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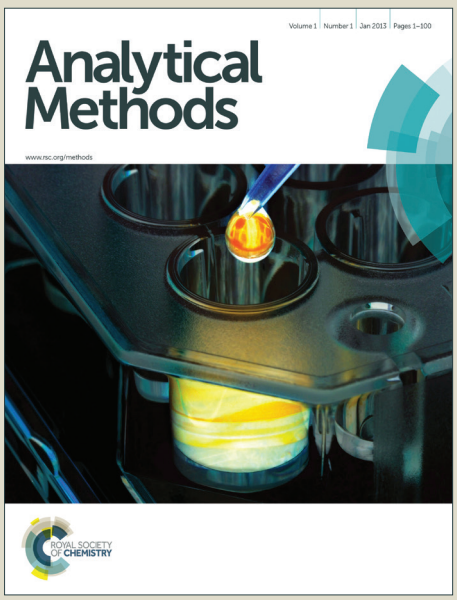

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A Novel Method Using ^{18}O and Metal Isobaric Labeling Combined with Multiple Reaction Monitoring Mass Spectrometry for the Absolute Quantification of a Target Proteome

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Abstract

Although many isobaric labeling-based targeted quantification methods by mass spectrometry (MS) have been developed and improved to meet proteome research requirements, some disadvantages limit their applications. For instance, some methods require expensive reagents and others have slight differences in chromatographic retention times due to the deuterium isotope effect when dimethylation labeling was performed with binary isotopic reagents of formaldehyde (d0, d2). Therefore, a novel absolute quantitation method for a target proteome using ^{18}O/metal isobaric labeling of a quantification concatamer protein (QconCAT) as internal standards combined with multiple reaction monitoring mass spectrometry (MRM MS) was established. The experimental results showed that the peptides can be very efficiently labeled with both ^{18}O+Ho and ^{16}O+Tm, and that the labeled peptides remained stable for 7 days. In addition, the labeled peptides with ^{18}O+Ho and ^{16}O+Tm displayed the nearly identical behaviors in high-performance liquid chromatographic and mass spectrometric analyses. Furthermore, using a tryptic digestion of a complex biological sample as a matrix, the linear range spanned two orders of magnitude, the relative errors of standard samples were less than 20% and the LOQ was 0.7 fmol/μL (RSD<10%) when the method was evaluated with two transitions of a randomly selected peptide (TATVDDIDNIYR) from a protein of a Thermoanaerobacter tengcongensis sample as a model. For further evaluation of our method, the absolute quantitation of 20 drug metabolic enzymes in 5 human liver microsomes was performed using the established method, and the results are consistent with those reported in the literature. These results suggest that this method using ^{18}O/metal isobaric labeling combined with MRM MS can be used as a new approach for the absolute quantitation of a targeted proteome in complex biological samples.

Key words: quantitation proteomics; ^{18}O labeling; metal labeling; QconCAT; MRM MS

1. Introduction

With the comprehensive development of proteomic technologies, the focus of proteomics research has gradually shifted from qualitative proteome profiles, post-translation modification maps and interaction networks to quantitative proteomics, which means that some key proteins in pathways, diagnostic markers and drug targets will be discovered by comparing the differential expression profiles of proteomes in biological and pathological states, and validated by targeted proteomics approaches. Although quantitative proteomics has recently become a rapidly growing area of research, the absolute quantitation of targeted proteomes faces immense challenges due to its requirements of high accuracy, high throughput and preparation of internal standard peptides.[1, 2] Therefore, it is urgent to develop absolute quantitation methods to meet proteomics research needs.

While many quantitation methods based on mass spectrometry (MS) have been developed for proteins or targeted proteomes, a novel technique for protein quantitation based on isobaric labeling combined with MS was only recently established. The basic principle for these new methods is complementary labeling at both the N-terminal and C-terminal of peptides digested from proteins; peptides with isobaric tags digested from different samples can pass the first filter (Q1) simultaneously and then fragment in a collision induced cell (Q2); finally, the product ions are captured in pairs in the third quadruple (Q3) in a triple quadruple mass spectrometer or similar instrument. According to the paired peak intensity ratios in a mass spectrum, the relative abundance of each peptide and its corresponding proteins between different samples can be obtained. If the concentrations of spiked peptides or proteins are known, the absolute amounts of endogenous peptides or proteins can be calculated. There are many merits to these methods, such as avoiding a wide ion selection window for the non-isobaric labeling of peptides, which leads to interfering ions and precursor ion fragmentation in the same spatial and temporal cells; a narrow ion selection window can increase accuracy and benefit ion selection in the first mass analyzer due to ion signal superposition from the same peptides produced from a mixture of different samples. Therefore, researchers hope to focus on new method development in this area. For example, Thiede et al. first developed the isobaric peptide termini labeling (IPTL) method, realizing the peptide quantitation with b and y ion pairs in MS/MS spectra.[3-5] In this method, the authors used two labeling methods: either 2-methoxy-4,5-dihydro-1*H*-imidazole (MDHI) or tetradeuterated

