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Application of liquid chromatography-tandem mass spectrometry (LC–MS/MS) for the analysis of stable isotope enrichments of phenylalanine and tyrosine

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ABSTRACT

Whole-body protein synthesis and breakdown are measured by a combined tracer infusion protocol with the stable isotope amino acids L-[*ring*-$^2H_5$]-phenylalanine, L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine that enable the measurement of the phenylalanine to tyrosine conversion rate. We describe a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the measurement of very low tracer–tracee ratios (TTR) of the amino acids L-phenylalanine and L-tyrosine in human plasma. TTR calibration curves of the tracers L-[*ring*-$^2H_5$]-phenylalanine, L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine were linear ($r^2 > 0.99$) in the range between 0.01% and 5.0% TTR and lowest measurable TTR for the tracers was 0.01% at a physiological concentration of 60 μM. The method was applied successfully to plasma samples from a clinical study reaching a steady state enrichment plateau (mean ± SD) of 3.33 ± 0.19% for L-[*ring*-$^2H_5$]-phenylalanine, 2.40 ± 0.43% for L-[*ring*-$^2H_2$]-tyrosine and 0.29 ± 0.07% for L-[*ring*-$^2H_4$]-tyrosine, respectively. The LC–MS/MS method can be applied for measurement of very low plasma enrichments of phenylalanine and tyrosine for the determination of whole-body protein synthesis and breakdown rates in humans.

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1. Introduction

Whole-body protein synthesis and breakdown (named protein turnover) can be measured by constant infusion of stable isotope labeled amino acids into the circulation [1,2]. Infusion of stable isotope labeled amino acids (^2H, ^{13}C or ^{15}N) can be used to trace the amino acids that are involved in protein metabolism into the bloodstream. Collection of blood samples before and during infusion enables determination of the enrichment of the labeled amino acid. These stable isotopically labeled amino acids ("tracers") have the same chemical properties and the ionization efficiency is equal to their natural amino acids ("tracees"). Because of a mass difference between tracer and tracee a mass spectrometer can distinguish both from each other. The relationship between tracer and tracee is called tracer–tracee ratio (TTR). During constant infusion of a tracer into the bloodstream via a vein in the arm of a subject, the TTR in plasma will increase from the natural abundance TTR to a new value. This increase in TTR can be calculated using Eq. (1) [2]:

$$TTR = TTR_{measured} - TTR_{natural} \quad (1)$$

TTR is conventionally measured in plasma by mass spectrometry in combination with a separation technique such as gas chromatography [3,4] or liquid chromatography [5,6].

The most common applied method to study whole-body protein turnover that required only blood sampling is via combined infusion of L-[*ring*-$^2H_5$]-phenylalanine and L-[ring-$^2H_2$]-tyrosine and measuring enrichment of phenylalanine and tyrosine [1]. Phenylalanine is an essential amino acid that is not synthesized endogenously [1] and is thus ideal for *in vivo* whole-body protein turnover studies. In addition, the only disposal route of phenylalanine is conversion to tyrosine by the enzyme phenylalanine hydroxylase. After infusion with L-[*ring*-$^2H_5$]-phenylalanine, this conversion route is followed by measuring the L-[*ring*-$^2H_4$]-tyrosine enrichment.

Sample preparation for amino acid analyses can be very laborious due to sample preparation steps such as protein precipitation, extraction and derivatization of the amino acids. All these sample preparation steps are necessary as well for GC–MS [7–11], LC–MS [12], LC–MS/MS [13] and HPLC analyses [14,15]. The analysis of amino acids is time consuming due to sample preparation and analysis time. The analysis of amino acids with HPLC and fluorescence detection has an analysis time of approximately 60 min [14] while analyses by an amino acids analyzer, analysis times up to 120 min [16] are common. Nowadays, analytical methods with short analysis times, easy and fast sample preparation are preferred

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by analytical chemist because these analytical methods are less laborious and assure a higher sample throughput.

The commercially available Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit is a less laborious sample preparation method [17]. The Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit consists of solid phase extraction (SPE) with pipette tips for the extraction of the amino acids from the plasma matrix (or urine) followed by derivatization of the amino acids and analysis by LC–MS. Analysis time is short (<20 min) and the method enables the measurement of approximately 50 different amino acids and dipeptides with detection limits of approximately 1 μM or less for most of the amino acids [17], making the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit very attractive for stable isotope tracer studies. Previously, we applied the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit to the determination of stable isotope enrichments of *d*3-3-methylhistidine [18] with LC–MS analysis. We therefore used the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit for the measurement of stable isotope enrichments of the amino acids such as L-phenylalanine and L-tyrosine needed for the determination of whole-body protein kinetics. During method development and validation, we concluded that straight forward LC–MS analysis was not accurate enough because of interfering matrix components influencing the TTR determination which resulted in high ratios.

