Published in final edited form as:
Anal Chem. 2010 March 15; 82(6): 2561–2567. doi:10.1021/ac1001433.

Measurement of human surfactant protein-B turnover *in vivo* from tracheal aspirates using targeted proteomics

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Abstract

We describe a method to measure protein synthesis and catabolism in humans without prior purification and use the method to measure the turnover of surfactant protein-B (SP-B). SP-B, a lung-specific, hydrophobic protein essential for fetal-neonatal respiratory transition, is present in only picomolar quantities in tracheal aspirate samples and difficult to isolate for dynamic turnover studies using traditional *in vivo* tracer techniques. Using infusion of [5,5,5-$^2H_3$] leucine and a targeted proteomics method, we measured both the quantity and kinetics of SP-B tryptic peptides in tracheal aspirate samples of symptomatic newborn infants. The fractional synthetic rate (FSR) of SP-B measured using the most abundant proteolytic fragment, a 10 amino acid peptide from the carboxy-terminus of proSP-B (SPTGEWLPR), from the circulating leucine pool was 0.035±0.005 hr^{-1} and fractional catabolic rate was 0.044±0.003 hr^{-1}. This technique permits high-throughput, sensitive measurement of turnover of low abundance proteins with minimal sample preparation.

Introduction

The development of modern shotgun proteomics methods has facilitated the qualitative and quantitative assessment of complex protein mixtures by measuring proteolytically digested peptides using mass spectrometry1–3. The use of selected reaction monitoring (SRM) to target specific peptides of interest further enhances analytical sensitivity and precision and provides a means to perform high throughput measurements4–6. However, quantifying the abundance of a peptide fails to provide understanding of the metabolic balance between the rate of protein synthesis and catabolism7, neither of which is measured routinely in proteomics experiments. To understand metabolic regulation of any biological system, kinetic measurements must be included as part of any systems analysis.

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The measurement of stable-isotopically labeled amino acid incorporation into proteins is one method to interrogate *in vivo* protein kinetics in humans.[8–11] Traditionally, to measure protein turnover in humans, a stable isotopically labeled amino acid is administered continuously, and the enrichment at sampled time points is determined by the purification of the protein, hydrolysis of the protein to amino acids, derivatization of the amino acids, and measurement of the amino acid enrichment by gas chromatography-mass spectrometry or gas chromatography-combustion-isotope ratio mass spectrometry[12]. This process is impractical for measurement of multiple time points in multiple patients required for genotype-phenotype correlation. Additionally, because protein specificity is lost during protein hydrolysis, any minor, non-specific protein contamination from inhomogeneous protein purifications will alter the accuracy of the kinetic measurement. Thus, the measurement of protein kinetics *in vivo* has been limited to single abundant proteins that are easily purified from tissue or plasma[8, 10, 13].

Surfactant protein-B (SP-B) is a lung-specific protein required for successful fetal-neonatal pulmonary transition. Its hydrophobicity and low abundance in accessible, low volume tracheal effluent samples from newborn infants with respiratory distress syndrome (RDS) make SP-B difficult to purify in sufficient quantities for traditional gel-based proteomic analysis. Synthesized in pulmonary alveolar epithelial type II cells as a 381 amino acid propeptide, proSP-B undergoes sequential proteolytic cleavages from both the N-terminal and C-terminal ends and glycosylation to yield a 79 amino acid mature SP-B peptide (Figure 1). Rare, loss of function mutations in the gene encoding SP-B (*SFTPB*) result in lethal RDS in newborns[14–16], and common coding and non-coding region variants have been statistically associated with non-lethal RDS[17, 18]. However, the regulation of SP-B by these variants is unknown. *In vivo* quantification of SP-B metabolism using a stable isotopically labeled amino acid infusion in newborn patients with RDS provides a means to assess dynamic SP-B expression.

Using a primed continuous intravenous infusion of [5,5,5-$^2H_3$] leucine, we demonstrate sufficient experimental sensitivity and precision to calculate the *in vivo* fractional synthesis and fractional catabolic rates of human SP-B in newborns with RDS. We performed this analysis directly on tryptic digests of tracheal aspirates by SRM without protein enrichment and with minimal sample preparation. This approach permits high throughput measurement of low abundance protein turnover *in vivo* from tracheal aspirates that can be extended to other proteins from the same samples during the same tracer infusion and can also be used as a metabolic phenotype for genetic association studies.

Methods

Subjects

Ten infants born at 25 to 37 weeks gestation were studied in the Newborn Intensive Care Unit at St. Louis Children's Hospital between 1 and 5 weeks of age. Table 1 presents the subject characteristics of the patients studied. All patients required mechanical ventilation for neonatal respiratory distress syndrome and were screened to ensure they met the appropriate inclusion criteria for the study. Parents were instructed of the purpose, benefits, and risks of the study and gave written consent in accordance with approved protocols. All protocols were approved by the Washington University Human Research Protection Office and by the University of Washington Institutional Review Board.

Infusion protocol

Each patient received a 5 minute intravenous priming dose of [5,5,5-$^2H_3$]leucine (Cambridge Isotope Laboratories, 98% ^2H) of 18 μmol/kg followed by a 6-hour continuous infusion of 18 μmol/kg/hr of [5,5,5-$^2H_3$]leucine, which was dissolved in 5% dextrose in water and prepared

in sterile fashion. Tracheal aspirates were collected during routine airway suctioning after instillation of 0.5 ml normal saline into the endotracheal tube and aspiration into a sterile trap at intervals of approximately 3 hours throughout the infusion and for approximately every 6 hours for up to 5 days or until extubation. Aspirates were collected, kept on ice, aliquoted into fractions of ~0.5 mL, and stored at −80°C until analysis.