MDHI-*d*4 was selected first to label the C-terminal lysine residues, and tetradeuterated succinic anhydride (SA-*d*4) or SA was then used to label the N-termini of the same peptides from proteins digested by Lys-C, resulting in isobaric masses for peptide originating from the same protein but different samples. Zou et al.[6] and Zhang et al.[7] extended the selective dimethyl-isobaric labeling method, in which the reaction procedures were simplified and the side reactions reduced simultaneously. Based on these ideas mentioned above, more duplex-isobaric methods were developed. For example, Yang et al.[8] developed a protein quantification method based on an MS/MS spectrum using in vivo termini amino acid labeling (IVTAL), in which one population of cells was grown in a culture medium containing $^{13}C_6$-arginine ($^{13}C_6$-R) and the other was cultured in a different culture medium containing$^{13}C_6$-lysine ($^{13}C_6$-K).After the two populations of cells were mixed, the extracted protein mixture was digested first by Lys-N and then by Arg-C. The resulting peptides had two forms: either starting with K and ending with $13C_6$-R or starting with $13C_6$-K and ending with R for the same peptides from different biological samples. These labeled peptides had the same mass in MS spectra, and the b and y ions appeared in pairs in the MS/MS spectra after fragmentation. The authors also developed another duplex-isobaric labeling method by combining dimethyl labeling and a form of enzyme-catalytic ^{18}O labeling that had been applied in the absolute quantification of proteins in mouse liver samples, and accurate results were achieved.[9] In addition, for the quantification of targeted proteomes by MS using isobaric labeling, Aebersold et al. [10] developed the index-ion triggered MS2 ion quantification (iMSTIQ) method, in which the acquisition of full MS2 spectra for isotopically light target peptides is reproducibly triggered by isotopically heavy "index" peptides, and the target peptides' ion intensities are not involved in their selection; therefore, the sensitivity and reproducibility are enhanced compared with inclusion list-based mass spectrometry approaches.

Although many isobaric labeling-based targeted quantification methods using MS have been developed and improved, some disadvantages limit their applications. For instance, some methods need expensive reagents, and others have slight differences in chromatography retention times due to the proton effect. Therefore, new isobaric labeling methods need to be developed. Meares et al. [11] first used metals as tags to label peptides for their quantification by mass spectrometry and demonstrated that metal labeling is highly stable, efficient and low-cost, and can be used for multiplex tags for protein quantitation by various types of mass spectrometry (ICP-MS or

ESI-MS); as a result, this metal labeling method has since been applied in proteomic research.[12-23] Another labeling method, enzymatic ^{18}O labeling, has been widely applied in protein quantitation by MS owing to its low cost and simple operation procedures.[24-26] Based on the merits of metal tags and enzymatic ^{18}O labeling, we developed a novel absolute quantitation method for targeted proteomes using duplex isobaric labeling combined with MS. In this method, peptides are first labeled at N-termini by metal labeling and then at C-termini by enzymatic ^{18}O labeling, resulting in a single mass peak of one peptide from different samples in MS spectra but paired peaks in MS/MS spectra.

In addition, internal standard peptides were prepared by duplex isobaric labeling of a digested QconCAT protein and applied to allow absolute quantitation of targeted proteomes using multiple reaction monitoring mass spectrometry (MRM MS). There are several advantages of this method. First, internal standard peptides and targeted peptides behave similarly in chromatographic separation and mass spectrometric fragmentation, which can reduce experimental error. Second, the MS signals of internal standard peptides and targeted peptides can be superposed in MS1 spectra due to their identical molecular weights, which enhances the MS sensitivity. Third, mass peaks for both b ions and y ions in MS/MS spectra appear in pairs that can be used for accurate protein quantitation. Fourth, the combination of QconCATs and the MRM technique realizes the simultaneous quantitation of multiple targeted proteins. In order to demonstrate the robustness of the method, we applied it to the quantification of drug metabolic enzymes (including the cytochrome P450 oxidase (CYP450) family and the uridine 5'-diphosphoglucuronosyltransferase (UGT) family) in human liver microsomes. Such quantification is very important for safety evaluations and clinical applications of medicines because different medicines have obvious induction and inhibition effects on P450 enzymes, and these in turn lead to variations in drug content.[27, 28]There are four methods for determining the concentrations of drug metabolic enzymes. The first one is the probe drug method, and most substrates for probe drugs are isozymes. The advantages of this method are that genes and circumstances are taken into consideration when determining enzyme activity. However, there are several disadvantages, such as complicated operation procedures, the time-consuming process, the cross contamination of substrates, and the lack of probe drugs specific to a substrate.[29] The second method is Western blotting, which has the merits of being highly specific and highly sensitive but suffers from complicated operation

procedures and needs very specific antibodies with very high purity due to the high sequence homology of drug metabolic isozymes.[30] The third method is mRNA assay, which can be used to estimate the content of its corresponding protein. However, the amount of mRNA cannot accurately represent the amount of drug metabolic enzymes because the amount of a protein is regulated by many factors in its expression process.[30] The fourth quantitation method is stable isotopic dilution MS, which can be employed to determine the drug metabolic enzymes with high specificity, high sensitivity and high accuracy.[31] However, the proper selection of a signature peptide for a target protein and its accurate quantitation are important preconditions for the quantitation of drug metabolic enzymes. Therefore, we established a method based on ^{18}O/metal isobaric labeling combined with MRM MS for the absolute quantitation of drug metabolic enzymes in human liver microsomes. The experimental results indicated that our method can be successfully applied in the absolute quantitation of targeted proteins in complex biological samples with high accuracy and high throughput.

2. Experimental

2.1 Materials and Reagents

Normal human liver microsomes are purchased from Ruide Hepatic Disease Research Center (Shanghai, china). Human liver microsomes, extraction kits and ampicillin are from Shenggong Biotech Co. Ltd (Shanghai, China). Competent *Escherichia coli* cells BL21 and isopropyl thio-D-galactoside are bought from TaKaRa Corporation (Japan). Yeast extracts are obtained from OXOID Ltd. (Basingstoke, Hampshire, United Kingdom). Glutathione sepharose packing materials are bought from GE Corporation (USA). Reduced glutathione is bought from Xinjing Biotech Co., Ltd (Beijing, China). Synthesized peptides are from GL Biochem Co., Ltd (Shanghai, China). Phosphate buffer solution (PBS), myoglobin, α-cayno-4-hydroxycinnamic acid (CHCA, 95% purity), $HoCl_3$, $TmCl_3$, triethyl ammonium bicarbonate (TEAB), ammonium bicarbonate and ammonium acetate are purchased from Sigma Corporation (USA). Sequencing grade modified trypsin is bought from Promega Corporation (USA). 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid mono-(N-hydroxysuccinimide ester) (DOTA-NHS ester) is obtained from Macrocyclics Co., Ltd (USA). $H_2{}^{18}O$ (97% purity) is from Shanghai Research Institute of Chemical Industry (China). Rapigest™ SF is purchased from

Waters Co., Ltd (USA). Water used in our experiments is ultrapure water prepared by Milli-Q water filtration station (Millipore Corporation, USA). Other reagents are all analytical pure (Sinopharm Chemical Reagent Co., Ltd, China).