We report the development, validation and application of a new LC–MS/MS method for the determination of very low stable isotope enrichments of phenylalanine and tyrosine in human plasma samples applying the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit used for sample preparation.

2. Materials and methods

2.1. Chemicals

Stable isotope labeled L-[*ring*-$^{2}H_5$]-phenylalanine, L-[*ring*-$^{2}H_2$]-tyrosine and L-[*ring*-$^{2}H_4$]-tyrosine were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA), methanol (HPLC grade), ammonium formate, sodium chloride, trichloroacetic acid (TCA), hydrochloric acid, glacial acetic acid, 1-propanol, ovalbumin and water (HPLC grade) from Fisher Scientific (Pittsburgh, PA, USA). L-phenylalanine, L-tyrosine and propylchloroformate (PCF) were purchased from Sigma–Aldrich (St. Louis, MA, USA).

Plasma sample preparation for LC–MS/MS analysis was carried out by using the EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit and was purchased from Phenomenex Inc. (Torrance, CA, USA). Argon and nitrogen gas (both highest purity) for LC–MS/MS analyses were purchased from Praxair (Danbury, CT, USA).

2.2. Experimental infusion protocol

The infusion protocol [19,20] involved a 2-h stable isotope measurement and started at 8 h after an overnight fast of at least 10 h. The first blood samples taken before start of the infusion with the stable isotope amino acids were used for baseline measurements study (0 min). Whole-body protein metabolism was assessed via infusion of stable isotope L-[*ring*-$^{2}H_5$]-phenylalanine (prime 3.65 $\mu mol\,kg^{-1}$; infusion 4.5 $\mu mol\,kg^{-1}\,h^{-1}$). Moreover, a bolus dose of L-[*ring*-$^{2}H_4$]-tyrosine (prime 0.52 $\mu mol\,kg^{-1}$), L-[*ring*-$^{2}H_2$]-tyrosine (prime 0.95 $\mu mol\,kg^{-1}$; infusion 0.77 $\mu mol\,kg^{-1}\,h^{-1}$) were administrated to prime the L-phenylalanine-derived plasma tyrosine pool. Blood was sampled at times 0, 60, 90, 105, and 120 min during the study.

2.3. Preparation of plasma samples

Immediately after sampling, blood was transferred into pre-chilled blood tubes (4 mL) on ice, containing lithium-heparin. The blood was centrifuged immediately for 10 min at 4000 rpm at a temperature of 4 °C, and plasma was collected and put on ice. For LC–MS/MS measurements 900 μL plasma was added to 90 μL of a 50% TCA solution, vortexed and snap frozen in liquid nitrogen and stored at −80 °C until analysis.

Sample preparation for LC–MS/MS analyses was as follows: an aliquot of 25 μL of the TCA deproteinized plasma was pipetted into a glass vial (1 mL) and diluted with 125 μL water. A solid phase extraction tip was attached to a 1.5 mL syringe and the diluted plasma sample was pulled slowly through the SPE tip by moving the piston of the syringe. After completion, 200 μL of the washing solution (solution 2) was added to the glass vial and slowly pulled through the SPE tip to remove urea and other matrix components. The syringe was removed leaving the SPE tip inside the glass vial. Then, 100 μL of a freshly prepared elution/extraction (solution 3A/3B: 3/2% (v/v)) buffer solution was pipetted into the vial, a syringe of 0.6 mL was attached to the pipette tip and the piston was pulled up approximately 1 cm and attached to the SPE tip. The elution/extraction buffer was drawn into the SPE tip and the solid phase sorbent material was then expelled into the glass vial by pushing the piston down. This step was repeated until all of the SPE sorbent material from the pipette tip was expelled.