Preparation of Tracheal Aspirates for SP-B tracer measurement

One fraction of the tracheal aspirate sample was prepared for immunoblotting using antisera directed against mature, and N- and C- terminal regions of proSP-B as described in the Supplemental materials. For preparation for mass spectrometry, 100 μL of 0.2% RapiGest SF (Waters Corporation, Milford) diluted in 50mM ammonium bicarbonate pH 7.8 was added to 100 μL of the unfractionated tracheal aspirate and heat denatured for 5 min at 100°C. Proteins were reduced with the addition of 2 μL of dithiothreitol (DTT) at 500 mM and incubated for 30 min at 60°C. The reduced proteins were then alkylated with the addition of 6 μl of 500 mM iodoacetamide. Reduced and alkylated aspirates were digested by adding 10 μl of a 0.2 μg/μl solution of trypsin (Promega, Madison) and incubating for 4 hours with constant mixing at 37° C. Following digestion, 10 μl of 5 M HCl was added to each sample and incubated for 45min at 37°C to hydrolyze the RapiGest and quench the digestion. The samples were centrifuged at 15,339 × g, 4 °C for 15 min and the supernatant was stored at −80 °C until analysis.

Liquid Chromatography-Tandem Mass Spectrometry

The digested aspirate mixture was loaded onto a microcapillary LC column (75 μm × 25 cm) packed with C12 reversed phase chromatography material (Phenomenex, Jupiter 4μ, Proteo 90Å) and interfaced with a triple quadrupole mass spectrometer (TSQ Ultra, Thermo Fisher) using an HPLC and an autosampler in a nanoflow configuration as described previously[19]. A high resolution selected reaction monitoring method (hSRM) was used to monitor the following precursor > product ion transitions for the unlabeled and labeled forms. The doubly charged precursor ions were monitored in Q1 with a resolution of 0.2 FWHM and three singly charge y-ions (y4, y5 and y6) for each peptide were monitored in Q3 with 0.7 FWHM. Thus, to measure the infused tracer into the peptide SPTGEWLPR we monitored the transitions *m/z* 521.7 >571.3, 700.4, 757.4 and *m/z* 523.3>574.4, 703.4, 760.4 for the tracee and tracer respectively. Product ion ratios and chromatographic retention time were used to confirm the peptide identity within the complex mixtures. Each sample underwent five technical replicates, which were used to compute the $^{2}H_{3}$-leucine enrichment in SP-B over the time course of collected tracheal aspirates (Supplementary Table 1).

Quantification of peptide abundance

Protein concentration of tracheal aspirates was determined using the BCA Protein Assay (Rockford, IL). Protein samples were denatured using 0.2% RapiGest SF (Waters Corporation, Milford, MA) in 50 mM ammonium bicarbonate pH 7.8 and digested as described above. Labeled and unlabeled forms of the peptides FPIPLPYCWLCR, DPLPDPLLDK and SPTGEWLPR were acquired from Sigma and initially diluted in DMSO (Table 2). Subsequent dilutions were performed to get a calibration curve ranging from 2 fmols to 2 pmols/μl. SRM transitions were acquired using 0.7 FWHM resolution in both, Q1 and Q3 and the transitions monitored for each peptide form are shown in table 2. SRM peaks were integrated using QuanBrowser (Xcalibur, Thermo Fisher Scientific) and quantification of the amount of target analyte determined from the mass spectrometry data as described previously[3, 20, 21]

Measurement of plasma alpha-ketoisocaproic acid (KIC) enrichment

The enrichment of alpha-ketoisocaproic acid in plasma was measured by gas-chromatography/mass spectrometry as the O-t-butyldimethylsilyl quinoxalinol derivatives as described previously[22].

Data Analysis

The mass spectrometry data were processed to produce a background subtracted peak area for the selected reaction monitoring transitions 521.7>757.4 and 523.7> 760.4 using the Xcalibur data-system. The first transition is for the peptide with natural abundance isotopes; whereas, the second transition is used to monitor the peptide enriched in $^{2}H_{3}$-leucine. These precursor and product ion transitions correspond to the doubly charged precursor ion and singly charged base peak product ion for the peptide SPTGEWLPR. The ratios of the labeled / unlabeled SRM transitions measured in the tracheal aspirate were used to calculate the tracee / tracer mole ratio (R_{TTR}) at each time t using the equation:

$$R_{TTR}=R_T - R_0 \quad \text{(Eq 1)}$$

where R_T is the area ratio for the labeled/unlabeled peptide signal at time t and R_0 is the area ratio at time 0 (i.e. prior to the start of the infusion). The R_{TTR} was then converted to a mole percent excess (MPE) enrichment using the equation:

$$MPE=\frac{R_{TTR}}{R_{TTR}+1}*100 \quad \text{(Eq 2)}$$

The fractional catabolic rate (FCR, pools/hour) was calculated by fitting the data from all the patients using a population model as described below. The fractional synthetic rate (FSR, %/hour) for SP-B was calculated by dividing the upslope of the time enrichment curve by the average plasma KIC enrichment using the circulating plasma KIC pool as a proxy for the precursor enrichment[23, 24].

Population Modeling

A five compartmental model was developed to describe tracer kinetics in the plasma amino acid and surfactant protein pools. All rate constants were first-order. A four compartment model with central plasma and three extra vascular exchange compartments has previously been used to describe plasma leucine kinetics [25, 26]. The surfactant protein pool was characterized by a single compartment and was the product of an extravascular leucine compartment (Supplementary Figure 1). For parameter estimation, the model had the following parameters: Scalar for tracer input (Inpt), the rate constant for the fraction of the precursor pool going into SP-B, and fractional catabolic rate (FCR) of SP-B. The model was fit to the plasma KIC and SP-B enrichment data using the Nonlinear Mixed Effects Modeling (NONMEM) software (version V, ADVAN3, TRANS4; NONMEM Project Group, University of California, San Francisco, San Francisco, CA), interfaced with PDx-Pop Version 1.1j Release 4 (GloboMax LLC, Hanover, MD)[27, 28].