2.2 Preparation of a Concatemer of Q Peptides (QconCAT) of 22 Drug Metabolic Enzymes in Human Liver Microsomes

A QconCAT plasmid was constructed of quantotypic peptides of 22 drug metabolic enzymes in human liver microsomes (quantotypic peptides are listed in the appendix of reference[32]); the detailed preparation procedures are described in the Supporting Information.

2.3 Digestion and ^{18}O Labeling of the QconCAT Protein

The expressed QconCAT protein was divided into two parts. One part was lyophilized and added to ^{16}O water containing 50 mmol/L NH_4HCO_3 and 0.1% Rapigest™ SF to a final concentration of 1 μg/μL. Trypsin was added at mass ratio of trypsin : protein = 1:50 before a digestion was performed at 37℃ for 18 h. The other part was lyophilized and added to ^{18}O water containing 50 mmol/L NH_4HCO_3 and 0.1% Rapigest™ SF, and digested using the same conditions as above. To inactivate the trypsin, each sample was added to TCEP dissolved in $H_2{}^{18}O$ at a final concentration of 100 mmol/L and put in 100℃ water bath for 10 min for denaturation. Then, IAA dissolved in $H_2{}^{18}O$ was added to each sample at a final concentration of 200 mmol/L, and immediately placed in the dark for 1 h. After the reaction, both tryptic digests were separately desalted, dried and stored for the next use.

2.4 Metal Labeling of $^{18}O/^{16}O$ Labeled Peptides

Guanidination was first performed to block the ε-amino groups of lysines of the C-termini of quantotypic peptides because partial peptides generated with trypsin are terminated with lysine amino acid residues that can react with the metal labeling reagent. O-methylisourea dissolved in ammonia solution (pH 11) was separately added to ^{18}O or ^{16}O labeled peptides at a final concentration of 1.5 mmol/L. The reactions were assisted by microwave for 1 min, and then the peptide solutions were desalted. The guanidinylated peptides were redissolved in 0.2 mol/L TEAB buffer (pH 8.5) at a concentration of 1 μg/μL. Acetonitrile and a DOTA-NHS-ester acetonitrile solution were sequentially added to each sample at a volume ratio of water : acetonitrile = 1:3 and a mass ratio of peptide : DOTA-NHS-ester = 1:50; then, the reaction between the peptides and DOTA-NHS-ester proceeded at 20℃ for 1 h. The mixture was dried before 0.1 mol/L

ammonium acetate buffer was added to adjust the final pH to 5.6. Finally, $HoCl_3$ and $TmCl_3$ (dissolved in ammonium acetate) were added to ^{18}O or ^{16}O labeled peptide solutions at a molar ratio of DOTA-NHS-ester : MCl_3 = 1:2, respectively, and the peptide solutions were incubated in a 37˚C water bath for 1 h. The labeled peptides were desalted, dried and stored for the next use after the reactions were complete. Partial samples were collected to be analyzed by Fourier transform mass spectrometry (FT-ICR MS) to determine whether the labeling reactions were complete.

2.5 Protein Extraction, Digestion and Metal Labeling of Human Liver Microsomes

The concentrations of the protein extracts of 5 normal human liver microsomes stored at -80℃ were determined by Bradford assay, and 100 μg of each sample was lyophilized and added to 8 mol/L urea to dissolve the samples. Then, each sample was processed by ultrasonication for 10 min and centrifuged at 14,000 g, and DTT was added to each supernatant to a final concentration of 10 mmol/L before incubating in a 37℃ water bath for 4 h. IAA was added to each sample at a final concentration of 50 mmol/L, and the samples were placed in the dark for 1 h. A solution of NH_4HCO_3 was added to each sample until the urea concentration reached 1 mol/L, after which trypsin was added at a mass ratio of trypsin : protein = 1:50 and digested at 37℃ for 18 h. Each digest was labeled with Tm, desalted and stored at -80℃ for the next use. Partial samples were collected to be analyzed by FT-ICR MS to determine labeling completeness.

2.6 MALDI-TOF-MS Analysis

A total of 1μL of each sample and 1 μL of a 5 mg/mL CHCA solution (dissolved in 50% ACN containing 0.1% TFA) were sequentially deposited on a MALDI plate and dried at room temperature. A 4800 Proteomics Analyzer (AB SCIEX, USA) was used to analyze samples; each time, it was first calibrated by tryptic digested myoglobin with error ≤ 10 ppm. The MS1 acquisition mode was MS-2KV reflective mode (accelerating voltage: 20 KV, scanning range: m/z 600-4000, laser intensity: 3900, recorded spectra: 1500 laser shots accumulated), and the MS2 acquisition mode was MS/MS 2KV mode (laser intensity: 5000, recorded spectra: 2000 laser shots accumulated). Peaks in MS1 spectra with S/N ≥ 20 were selected for MS/MS analysis. All spectra were analyzed by GPS software (AB SCIEX, USA).

2.7 HPLC-ESI-LTQ-FT MS Analysis

An Agilent 1100 capillary high performance liquid chromatograph with an autosampler was coupled to an LTQ-FT MS (Thermo Fisher Scientific, USA) through an ESI source for sample

analysis. Chromatographic conditions: Loading amount: 20 μL. Flow rate: 300 nL/min. Mobile phase A: 2% ACN in 0.1% FA aqueous solution; mobile phase B: 80% ACN in 0.1% FA aqueous solution. Elution gradient: 0-90 min, 6%-40% B; 90-100 min, 40%-100% B; 100-110 min, 100% B; 110-120 min, 100% -6% B, 120-135 min, equilibrating the column with 6% B.