Derivatization agent (solution 4: 50 μL) was added and the vial was vortexed vigorously for 10 s. The derivatization agent was allowed to react for 1 min and 100 μL of organic extraction solvent (solution 5: propylchloroformate in chloroform) was added and the vial was vortexed vigorously for 20 s and was allowed to stand for 1 min for organic solvent phase separation. In case the organic solvent phase separation was not complete, 100 μL of a saturated sodium chloride solution was added and the glass vial was vortexed one more time for 10 s and after 1 min of organic solvent phase separation an aliquot of 100 μL of the upper organic phase was transferred into a new glass vial and the organic solvent was evaporated until dryness at ambient temperature with high purity nitrogen gas. The residue was dissolved with 100 μL of a mixture of methanol/water (62/38) and pipetted into a plastic spring-loaded micro insert and placed into an auto sampler vial with a septum cap.

2.4. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

The liquid chromatographic separation was performed on the Phenomenex LC AAA-MS column (250 mm × 3 mm ID, 4 μm particles) using a Waters 2695 HPLC system (Milford, MA, USA) with integrated auto sampler and sample chiller set at a temperature of 10 °C and a Waters column heater module controlled by a Waters TCM (temperature control module). Separation of the amino acid derivatives was achieved by gradient elution using the following gradient: 0 min 38% A, 13 min 17% A, 13.01 min 38% A and 20 min 38% A at a flow of 0.5 mL/min at a column temperature of 35 °C. Eluent A consisted of 100% water containing 10 mM ammonium formate and B consisted of 100% methanol. The sample injection volume was 10 μL.

A Thermo Quest TSQ triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an API 2 electro spray ionization (ESI) probe was employed for analyses of the amino acid enrichments. The mass spectrometer conditions were 1 s scan time; heated capillary temperature: 325°C; spray voltage 4.5 kV; conversion dynode: 15 kV; electron multiplier voltage 1600 V and sheath gas pressure: 0.62 MPa. Collision-induced dissociation (CID) spectra of the (sta-

Table 1
LC–MS/MS conditions for the determination of TTRs.

Amino acid/stable isotope labeled amino acid	Parent ion *m/z*	Product ion *m/z*	CID energy (eV)
Phenylalanine	294.3[a]	206.3[a]	17.5
L-[*ring*-$^2H_5$]-phenylalanine	299.3[a]	211.3[a]	17.5
Tyrosine	413.2[b]	222.2[b]	30.0
L-[*ring*-$^2H_2$]-tyrosine	415.2[b]	224.2[b]	30.0
L-[*ring*-$^2H_4$]-tyrosine	417.2[b]	226.2[b]	30.0

[a] $(M+H)^+$-adduct.
[b] $(M+NH_4)^+$-adduct.

ble isotope labeled) amino acids derivatives were obtained at an argon collision cell pressure of 8.27×10^{-6} MPa and different collision energies were used (Table 1).

A Waters 510 isocratic HPLC pump added post column to the eluent continuously (0.1 mL/min) a methanol/water (2/8) solution containing 1% acetic acid through a mixing tee for enhancing ionization of the amino acid derivatives.

The HPLC system was controlled by the Waters Corporation Empower™ 2 Software package and collection of mass spectrometric data and control of the mass spectrometer was performed by the Xcalibur software version 1.3. from Thermo Finnigan (San Jose, CA, USA).

3. Results and discussion

3.1. Method validation

3.1.1. The EZ:faast derivatization reaction

Analysis of amino acids in plasma samples is possible after derivatization of the amino acids with various derivatization reagents. Amino acids are polar compounds and therefore reverse phase separation is hampered. To overcome this separation problem, derivatization of the polar amino acids into less polar amino acids derivatives is usably done. In addition, mass spectrometric behavior (e.g. better ionization) and better mass spectrometric resolution are the result of derivatization.

The most popular derivatization reagents are a mixture of *o*-phthaldialdehyde (OPA)/3-mercaptopropionic acid (MPA) [15], phenylisothiocyanate (PITC) [21] and FMOC [22]. The EZ:faast amino acid commercial kit contains alkyl chloroformate as derivatization reagent and the method was especially developed for quick analysis and measurement of numerous amino acids and their concentrations in plasma and urine samples using LC– and GC–MS analyses. The method was used previously by Badawy et al. [11] for the rapid measurement of tryptophan and brain uptake competitors and was described as being a simple, rapid and elegant method for amino acid concentration determination.