Immunoblotting

We used immunoblotting of unfractionated tracheal aspirate samples on nitrocellulose membranes transferred after SDS polyacrylamide gel electrophoresis under non-reducing conditions and probed first with anti-serum to mature SP-B (Chemicon, Inc, Temecula, CA; #AB3780), and, secondly, after stripping nitrocellulose membranes using Re-Blot Plus Strong Antibody Stripping Solution (Chemicon # 2504), re-probed with primary anti-serum to pro-

SP-B (Chemicon #AB3430). Each sample was adjusted to contain a mean of 3.4 ng (± 1.9 ng S.D.) of SP-B based on prior slot blot analysis with antibody to mature SP-B. Chemiluminescent detection with ECL(™) Western Blotting Detection Reagents and Hyperfilm(™) ECL (GE Healthcare #RPN2106, #28-9068-39) was performed. A separate immunoblot was prepared and probed first with primary anti-serum to the N-terminal portion of proSP-B (Ser 145-Leu 160; gift of Susan Guttentag, MD), and secondly, after stripping, re-probed with primary anti-serum directed against the 102 residue C-terminal fragment of proSP-B (Chemicon # AB3432).

Results

Selection of Peptide Transitions for the Measurement of Tracer Enrichment

With protein immunoblotting of tracheal aspirate samples, we identified SP-B peptides recognized by antibodies directed against the mature SP-B, and the N– and the C-terminus, respectively, of proSP-B[18] (Supplementary Figure 2). We consistently detected ions that represent SP-B specific proteolytic fragments from mature SP-B (FPIPLPYCWLCR, amino acids 1–12 of the mature peptide and 201–212 of proSP-B) and from the C-terminus of proSP-B (SPTGEWLPR corresponding to amino acids 287–295 of proSP-B) in tracheal aspirate samples. We also detected a fragment from the N-terminus (DPLPDPLLDK, amino acids 166–175 of proSP-B) but in significantly lower quantities and thus confirmed the protein blot findings (Figure 3).

Figures 2A and 2B show the full scan MS spectra of the precursor ions for the endogenous unlabeled (SPTGEWLPR) and labeled (SPTGEW($^2H_3$-L)PR) forms of the SP-B peptide. To achieve the isotopic resolution of the doubly charge precursor, we used a full width at half-maximum (FWHM) resolution setting of 0.2 *m/z* in Q1. The product ion spectrum for the target peptide (Figure 2C) showed the y6 ion at *m/z* 757.4 to be the most abundant fragment. Therefore, we selected the SRM transition 521.7>757.4 for the 2+ charged precursor and 1+ charged y6 fragment for all subsequent quantitative measurements. As expected, there is an overlap in signal between the monoisotopic peak from the labeled SP-B peptide with the M+3 isotope of the endogenous unlabeled isotopomer. As a result, there would be an expected contribution from the unlabeled peptide into the 523.2>760.4 SRM transition (Figure 4). In addition, we monitored two other SRM transitions, 521.7>571.3 and 521.7>700.4, from the y4 and y5 ions respectively, as confirmatory for ion ratio and retention time.

Time Course Measurement of $^2H_3$-Leucine Enrichment

We calculated the tracer:tracee mole ratio (TTR) by subtracting the measured M+3/M+0 isotope ratio by the endogenous isotope ratio calculated from the average ratio measured at t=0 (Supplementary Table 1). We then converted the TTR values to mole percent excess (MPE) enrichment values as described previously.[9] We detected enrichment above background in proSP-B within 5 hours of the start of the infusion which peaked at approximately 10 hours, and decreased toward baseline by 24 hours suggesting rapid turnover of newly synthesized proSP-B (Figure 5). The mean (± SEM) fractional catabolic rate (FCR), a measure of turnover of the pool of interest, was 0.044 ± 0.003 $hour^{-1}$ or 1.06 pools per day in this heterogeneous group of infants (Table 1). The FCR was computed using a population modeling approach to fit the peptide and plasma KIC enrichment data to a five pool compartmental model. For the infants for whom sufficient time points were available (n=7), the mean (± SEM) fractional synthetic rate (FSR) for SP-B derived from the circulating leucine pool was 0.035 ± 0.005 $hour^{-1}$.

Discussion

While methods exist for the measurement of individual protein production and disposal rates, the application of these analyses has been limited to a few relatively abundant proteins sampled from easily accessible compartments and purified for mass spectrometry. These methods, while precise and capable of measuring small enrichments from an infused tracer, are laborious, intolerant of proteins not purified to homogeneity, and often require nanomoles of proteins for analysis, which is several orders of magnitude greater than the amount of SP-B measured in our unfractionated tracheal aspirate samples.

Recently, Bateman and colleagues described the use of liquid chromatography-tandem mass spectrometry for the measurement of human amyloid-β synthesis and disposal rates in cerebrospinal fluid[29]. This method used immunoprecipitation to enrich for amyloid-β, followed by tryptic digestion of the precipitated mixture, and analysis of the resulting peptides by LC-MS/MS using either an LCQ or LTQ ion trap mass spectrometer. With an ion trap mass spectrometer only a tiny fraction of the total measurement time is actually spent measuring the ions of interest. While ion trap mass spectrometers have extremely high full-scan sensitivity for qualitative analyses, the total number of ions measured is often relatively limited making their quantitative precision not particularly high. Our method builds on that of Bateman et al. by improving the sensitivity of the analysis and eliminating the need for most of the sample preparation. Furthermore, using a triple quadrupole mass spectrometer operated in selected reaction monitoring mode permits the selective analysis of the targeted peptide within a complex matrix with sufficient precision for a robust quantitative analysis.

To detect the incorporation of low levels of an infused tracer into protein, the precision of the peptide isotope ratio measurement must be sufficient so that the difference between the ratio prior to and following the administration of the tracer can be distinguished. Therefore, to measure “tracer quantities” of labeled amino acids in peptides, we first used a triply labeled amino acid tracer, $^{2}H_{3}$-leucine. The theoretical natural abundance of M+3/M+0 isotope ratio for the peptide SPTGEWLPR is 4.7%. This relatively low background isotope ratio makes the measurement of even a 1% $^{2}H_{3}$-leucine enrichment possible because it would contribute to a >20% increase in the measured isotope ratio – a precision that is readily achievable with most triple quadrupole mass spectrometers. In contrast, the same enrichment measured using a ^{13}C-leucine tracer would increase the M+1/M+0 isotope ratio by <2% because the endogenous background isotope ratio is 57.8% – a precision that is beyond the capabilities of conventional LC-MS instrumentation. Thus, measuring a 1% increase over a 4.7% background is more reliable than measuring the same enrichment over a 57.8% background.