MS settings: Acquisition mode: positive mode; scan range: m/z 375.0 - 1500.0; acquisition time range: 0-110 min. When data dependent acquisition mode (DDA) was selected for tandem MS analysis, the top 10 ions with the strongest intensity in MS1 spectra were sequentially sent to collision induced dissociation (CID) with a collision energy of 35V followed by MS2 analysis. The dynamic exclusion time was set as 30s.

2.8 NanoLC-ESI4000 Qtrap MS Analysis

An Eksigent nanoLC-2D capillary high performance liquid chromatograph equipped with a Magic TM C18 column (10cm×75μm, i.d., 5μm) was coupled to a 4000 Qtrap MS (AB SCIEX, USA) for peptide quantification. Mobile phase A and mobile phase B were the same as above. Elution gradient: 0-2 min, 0-20% B; 2-40 min, 20%-50% B; 40-42 min, 50%-95% B; 42-55 min, 95% B; 55-60 min, 95% -100% B; 60-65 min, 100% B-100% A; 65-80 min, equilibrating the column with 100% A. The flow rate was set as 300 nL/min, and the loading amount was 20 μL.

MS settings: when MRM and positive mode were chosen for peptide quantification, the MS parameters were set as follows: nano-spray voltage: 2000 V; curtain gas: 20.00 L/min; nebulizer gas: 15.00 L/min; interface heater temperature: 150˚C; collision gas: medium; entrance potential: 10 V; collision cell exit potential: 15 V; dwell time: 100 ms; resolution Q1: 0.7 m/z; and all gas used: nitrogen.

2.9 NanoLC-ESIQtrap 5500 MS Analysis

An Eksigent nanoLC-2D capillary high performance liquid chromatograph equipped with a Magic TM C18 column (10cm×75μm, i.d., 5μm) was coupled to a Qtrap 5500 MS (AB SCIEX, USA) for peptide quantification. The chromatographic conditions were the same as above.

MS settings: when MRM and positive mode were chosen for protein quantification, the MS parameters were set as follows: ion spray voltage: 2000 V; curtain gas: 15.0 L/min; declustering potential: 80.0 V; entrance potential: 10.0 V; collision cell exit potential: 30.0 V; collision gas: advanced; dwell time: 30ms; resolution Q1: 0.7 m/z; and all gas used: nitrogen.

3. Results and Discussion

3.1 The Principle of Absolute Quantitation of a Targeted Proteome Based on ^{18}O/Metal Isobaric Labeling Combined with MRM MS

As shown in Figure 1, quantotypic peptides of targeted proteins that are to be absolutely quantified are selected to compose a corresponding QconCAT protein using the QconCAT technique.[33] A small amount of the QconCAT protein was first digested with trypsin, and then analyzed by MS to confirm its components. Finally, the QconCAT protein solution was divided into two identical aliquots. One is digested in $H_2{}^{18}O$ and the other in $H_2{}^{16}O$ with trypsin. ^{18}O labeling of the C-termini of peptides through the digestion in $H_2{}^{18}O$ resulted in a 4 Da mass shift compared with those peptides digested in $H_2{}^{16}O$ with the same enzyme. The ^{18}O labeled peptides were further labeled with the metal element Ho at the N-termini, which increased the molecular weight of each peptide by 548 Da. The ^{16}O labeled peptides were labeled by Tm at the N-termini, which increased the molecular weight of each peptide by 552 Da. The labeling of both termini of each quantotypic peptide of the QconCAT protein and endogenous proteins with complementary labels resulted in the same molecular weights because ^{18}O+Ho and ^{16}O+Tm have the same mass. The peptides from the same proteins of healthy and diseased samples appear as one peak in MS1spectra, but after fragmentation in CID, b ions or y ions appear in pairs with a 4 Da mass shift when singly charged. The ^{18}O+Ho labeled peptides as internal standards were mixed with a series of ^{16}O+Tm labeled internal standard solutions at different concentrations, and then, the digest of a real sample was added to each standard solution as a matrix; the results were used to plot calibration curves of the transitions of quantitypic peptides, with the mass peak area ratios of ^{16}O+Tm to ^{18}O+Ho labeled internal standards on the y-axis and the corresponding concentration ratios on the x-axis. The ^{18}O+Ho labeled standard peptides were also separately added to ^{16}O+Tm labeled samples, and the prepared samples were analyzed by HPLC-MRM MS. The concentration ratios of endogenous peptides to their corresponding standard peptides can be calculated based on the determined mass peak intensity ratios of paired endogenous to corresponding standard peptides from the established calibration curves. Finally, the concentrations of the endogenous peptides of a target protein could be determined using the known concentrations of the added standard peptides, and thus, the absolute amount of the targeted protein could be obtained from the stoichiometric relation of quantotypic peptides and the corresponding target protein. Similarly,

absolute quantities of a targeted proteome in a biological sample can be achieved using the established method.

3.2 Establishment of an Absolute Quantitation Method for Determining a Targeted Proteome Using ^{18}O/Metal Isobaric Labeling Combined with MRM MS

3.2.1 Examination of ^{18}O/Metal Isobaric Labeling Efficiency

An important premise of protein absolute quantitation is that both peptide termini of each quantotypic peptide of a protein are complementarily labeled with^{18}O and metal elements with high efficiency. Therefore, we first randomly selected and synthesized three peptides (VDDGNDLDAIR, TATVDDIDNIYR and AGSDVAAFTESAIAR) from the tryptic digested proteins of a *Thermoanaerobacter tengcongensis* sample (BGI, Beijing, China). These peptides were labeled with ^{18}O+Ho and ^{16}O+Tm, and analyzed by MALDI-TOF MS. As shown in Figure 2, the labeling efficiency of the peptide VDDGNDLDAIR with ^{18}O was 95.1%, and the labeling efficiencies with Tm and Ho were 99.9% and 99.8%. The labeling efficiency of the peptide TATVDDIDNIYR with ^{18}O was 94.5%, and both labeling efficiencies with Tm and Ho were 99.9%. The labeling efficiency of the peptide AGSDVAAFTEAIAR with ^{18}O was 95.3%, and both labeling efficiencies with Tm and Ho were 99.8%. These results showed that high labeling efficiencies of both peptide termini of each peptide with ^{18}O and metal elements can be achieved and can meet the demands of protein absolute quantitation by MRM MS.