That the method has a high applicability was demonstrated by Fonteh et al. [17] who used the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit for the determination of amino acids and dipeptides concentrations by LC–MS/MS analyses. Because of the short analysis time and the easy application of the method to sample preparation we used the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit for the measurement of TTR in plasma samples on amino acids from clinical studies studying protein metabolism. The recoveries of the amino acids L-phenylalanine and L-tyrosine were determined by spiking the ovalbumine solution (1% in (99/1) water/0.5N HCl) with known concentration L-phenylalanine and L-tyrosine (20, 50 and 100 μM). The spiked ovalbumine solutions were prepared for LC–MS/MS analysis as described in Section 2.3. Obtained recoveries were $92.9 \pm 5.5\%$, $105.6 \pm 5.1\%$ and $98.7\% \pm 3.1\%$ for L-phenylalanine at 20, 50 and 100 μM, respectively while recoveries for L-tyrosine were $104.0 \pm 13.1\%$, $89.9 \pm 2.7\%$ and $102.6 \pm 2.3\%$ at 20, 50 and 100 μM, respectively. To our knowledge our application of the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit has not been previously published.

We applied the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit for the measurement of TTRs of the amino acids phenylalanine and tyrosine, but application of the method as recommended by the manufacturer confronted us with analytical problems. The major problem was the measurement of the natural TTRs of L-phenylalanine and L-tyrosine from a standard solution. The natural TTR of L-phenylalanine and L-tyrosine were found to be 2.97% for the natural $m+5$ enrichment ($m/z = 299.3$) of L-phenylalanine and 0.31% for the L-tyrosine ($m+4$) enrichment ($m/z = 417.2$). Both natural TTR were significant higher then the theoretical values of 0.003% for L-phenylalanine ($m+5$) and 0.06% for the L-tyrosine ($m+4$), respectively. The high natural TTR was caused by interfering matrix components probably formed during derivatization because the high natural enrichment was not only noticed with standards but also with plasma samples (pre-infusion).

Because the TTR measurement by LC–MS failed, we decided to refine the method and to develop a LC–MS/MS analysis with better accuracy, more selectivity and sensitivity. However, the application of a LC–MS/MS method required knowledge of the derivatization agent from the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit.

Measurement of TTR of phenylalanine and tyrosine derivatives using LC–MS/MS is only possible if fragmentation reactions and formed product ions can be elucidated. Understanding the fragmentation pattern is only possible when the structure of the amino acid derivatives is known. The Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit manual gives the user some information about the derivatization reaction type applied, but it does not specify which derivatization reagent is used. For the LC–MS analysis of the amino acid derivatives a table with the molecular ion of each amino acid is presented. As mentioned before the method was developed for the measurement of amino acid concentrations by LC–MS but that method was not accurate enough for TTR analysis. Moreover, we had to elucidate the structures of the phenylalanine and tyrosine derivatives.

Zampolli et al. [8] previously described a general derivatization scheme of amino acids containing additional functional groups (amino and carboxyl) with the derivatization agent methylchloroformate (MCF). Combination of results from previous LC–MS and LC–MS/MS experiments (data not shown) and the derivatization reaction schemes described by Zampolli et al. [8] made it possible to identify the derivatization agent to be propylchloroformate. That PCF was indeed the derivatization reagent used as derivatization reagent in the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit was confirmed by LC–MS and LC–MS/MS analyses of (stable isotope labeled) amino acids after derivatization with purchased PCF after being dissolved in 1-propanol. The confirmation to PCF was done by comparison of retention times of a mixture of amino acids derivatized by the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit and by the purchased PCF. Retention times of amino acids from both mixtures were identical while LC–MS spectra and LC–MS/MS fragmentation of both derivatized amino acid mixtures obtained identical mass spectra with equal abundances for all fragments, leading us to the conclusion that the applied derivatization agent in the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit was PCF.

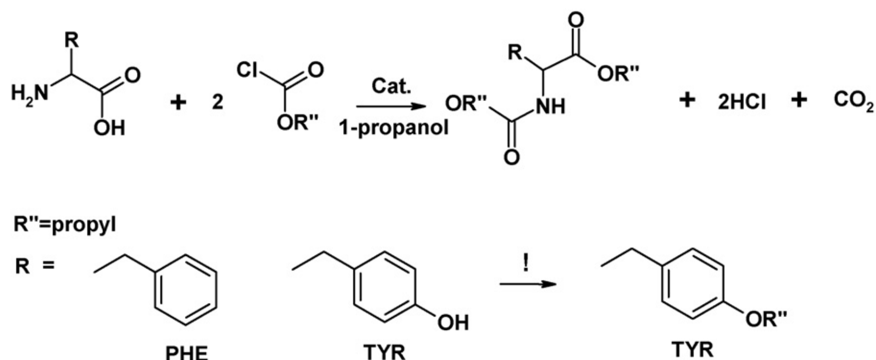

Fig. 1. Derivatization reaction of L-phenylalanine and L-tyrosine with propylchloroformate (PCF) and their derivatives.