In using this mass spectrometry technique to measure SP-B turnover, we have controlled the two main limiting factors in the measurement of low levels of tracer enrichment: the background isotope ratio upon which the enrichment is superimposed, and the number of ions that are measured by the mass spectrometer to calculate the isotope ratios[21, 30]. While the background isotope ratio can be controlled by the number of labels on the infused tracer, the number of ions that are measured by the mass spectrometer is limited by the abundance of the protein in the sample and the mass spectrometry approach used to measure the isotope ratio.

Another important consideration of the measurement of low abundance proteins in a complex mixture is the sensitivity of the method. While a protein or peptide can be quantified by measuring the most abundant isotope peak relative to an internal standard, to detect a small amount of a rare isotopomer during the infusion of a tracer requires substantially greater sensitivity because the intensity of the heavy isotope peak will be small relative to the monoisotopic or base isotope peak. In this case, the M+3 isotope peak is only 4.7% the intensity of the monoisotopic peak. This requires a method with >20x sensitivity compared to a method

to simply quantify the level of the peptide. Because of the increased demands on the sensitivity to measure tracer enrichments over the quantification of a peptide abundance, all tracer measurements were performed on the C-terminal peptide – the most abundant peptide on average across the 10 patients in this study.

Previously we found multiple SP-B peptides in tracheal aspirate samples of newborns that were recognized by a polyclonal antibody directed at both the carboxy- and amino-termini of proSP-B and by a polyclonal antibody directed at mature SP-B[18]. Here we extend those initial Western blot findings by identifying the presence of these peptides using antibodies specific for the N- and C-terminal ends of proSP-B, respectively, and confirm those findings with mass spectrometry by identifying and quantifying specific tryptic peptides from each end of proSP-B. We were surprised that the most abundant peptide found was frequently derived from the carboxy terminus, the physiologic significance of which is unclear.

The FCR value of 1.06 $days^{-1}$ for the proSP-B peptide translates into a half-life of 16.50 ± 1.32 hours (mean ± SE) in the 10 patients studied. It is important to note that this value is indistinguishable from the measurement of 8–35 hours reported previously by Cogo et al. for mature SP-B. Cogo et al. measured their tracer enrichment by enriching for the mature SP-B and measuring the infused amino acid by gas chromatography–combustion–isotope ratio mass spectrometry[31]. Their analysis was extremely laborious requiring approximately 2 days of work for each time point. In contrast, the approach described here permits preparation of all the samples from several patients in parallel in <6 hours followed by <1 hour of instrument time per time point. Ultimately we obtained this increase in throughput by performing the analysis on the peptide level – eliminating the need to purify the protein. Thus, our protocol reduced the number of steps in the sample preparation process from five to two (Figure 6).

An important consideration in the interpretation of these findings and comparing the results of our analysis is that our turnover kinetics represent the turnover of the proSP-B protein. In contrast, the work of Cogo et al. measured the turnover of the mature SP-B protein. Further studies are underway to determine if each SP-B peptide exhibits unique kinetics, to determine the physiological and clinical significance of differences in SP-B turnover, to determine the correlation between *in vivo* SP-B and surfactant phospholipid turnover, and finally, to determine the kinetics of other proteins that are present in tracheal aspirate samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support for this work was provided from National Institutes of Health grants R01 HL65385 (AH), R01 HL65174 (FSC, AH), R01 DK069386 (MJM), R01 HL82747 (FSC,AH), K23 HL83091 (KLS), P30 DK56341 (BWP, AH), and support from the Saigh Foundation (AH, FSC). We would like to thank Susan Guttentag, MD, University of Pennsylvania for generously providing antisera and baculovirus constructs of proSP-B.

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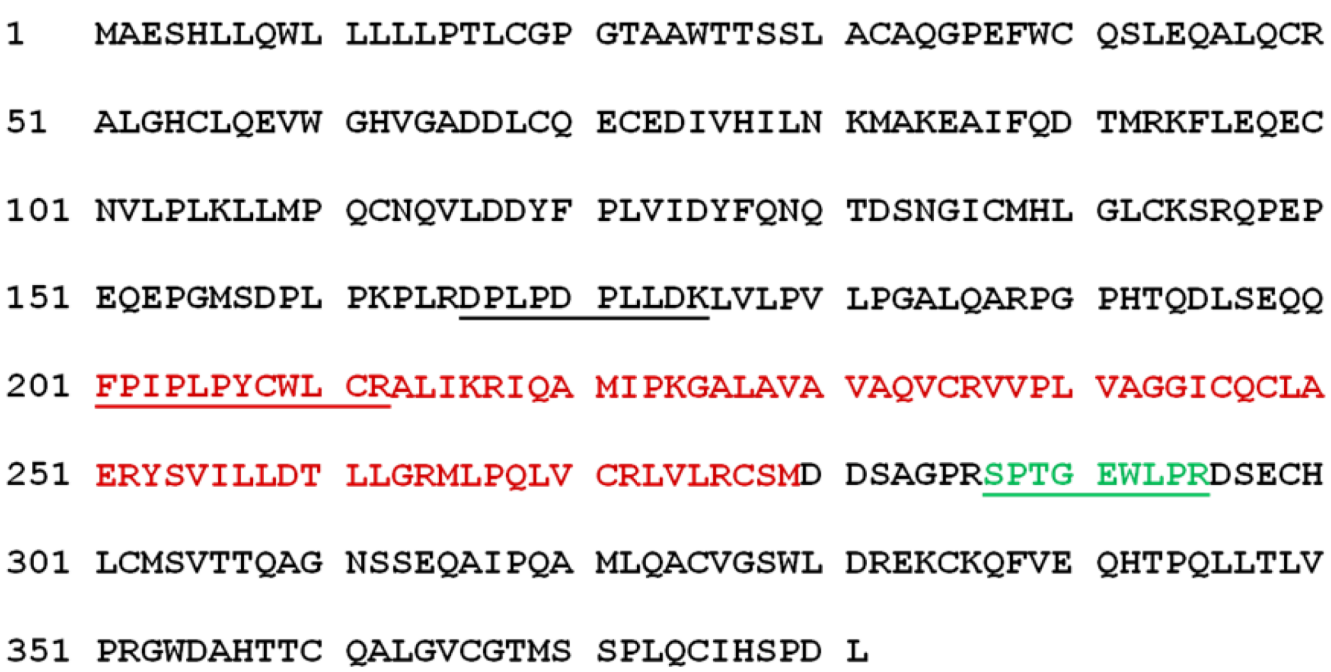