3.2.2 Stability of ^{18}O/Metal Labeled Peptides

The accuracy of protein quantitation is directly affected by the stability of the ^{18}O/metal labeled peptides of a target protein. To examine the stability of labeled peptides, two peptides (TATVDDIDNIYR and AGSDVAAFTEAIAR)were selected and labeled separately with ^{18}O+Ho and ^{16}O+Tm. one portion of each sample was analyzed by MALDI-TOF MS, and the other portion was stored at -80℃ for 7 days and then analyzed by MALDI-TOF MS. As shown in Figure 3, the peak intensities of both labeled peptides before and after storage changed slightly, which means that no reversible exchange occurred. This result indicated that ^{18}O/metal labeled peptides are very stable which can guarantee an established protein quantitation method with high accuracy when the samples are quantitatively determined.

3.2.3 Fragmentation Behavior of ^{18}O/Metal Labeled Peptides in CID

To examine the fragmentation behavior of ^{18}O/metal labeled peptides in CID, one peptide

(VDDGNDLDAIR) was randomly selected and labeled with ^{18}O+Ho and ^{16}O+Tm, and these two labeled peptides were mixed (mole ratio, 1:1) and analyzed by MALDI-TOF MS/MS. As shown in Figure 4, after fragmentation of the labeled peptides in CID, the mass peaks of b ions and y ions appeared in pairs with a 4 Da mass shift, which indicated that the fragmentation mode of the labeled peptides was unchanged when the peptide was labeled with ^{18}O/metal. The intensity ratios of paired b ions and y ions were approximately 1:1, which suggested that no exchanges of oxygen isotopes or metal elements occurred between labeled peptides. This will ensure that the method has high accuracy when it is used for absolute quantitation of a target protein.

3.2.4 Selection of Transitions of Labeled Quantotypic Peptides and Their Chromatographic Retention Behavior

One peptide TATVDDIDNIYR of a protein of a *Thermoanaerobacter tengcongensis* sample was selected as a model to examine chromatographic retention behavior of the labeled quantotypic peptide when analyzed on a NanoLC-ESI-4000Qtrap MS. The transitions of the labeled peptide and optimal MS parameters were selected including tune mode and product ion scan mode. The labeled peptide solution was continuously injected into the electrospray source coupled to a 4000Qtrap MS by a syringe pump, and the intensities of different fragment ions were compared by tuning collision energy (CE) and declustering potential (DP). The transitions with high response factors were chosen for protein quantitation. In our method, the b ions and y ions with the highest intensities could be picked for protein quantitation because quantotypic peptides were complementarily labeled with^{18}O and metal elements on their N-termini and C-termini, respectively. Transitions such as the parent ion (TATVDDIDNIYR)/product ions b2 and y5 and optimal DP and CE values were shown in Table S1. ^{18}O+Ho labeled peptides and ^{16}O+Tm labeled peptides are mixed at a mole ratio of 1:1 and analyzed on NanoLC-ESI-4000Qtrap MS. As shown in Figure S2, the resulting selected transitions of both labeled peptides had nearly the same chromatographic retention time, which indicates that both labeled peptides can be co-eluted on the used capillary chromatographic column and can serve as internal standards for each other.

3.2.5 Evaluation of the Established Protein Quantification Method

A total of 20 μLof each of seven standard solutions was prepared by mixing a 75 fmol/mL ^{18}O+Ho labeled peptide solution as an internal standard with a series of ^{16}O+Tm labeled peptide solutions at concentrations of 0.7 fmol/μL, 1.5 fmol/μL, 3 fmol/μL, 6 fmol/μL, 12 fmol/μL, 50

fmol/μL, and 100 fmol/μL of ^{16}O+Tm labeled peptides as standards and 0.05 μg/μL of tryptic digested proteins of *Thermoanaerobacter tengcongensis* as a sample matrix. Each sample was analyzed twice by NanoLC-ESI-4000Qtrap MS. The calibration curves were plotted as shown in Figure S1 with the ratios of the average values of two XIC areas on the y axis and the concentration ratios of ^{16}O+Tm labeled peptides to a ^{18}O+Ho labeled peptide on the x axis. The linear ranges of transitions of b2 and y5 ions were within two orders of magnitude, namely, between 0.7 fmol/μL and 100 fmol/μL, and the linear correlation coefficient R2 was 0.999. The lowest concentrations of the calibration curves were determined as the limit of quantitation (LOQ) when RSD was less than 20% for concentration data points.[34] Therefore, in our quantitation method, the LOQ was 0.7 fmol/μL (RSD<10%) for these two transitions. In addition, the values measured by our method for the seven standard solutions were substituted into the calibration curves to calculate the determined concentration of each standard solution. The relative errors (RE%) were calculated and are shown in Table S2; the majority of the relative errors of standard samples were less than 10%, except for a few samples at the lowest concentration that had values less than 20%. These results suggest that the protein quantitation method based on ^{18}O/metal labeling combined with nano-LC-MRM MS has high accuracy, good reproducibility and a wide linear range and could meet the demands of micro-quantity determination of proteins in a complex biological sample by nano-LC MRM MS.