The derivatization of amino acids with PCF and amino acid derivatives are illustrated in Fig. 1.

3.1.2. Liquid chromatography

The liquid chromatography of the plasma samples from the clinical study were analyzed using a different gradient, we started at higher water content (38%) instead of the 32% recommended by the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit manual. The higher water content of 38% improved the LC-separation and enabled a separation between co-elution interfering substances and the amino acids of interest. Baseline separation of the amino acids was important because we preferred to have a LC-separation with no interference between the amino acids and with matrix components and the amino acids.

The present method showed baseline separation of L-phenylalanine and L-tyrosine with retention times of 13.09 and 15.49 min, respectively (Fig. 2) when our gradient was applied.

3.1.3. MS/MS experiments

Prior to the start with MS/MS experiments, parameters such as temperature of the heated capillary, sheath gas pressure and spray voltage were tested for their effect on the ion current and to operate the LC–MS system with maximum sensitivity. Increase of the heated capillary temperature (375 °C) resulted in a decrease of the ion current of the PCF derivatives of L-phenylalanine and L-tyrosine, probably due to pyrolysis of the derivatives. Decrease of the temperature caused a similar decrease in ion current, probably due to the lower efficiency of the evaporation of the solvent to release the charged ions, optimal capillary temperature was 325 °C. Changing the sheath gas pressure gave similar results; the sheath gas pressure was either too high or too low and both resulted in a decrease of the ion currents of the PCF derivatives. The optimal spray voltage was approximately 4.5 kV. A higher spray voltage did not further increase the ion currents of the PCF derivatives but created a high and difficult to control spray current, while a lower spray voltage (3 kV) decreased the ion currents by approximately 80%.

The only significant increase in ion current for the PCF derivatives was observed with post column addition of 0.1 mL/min of a solution MeOH/water (2/8) containing 1% HAc. This addition increased the ionization efficiency of the PCF derivatives or decreased ionization suppression of the PCF derivatives by matrix components. The process responsible for the increase in ion current is not clear.

MS/MS experiments with solutions that contain high tracer–tracee ratios of the amino acids (TTR = 50%) were done with a CID energy (20 eV) to obtain a general information about fragmentation. This approach gave sufficient information about the dissociation amino acid derivatives and presented us with valuable information about conservation or loss of the stable isotope labeling within the amino acid tracers. Fig. 2 depicts a typical LC–MS/MS chromatogram of the separation of a stable isotope labeled L-phenylalanine and L-tyrosine mixture showing the mass traces of the MS/MS parent ion transitions of the stable isotope amino acids. Esters typically undergo α-cleavage and may