Figure 1.
Surfactant protein-B sequence predicted from the gene transcript. The functional form of the mature protein is highlighted in red and the three peptides used in the measurement of protein abundance are underlined. The peptide highlighted in green was used for the measurement of the $^{2}H_{3}$-leucine tracer enrichment.

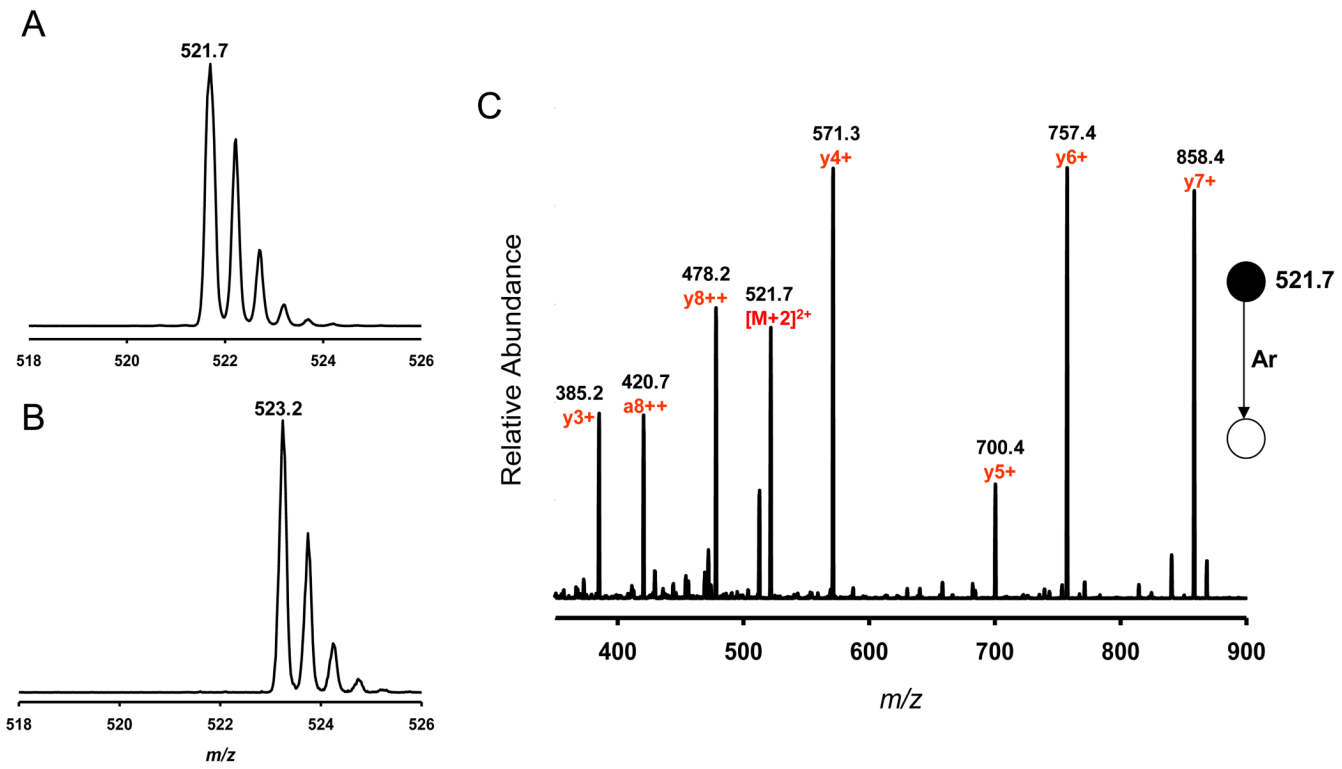

Figure 2.

A) Full Scan MS of the doubly-charged precursor ion, $[M+2H]^{+2}$, of the endogenous unlabeled SP-B peptide SPTGEWLPR. Naturally occurring isotopomers m+1 through m+4 of this peptide are resolved and detectable. B) Full Scan MS of the doubly-charged precursor ion, $[M+2H]^{+2}$, of the labeled SP-B peptide SPTGEW[$^{2}H_{3}$-L]PR. C) Collision Induced Dissociation (CID) spectrum of the doubly-charged precursor ion of the endogenous unlabeled SP-B peptide SPTGEWLPR at *m/z* 521.7.