3.2.6 Applications of the Protein Quantitation Method of ^{18}O/Metal Isobaric Labeling Combined with MRM MS

To further demonstrate our method, we applied this method to the quantitative determination of a target proteome, such as various drug metabolic enzymes. Because the abundance of some drug metabolic enzymes in human liver microsomes is too low to be detected, our established method was transplanted onto a Qtrap 5500 MS with a higher sensitivity and scanning rate. A solution of the QconCAT protein composed of 57 quantotypic peptides of 22 drug metabolic enzymes was divided into two aliquots. One was digested in ^{18}O water and the other in ^{16}O water. The ^{18}O labeled peptides of the QconCAT protein were first analyzed by FT-MS to ensure their complete digestion and labeling. Then, one signature peptide with a known concentration of the GST as a part of the QconCAT (GST) fusion protein was used to quantify the ^{18}O labeled counterpart of the QconCAT (GST) fusion protein, and its concentration was calculated. Finally,

^{18}O labeled peptides of the QconCAT protein were further tagged with Ho as an internal standard. The other portion of the QconCAT protein and the extracts of 5 human liver microsomes were digested in ^{16}O water and labeled with Tm, and then the labeled peptide solutions were analyzed by FT-MS to ensure their complete digestion and labeling before protein quantitation was performed.

^{18}O+Ho labeled peptide solution with a concentration of 300 fmol/μL as an internal standard was mixed separately with a series of ^{16}O+Tm labeled peptide solution with concentrations of 2 fmol/μL, 5 fmol/μL, 10 fmol/μL, 20 fmol/μL, 40 fmol/μL, 80 fmol/μL, 150 fmol/μL, 300 fmol/μL, 600 fmol/μL, and 1200 fmol/μL as standards containing a 3 μg/μL unlabeled peptide mixture of the tryptic digested protein extract of a human liver microsome as a matrix. The prepared standard peptide solutions were analyzed on a Qtrap 5500 MRM MS. As shown in Table S3, when the mass signals were more than 10 times the background noise and in the quantitation range, the linear correlation coefficients (R2) of 39 quantotypic peptides of 20 drug metabolic enzymes were no less than 0.98, but the S/N values of 18 other quantotypic peptides were less than 10 and were not suitable for the establishment of working curves for protein quantitation.

Each sample containing 3 μg ^{16}O+Tm-labeled peptide mixture from the tryptic-digested protein extract of one of 5 human liver microsomes and with 300 fmol of ^{18}O+Ho-labeled peptides as internal standards was analyzed in MRM mode twice on a Qtrap 5500. Each concentration ratio of a transition of an endogenous peptide to the transition of the corresponding internal standard peptide was obtained by substituting its corresponding peak area ratio to its working curve, and the protein content was calculated through its quantotypic peptides as each concentration ratio times the concentration of the corresponding ^{18}O+Ho-labeled internal standard. As shown in Table1, except for 3 RSD values, most RSD values of the measured contents of 20 drug metabolic enzymes in 5 human liver microsomes were less than 20%, and except for UGT 1A1, the contents of the other 19 drug metabolic enzymes in the 5 samples showed no obvious differences. In addition, compared with the previously reported data shown in Table S4, most results are in good agreement with those from the literature except that the content of CYP 2C19 was higher than that reported in the literature.[35, 36] In summary, these results indicate that absolute protein contents with high accuracy in complex biological samples can be determined using our protein quantitation method based on ^{18}O/metal isobaric labeling combined with MRM MS.

4. Conclusions

A new method using ^{18}O/metal isobarically labeled quantotypic peptides digested from a QconCAT protein with trypsin as internal standards combined with MRM MS for high-throughput absolute quantitation of a targeted proteome has been established. The experimental results showed that the peptides can be highly efficiently labeled with both ^{18}O+Ho and ^{16}O+Tm and that the labeled peptides remained stable for 7 days. In addition, the peptides labeled with ^{18}O+Ho and ^{16}O+Tm showed nearly the same behavior in high-performance liquid chromatographic and mass spectrometric analyses. With a tryptic digest of a complex biological sample as a matrix, the linear ranges spanned two orders of magnitude, the relative errors of standard samples were less than 20% and the LOQ was 0.7 fmol/μL (RSD<10%) when the method was evaluated with two transitions of a randomly selected peptide (TATVDDIDNIYR) of a protein of a *Thermoanaerobacter tengcongensis* sample as a model. For further evaluation of our method, the absolute quantitation of 20 drug metabolic enzymes in 5 human liver microsomes was performed using the established method and the results are consistent with those reported in the literature. These results suggest that this method based on ^{18}O/metal isobaric labeling combined with MRM MS can be used as a new approach for the absolute quantitation of a targeted proteome in complex biological samples.

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Figure 1 A schematic diagram of absolute determination of targeted proteins based on ^{18}O/metal isobaric labeling combined with MRM MS

Figure 2 MALDI TOF MS spectra of ^{18}O/metal labeled peptides

a, unlabeled; b,^{18}O labeled; c, ^{18}O+Ho labeled; d, ^{16}O+Tm labeled

Figure 3 MALDI TOF MS spectra of ^{18}O/metal labeled peptides for examining its stability

a, ^{18}O+Ho labeled (before storage); b,^{18}O+Ho labeled (after storage);

c, Tm labeled (before storage); d, Tm labeled (after storage)

Figure 4 MALDI-TOF MS/MS spectra of a labeled peptide mixture of VDDGNDLDAIR (^{16}O+ Tm) and VDDGNDLDAIR (^{18}O+Ho) at molar ratio of 1:1