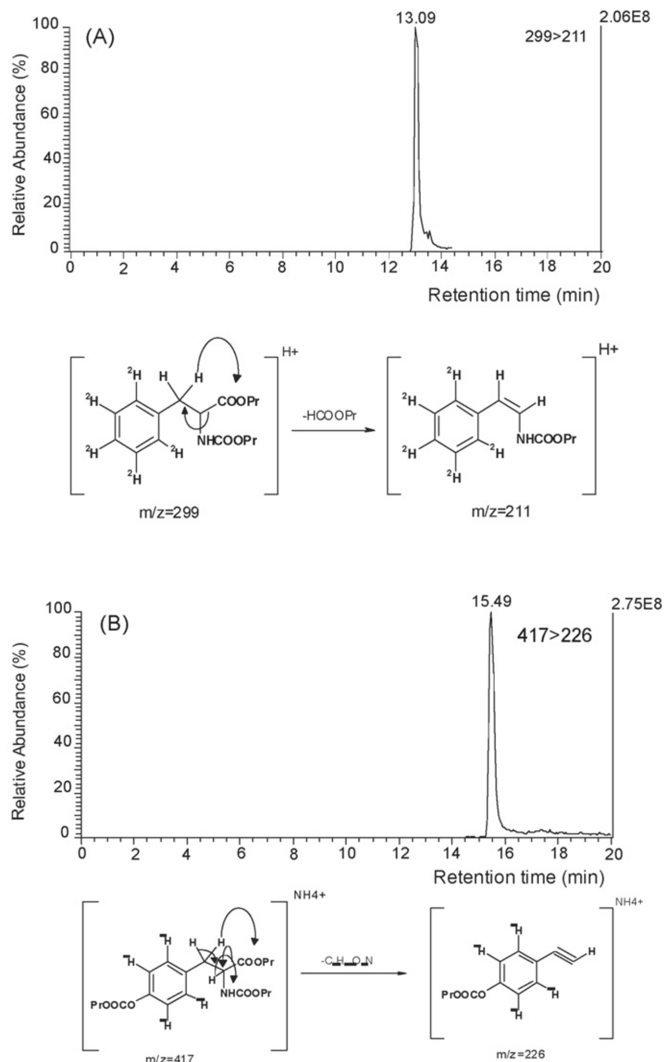

Fig. 2. LC–MS/MS separation of the PCF derivatives of the stable isotope amino acids amino acid (A) L-[*ring*-$^2H_5$]-phenylalanine and (B) L-[*ring*-$^2H_4$]-tyrosine and transition reactions.

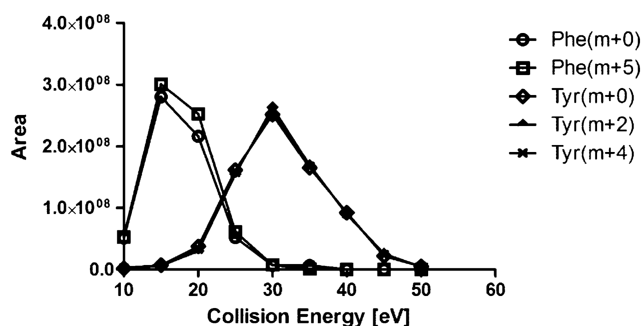

Fig. 3. Areas of fragment ions from CID reactions with argon gas at different collision energies.

also demonstrate McLafferty rearrangements resulting in the loss of a neutral fragment [23]. The product ions with the highest abundances for L-phenylalanine and (L-[*ring*-$^2H_5$]-phenylalanine) PCF derivatives are m/z = 206.3 and 211.3, respectively. Both high abundance fragment ions are the result of α-cleavage and McLafferty rearrangement where a hydrogen atom from the neighboring alkyl group is transferred to the carbonyl oxygen group of the propyl portion of the esters resulting in loss of the neutral HCOOPr fragment (Fig. 2).

Although the MS/MS spectrum of tyrosine showed beside this α-cleavage a fragment ion (m/z = 222.2) with much higher abundance than the α-cleavage fragment (m/z = 325.2), this fragment was identified as a fragment ion resulting from the α-cleavage of the propyl ester in combination with extra loss of the NHCOOPr-group (Fig. 2) also due to rearrangement reaction with the molecule. For the tyrosine tracers, L-[*ring*-$^2H_2$]-tyrosine resulted in fragment ion with m/z = 224.2 and L-[*ring*-$^2H_4$]-tyrosine in a fragment ion with m/z = 226.2, respectively.

Confirmation of α-cleavage was confirmed by fragmentation of a stable isotope labeled amino acid with an α-^{13}C atom (e.g. 1-^{13}C-leucine). Fragmentation of the non-labeled leucine as well 1-^{13}C-leucine resulted in a transition m/z 261 → 172 and not as expected m/z 261 → 173. This transition confirmed the dissociation of the $H^{13}COOPr$-group attached to the α-carbon of the amino acid and loss of the stable isotope marker within the amino acid leucine.

After the fragmentation reactions of phenylalanine, tyrosine as well as their stable isotope labeled analogues were elucidated, the next step was precursor ion-product ion transition optimization. The applied CID energy for every amino acid was optimized by application of different CID energies ranging from 10 to 50 eV (Fig. 3) by a solution with a TTR of 100% for both amino acids. Areas of parent- and fragment ions were determined from selected fragment ions at CID energies with the highest transitions rates (highest areas, Table 1) for phenylalanine at m/z 294.3 → 206.3, L-[*ring*-$^2H_5$]-phenylalanine at m/z 299.3 → 211.3, tyrosine m/z at 413.2 → 222.2, L-[*ring*-$^2H_2$]-tyrosine at m/z 415.2 → 224.2 and L-[*ring*-$^2H_4$]-tyrosine at m/z 417.2 → 226.2, respectively. The results of the CID optimization are illustrated in Fig. 3.