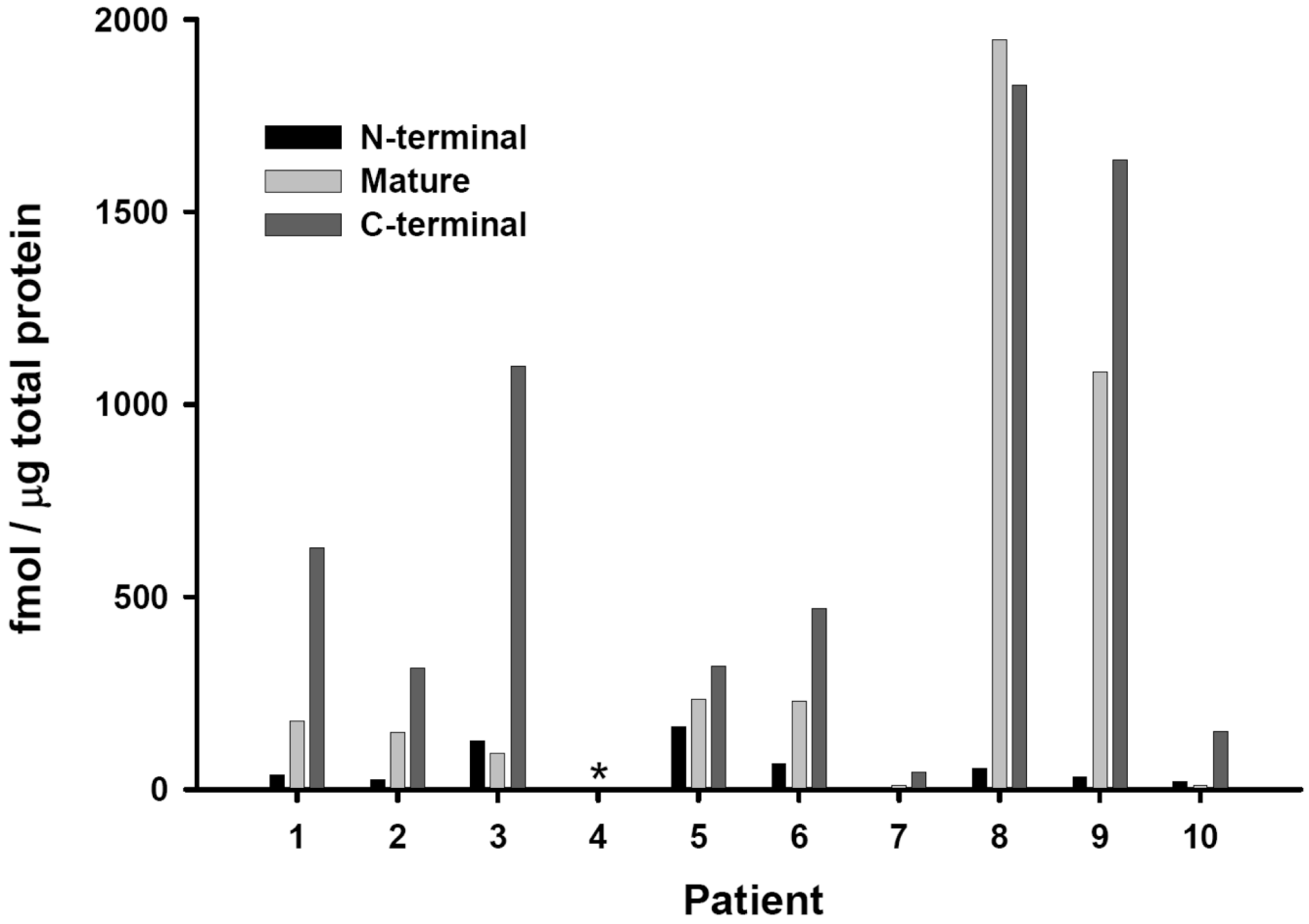

Figure 3.

Quantification of peptide fragments from mature SP-B (FPIPLPYCWLCR), N-terminal (DPLPDPLLDK) and C-terminal (SPTGEWLPR) regions of proSP-B. The C-terminal peptide was most consistently detectable in tracheal aspirate samples and thus was chosen for selected reaction monitoring for measuring new SP-B synthesis.

* Peptide quantity not determined.