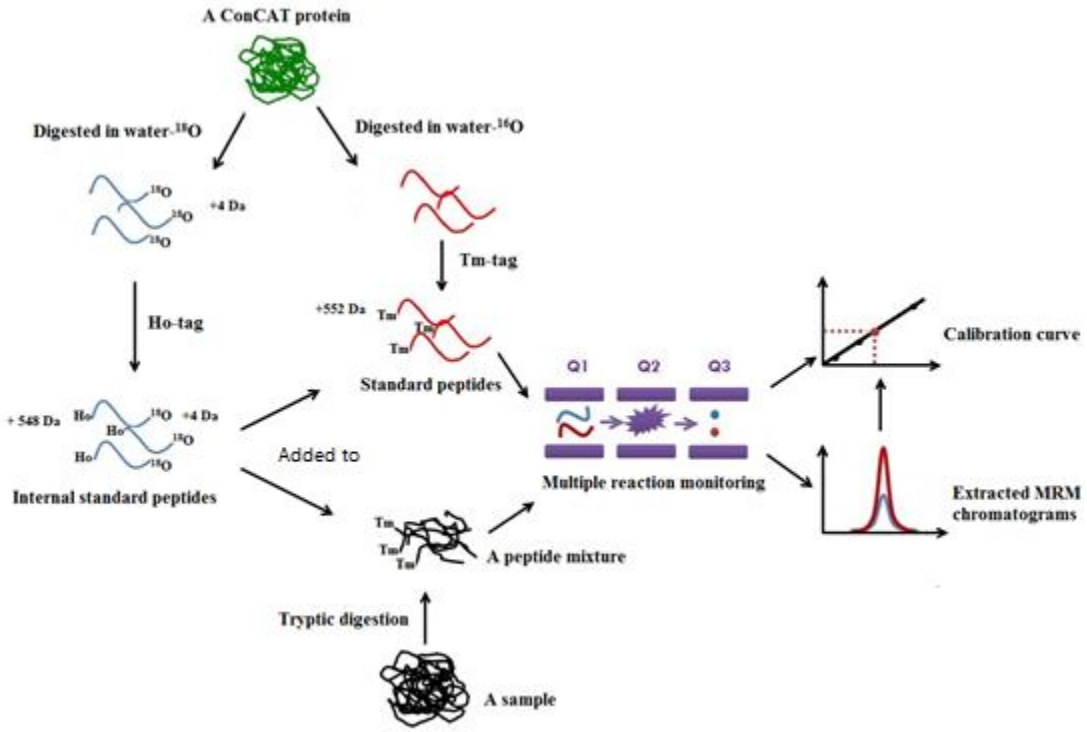
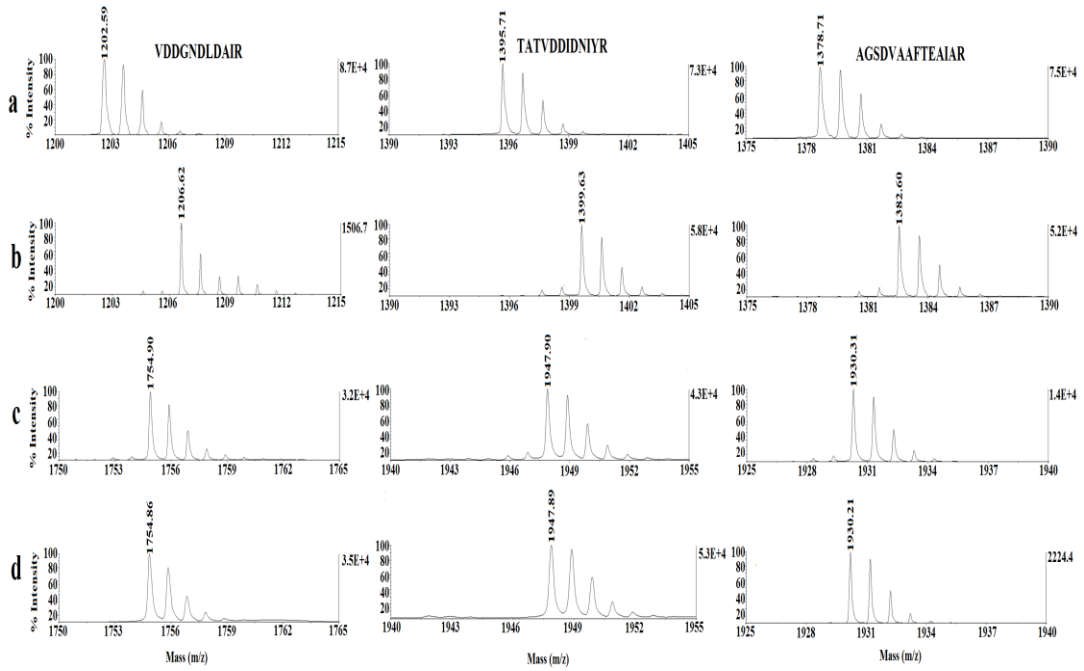


Figure 1 A schematic diagram of absolute determination of targeted proteins based on ^{18}O/metal isobaric labeling combined with MRM MS



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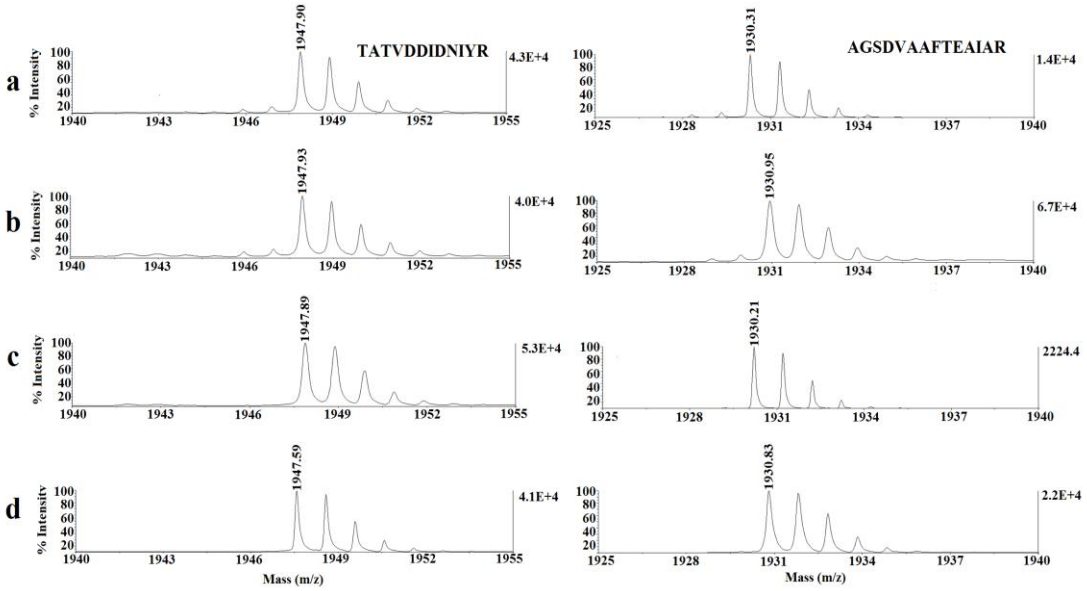