3.1.4. Validation of method and measurement by LC–MS/MS

Accuracy of the TTR measurement was validated by the analyses of TTR calibration curves prepared in an ovalbumin solution (1% in (99 + 1) water/0.5N HCl) with known TTR. Calibration solutions with ratios of 0%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0% and 5.0% TTR and above the natural enrichment at a physiological concentration level of 60 μM were used. Before sample preparation (Section 2.3) of the calibration solutions and LC–MS analysis (Section 2.4) the calibration solutions were deproteinized with 50% TCA solution to mimic matrix effects caused by deproteinization. Measured TTRs were plotted against the calibrator TTRs and calibration lines were analyzed by linear regression analysis. Equations of the TTR calibration curves were $y = 1.0814x$, $y = 1.0861x$ and $y = 1.0866x$ for L-[*ring*-$^2H_5$]-phenylalanine, L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine, respectively while correlation coefficients (r^2) were 0.9997,0.9997 and 0.9998 for L-[*ring*-$^2H_5$]-phenylalanine, L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine, respectively. The observed TTRs were corrected for the natural TTR of tyrosine ($m+2$) and ($m+4$) and for phenylalanine ($m+5$) (Section 1), respectively. The lowest TTR calibrator solution (0.01%) could still be quantified because of the signal/noise ratio (S/N) >10 and was defined as the methods limit of TTR detection for both amino acids. A calibrator solution with a TTR of 0.005% was also measured but due to the high background noise (S/N <3) accurate TTR determination at this ratio was not possible. The results showed that to be able to measure an accurate TTR, the TTR had to be at least 0.01% (0.01 mol%). The variance was less then 10% (5.8%, n = 5) while the variance for the 0.05% TTR and 0.10%TTR were even smaller, 0.77% and 0.51%, respectively. Fig. 4 illustrates the measurement of some of the TTR calibration solutions spiked with 0.01%, 0.05% and 0.1% TTR above the natural TTR of L-phenylalanine and L-tyrosine.

The repeatability of the TTR measurement by the method was investigated by intra- and interday validation by the means of five replicates of an ovalalbumin solution (Section 3.1.4) spiked with a TTR of 6.00% Phe ($m5/m0$), 2.00% Tyr ($m2/m0$) and 0.25% Tyr ($m4/m0$), respectively and prepared for analysis as described in

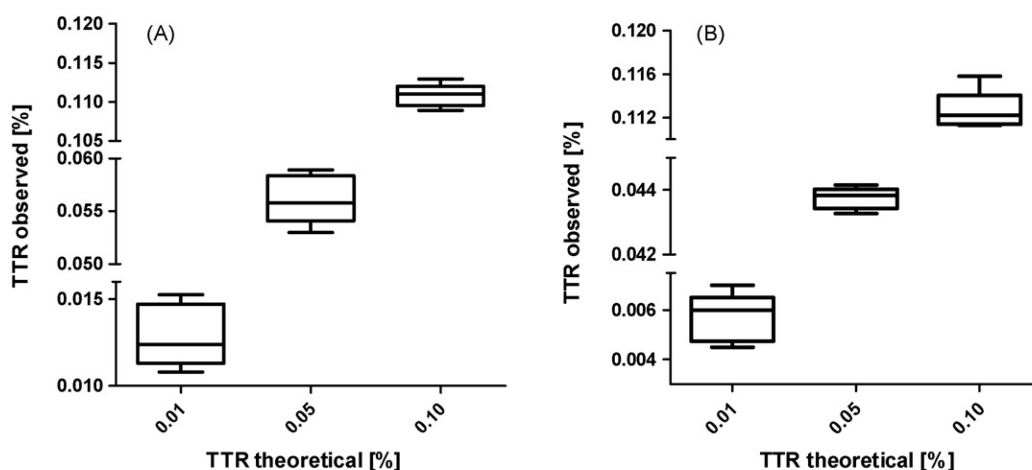

Fig. 4. Measurement (n = 5) of TTR calibration solutions with minimum and maximum range for: (A) L-[*ring*-$^2H_5$]-phenylalanine and (B) L-[*ring*-$^2H_4$]-tyrosine.

Table 2
Validation and ANOVA of the LC–MS/MS method for the determination of spiked TTR in ovalbumin at a physiological concentration of 50 μM ($n = 5$).