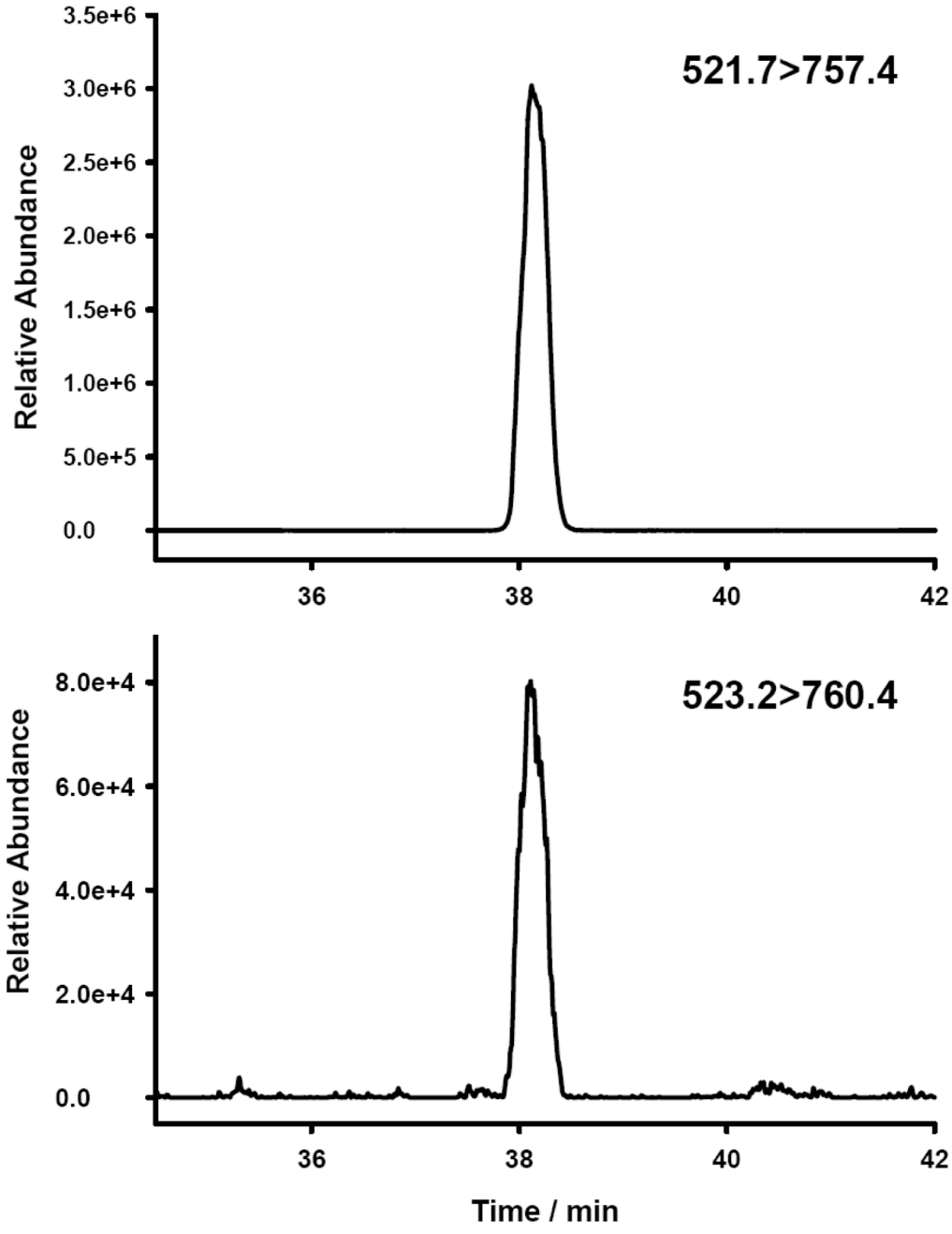

Figure 4.

SRM chromatograms of the transitions *m/z* 521.7>757.4 (y6) and *m/z* 523.2>760.4 (y6), for the endogenous unlabeled (SPTGEWLPR) (top) and labeled (SPTGEW[$^{2}H_{3}$-L]PR) (bottom) peptide from SP-B for Patient 9 at 22 hours after infusion start.

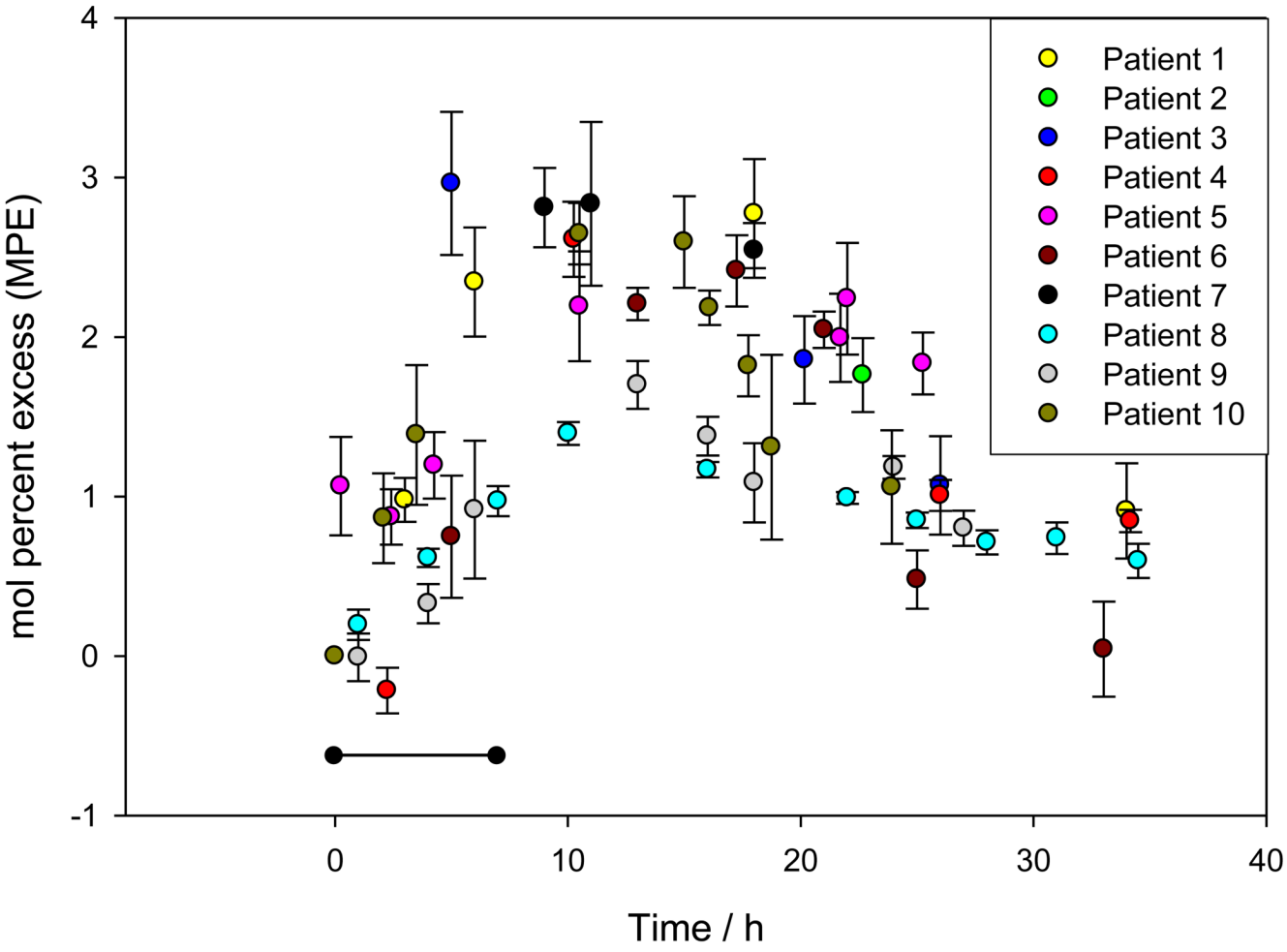

Figure 5.

Time-dependent measurement of SP-B enrichment expressed as a mole percent excess (MPE) in 10 newborns from a 6 hour, primed, continuous infusion of $^{2}H_{3}$-leucine denoted by the line from 0–6h. Points represent the mean value of five technical replicates ± 95% confidence intervals. The linearity and accuracy of the enrichment measurements were validated using an enrichment curve from synthetic peptide standards (Supplementary Figure 3).

