	0.14
Proopiomelanocortin	RPVKVYPNVAENESAEAFPLEF	2505.31	2505.26	213	2	1.12	0.89	0.97	1.03	0.08
Proopiomelanocortin	RPVKVYPNVAENE-phosphoSAEAFPLEF	2585.24	2585.23	63	2	0.81	1.16	1.02	1.02	0.12
Propeptidyl-amidating-monooxygenase	FRSPLSVF	951.52	951.53	−92	1	1.08	0.94	0.92	1.06	0.07
ProSAAS	SLSAASAPLVETST -36 Da (from VETST)	1296.66	1296.68	−132	1	0.92	0.82	1.08	1.20	0.15
ProSAAS	SLSAASAPLVETSTPL	1542.83	1542.81	132	1	1.14	0.99	0.95	0.93	0.08
ProSAAS	LENPSPQAPARRLLPP	1755.00	1754.98	113	1	1.08	0.85	0.97	1.11	0.10
ProSAAS	SLSAASAPLVETSTPLRL	1812.01	1812.01	42,3	1	1.06	0.92	0.94	1.08	0.07
ProSAAS	SVDQDLGPEVPPENVLGALL	2061.10	2061.06	162	1	1.04	0.85	1.03	1.09	0.09
ProSAAS	SVDQDLGPEVPPENVLGALLRV	2316.23	2316.23	12,3	1	1.15	0.85	1.03	0.98	0.11
ProSAAS	ARPVKEPRSLSAASAPLVETSTPLRL	2745.59	2745.55	145	2	1.01	0.89	0.96	1.15	0.10
Prosomatostatin	SANSNPAMAPRE	1243.58	1243.56	172	1	0.94	1.14	1.01	0.91	0.09
Protachykinin A	RPKPQQFFGLM-amide	1346.74	1346.74	43	2	0.92	0.93	1.07	1.09	0.08
Protachykinin A	ALNSVAYERSAMQNYE	1844.88	1844.84	203	1	1.13	0.91	0.81	1.17	0.15
Protachykinin A	DADSSVEKQVALLKALYGHGQISH	2565.34	2565.32	84,5	3	1.36	0.84	1.00	0.84	0.21
Prothyrotropin Releasing Hormone	GDLGEVGAWRPH	1292.68	1292.63	393	1	1.05	0.90	1.03	1.01	0.06
Prothyrotropin Releasing Hormone	EEKEEDVEAEERGDLGEVGAWRPH	2765.30	2765.25	165	2	1.08	0.95	1.01	0.97	0.05
Prothyrotropin Releasing Hormone	LLEAAQEEGAVTPDLPGLEKVQVRPE	2787.50	2787.48	73,4	2	0.91	0.98	1.06	1.06	0.06
ProVasoactive Intestinal Peptide	ISSSISEDPVPI	1242.67	1242.63	312	1	1.03	1.13	0.84	1.01	0.10
Provasopressin	VQLAGTRESVDSAKPRVY	1975.06	1975.05	84	2	1.24	0.82	0.97	1.00	0.15
Pyruvate kinase isozymes M1/M2	FTNTMRVVPVP	1259.67	1259.67	42	1	1.39	1.01	0.83	0.83	0.23
Ribosomal protein S21	AKADGIVSKNF	1148.63	1148.62	63	3	1.24	0.88	0.98	0.91	0.14
Secretogranin II	ESKDQLSEDASKVITYL	1924.97	1924.96	63	3	1.14	0.83	0.98	1.06	0.11
Secretogranin III	FPKPEGSQDKSLHN	1582.77	1582.77	−14	3	1.24	0.81	1.02	0.96	0.16
Synaptosomal-associated protein (Snap91)	SPSPTPATQSPKKPPAKDPLADLNIKDFL	3072.67	3072.65	55	5	1.03	0.77	0.88	1.37	0.22
Thioredoxin 1	VKLIESKEAFQEAL	1603.88	1603.88	−43	3	1.01	0.91	1.00	1.09	0.06
Thioredoxin 1	VKLIESKEAFQEALAA	1745.96	1745.96	33	3	1.22	1.00	0.97	0.83	0.14
Thymosin beta-10	Ac-ADKPDMGEIASFDKAKLKKTETQEKN TLPTKETIEQEKRSEIS	4936.44	4936.49	−99	8	1.06	0.88	1.02	1.05	0.07
Thymosin beta-4	Ac-SDKPDMAEIEKFDKSKLKKTETQEKN PLPSKETIEQEKQAGES	4963.52	4963.54	−47	9	0.97	0.86	1.00	1.18	0.12
Triose-phosphate isomerase	KVIADNVKDWSKVVL	1712.96	1712.98	−114	4	0.99	0.94	1.01	1.07	0.04
Tubulin beta	MREIVH(I/L)QA	1095.59	1095.59	03	1	1.14	0.84	1.05	0.98	0.11
Tubulin beta	MREIVH(I/L)QAGQ	1280.67	1280.67	03	1	1.17	0.89	0.95	1.01	0.10

Protein	Sequence	Obs M	Thr M	Diff ppm	z	#T	D0/others	D3/others	D6/others	D9/others	Stdev
Ubiquinol-cytochrome C reductase hinge protein	CVAHKLFKNLK +25 Da modification on Cys (cyano)	1324.74	1324.75	−7	3,4	3	1.22	0.81	0.74	1.31	0.25
Ubiquinol-cytochrome C reductase hinge protein	GDPKEEEEEELVDPLTTVREH	2450.17	2450.15	10	4	2	1.77	0.73	0.72	0.94	0.43
Vacuolar ATP synthase subunit 2	EVRPQVHPNYRVTV	1692.92	1692.91	2	4	1	1.26	0.81	1.07	0.89	0.17
VGF	NAPPEPVPPPRAAPAPTHV	1914.03	1914.01	7	3	1	1.17	1.05	0.73	1.08	0.17
VGF	KKNAPPEPVPPPRAAPAPTHV	2170.25	2170.20	23	5	3	1.04	0.96	0.89	1.12	0.09
Voltage-dependent anion channel protein 1	AGGHKLGLGLEFQA	1396.75	1396.75	5	3	2	1.07	0.93	0.96	1.04	0.06
Voltage-dependent anion channel protein 2	AGGHKLGLALELEA	1377.78	1377.76	11	3	2	0.99	0.92	0.89	1.21	0.12

Abbreviations: Obs M, observed mass of the unprotonated peptide after subtraction of the mass of the isotopic tags (monoisotopic, except for the 4.9 kDa Thymosin peptides, which are average masses); Thr M, theoretical mass of the unprotonated and un-tagged peptide (monoisotopic for all peptides below 4.9 kDa); Diff ppm, difference between observed and theoretical masses (in parts per million); z, charge; #T, number of isotopic tags incorporated into the peptide; D0/others, the peak height of the D0 form (including the monoisotopic and first two ^{13}C-containing peaks) relative to the peak heights of the other TMAB-labeled peptides; D3/others, as above, but for the D3 form relative to the average peak heights of the others; D6/others, as above, but for the D6 form; D9/others, as above, but for the D9 form; Stdev, standard deviation of the variation in peak height.