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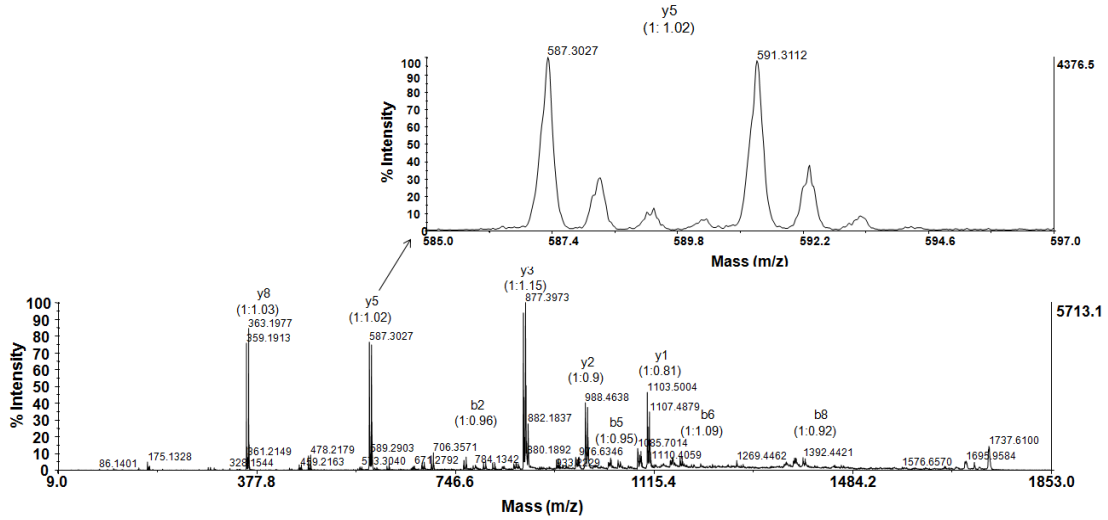

Figure 4 MALDI-TOF MS/MS spectra of a labeled peptide mixture of VDDGNDLDAIR (^{16}O+ Tm) and VDDGNDLDAIR (^{18}O+Ho) at molar ratio of 1:1

Table 1 Measured content of 20 drug metabolic enzymes in human liver microsomes and their RSD values

Protein	HM-03-010[#]		HM-03- 011		HM-03-012		HM-03-014A		HM-03-014B	
	Mean (f/μg)	RSD (%)	Mean (f/μg)	RSD (%)	Mean (f/μg)	RSD (%)	Mean (f/μg)	RSD (%)	Mean (f/μg)	RSD (%)
CYP 1A2	51.1	9.8	93.3	11.8	47.6	4.5	57.9	4.0	126.7	13.4
CYP 2A6	80.3	11.2	72.5	11.6	112.9	18.7	66.3	19.2	114.7	10.7
CYP2B6	16.6	18.8	20.0	22.5	21.4	18.0	23.4	13.7	25.2	21.5
CYP 2C8	65.6	10.8	73.3	8.5	72.0	17.2	63.1	18.7	143.2	8.2
CYP 2C9	105.6	11.1	122.9	4.9	147.8	6.8	93.6	7.0	184.5	10.8
CYP2C19	137.2	8.5	106.2	10.4	111.2	5.6	108.5	8.5	143.4	9.5
CYP 2E1	71.7	10.5	72.8	11.7	86.6	13.6	52.0	16.6	79.4	13.5
CYP 2D6	13.8	11.7	23.5	9.1	14.1	3.8	11.5	9.3	20.9	6.2
CYP3A4	39.0	19.0	41.0	13.8	57.5	13.0	88.5	13.5	111.4	18.6
CYP 3A5	43.9	12.6	124.4	7.1	52.8	10.7	42.7	19.4	63.7	7.8
UGT 1-1	32.8	5.5	15.9	22.4	3.8	8.4	26.3	6.0	64.0	9.7
UGT 1-4	66.7	22.6	77.1	19.6	142.5	18.0	88.2	13.4	132.0	17.8
UGT 1-6	18.3	19.4	17.3	21.3	17.7	15.1	18.8	10.9	35.9	18.7
UGT 1-9	16.9	3.7	16.9	4.1	15.7	5.1	19.0	6.9	23.8	14.7
UGT 2B4	92.9	6.6	90.6	15.8	82.9	18.1	96.9	15.5	195.9	7.4
UGT 2B10	120.3	11.3	133.1	8.9	198.8	7.4	205.5	11.8	365.5	6.6
UGT 2B11	8.4	4.0	10.5	8.3	9.5	11.2	9.4	4.2	13.2	7.8
UGT 2B15	110.3	13.0	102.4	14.5	54.6	6.5	38.9	14.8	71.0	12.8
POR	38.7	16.6	40.7	14.7	62.8	5.1	58.9	11.4	108.3	10.5
cyt b5	410.5	19.4	458.6	19.1	473.0	14.7	348.9	18.3	634.1	15.9

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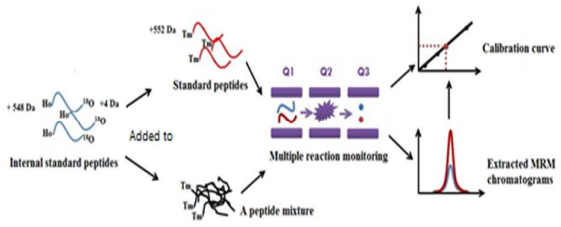

A novel method using ^{18}O/metal isobaric labeling combined with multiple reaction monitoring mass spectrometry for absolute quantification of target proteomes