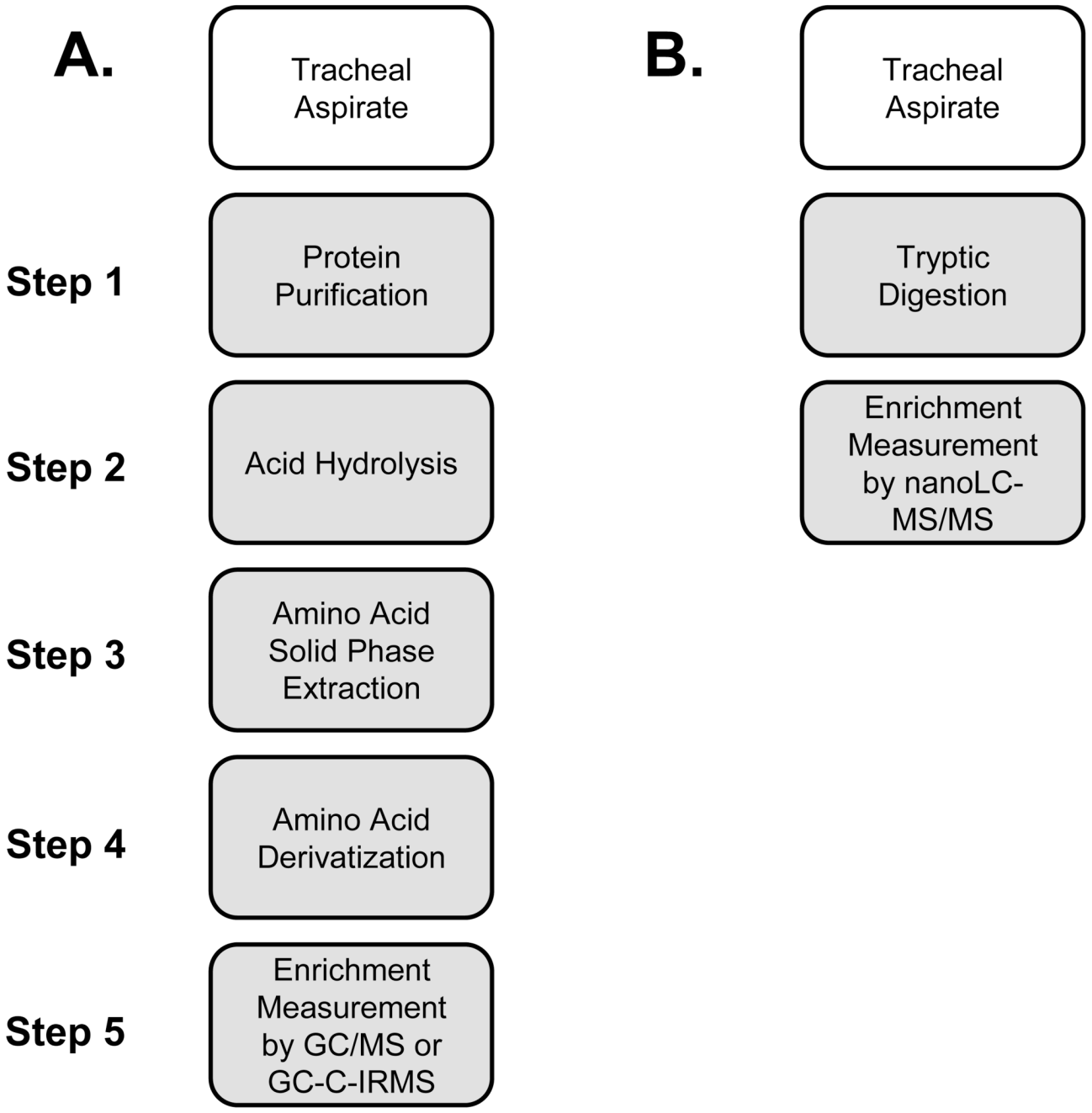

Figure 6.
Comparison of traditional procedures for measurement of metabolically labeled proteins (Panel A) and the techniques outlined in this manuscript (Panel B).

Table 1

Characteristics of the patients studied and kinetic results.

Patient	Gestational age at birth	Age at study (weeks)	FSR (hour^{-1})	FCR$_t$ (hour^{-1})	Half-life (h)
1	25	5	0.028	0.028	24.667
2	37	1	N/A	0.040	17.504
3	32	<1	N/A	0.043	16.008
4	37	1	N/A	0.051	13.618
5	26	2	0.04	0.031	22.216
6	37	<1	0.023	0.058	11.992
7	34	1	0.054	0.050	14.003
8	26	1	0.029	0.042	16.348
9	25	4	0.022	0.041	16.906
10	36	<1	0.051	0.059	11.768
Mean +/−	31.5 +/−	1.5 +/−	0.035 +/−	0.044 +/−	16.503 +/−
SE	1.5	0.5	0.005	0.003	1.325

N/A – insufficient data to calculate FSR

Table 2

Unlabeled and labeled peptides used for peptides quantification.

Peptide Sequences	**Precursor *m/z***	**Fragment**
SPTGEWLPR	521.7	571.3 (y_4), 757.4 (y_6), 858.4 (y_7)
SPTGEW[$^{13}C_6$,^{15}N]LPR	525.3	578.3 (y_4), 764.4 (y_6), 865.5 (y_7)
DPLPDPLLDK	561.8	585.4 (y_5), 700.4 (y_6), 797.4 (y_7)
DPLPD[$^{13}C_5$,^{15}N]PLLDK	564.8	591.4 (y_5), 706.4 (y_6) 803.4 (y_7)
FPIPLPYCWLCR	811.4	634.3 (y_4), 794.3 (y_5), 1054.4 (y_7)
FPIPL[$^{13}C_5$,^{15}N]PYCWLCR	814.4	634.3 (y_4), 794.3 (y_5), 1054.4 (y_7)