TTR	L-[*ring*-$^2H_5$]-phenylalanine	L-[*ring*-$^2H_2$]-tyrosine	L-[*ring*-$^2H_4$]-tyrosine
Intraday validation			
Spiked TTR (%)	6.00	2.00	0.25
Mean measured TTR (%)	6.07	2.04	0.27
Accuracy (%)	1.17	2.00	16.0
Interday validation (16 h later)			
Spiked TTR (%)	6.00	2.00	0.25
Mean measured TTR (%)	6.08	2.06	0.30
Accuracy (%)	1.33	3.00	24.0

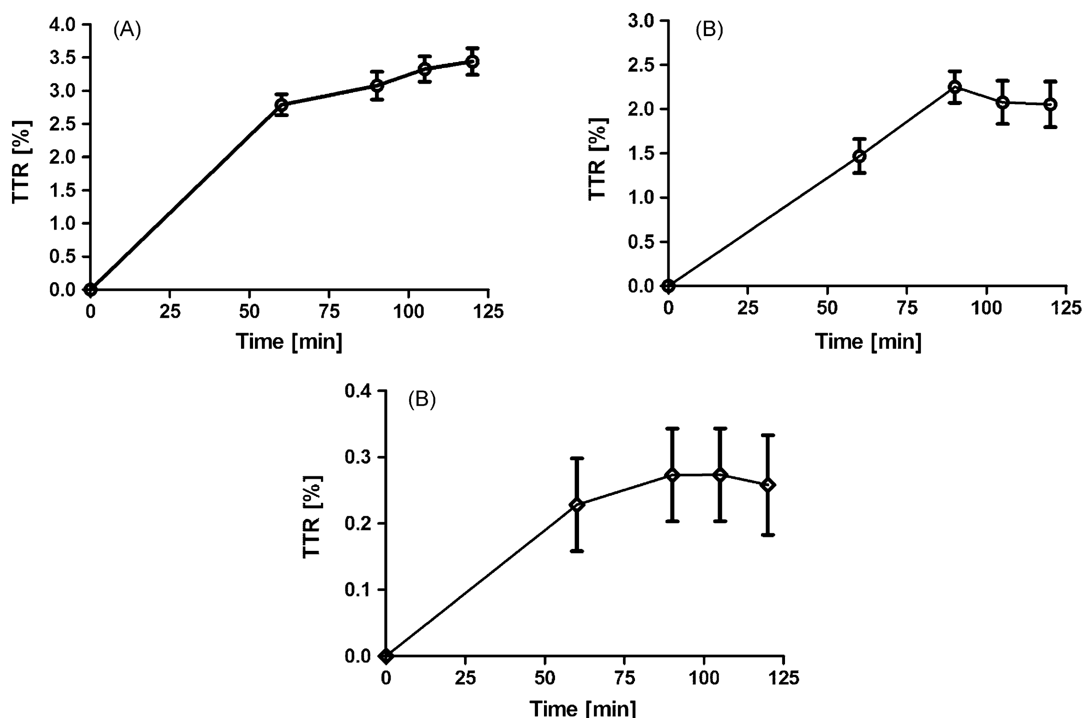

Fig. 5. Measured TTR for (A) L-[*ring*-$^2H_5$]-phenylalanine, (B) L-[*ring*-$^2H_2$]-tyrosine and (C) L-[*ring*-$^2H_4$]-tyrosine in plasma samples from a volunteer subject.

Section 2.3. The accuracy of the method is expressed as the relative standard deviation calculated from the multiple replicate measurements from these spiked ovalbumin solutions. All obtained intra- and interday accuracy values were acceptable for our human study purposes.

The method's repeatability was calculated from analysis of variance (ANOVA) [24] from obtained results intra- and interday validation (Table 2).

The calculated repeatability of the method was 0.999 and proved the excellent repeatability of the new method for the determination of protein kinetics in metabolism studies with human subjects.

The developed method is compared to a GC–MS/MS [3] method that reported a detection limit of 0.1% TTR. Our method is 10 times more sensitive and used substantially less plasma (25 μL instead of 500 μL). Our new developed method is also less laborious and time consuming due to short derivatization time (1 min instead of 1 h), no multiple lyophilization steps of the plasma sample and just one simple derivatization step instead of two applied for the GC–MS/MS analysis.

3.2. Application of the method

After validation of the method, plasma samples from a clinical study were analyzed ($n = 5$) for the stable isotope enrichment of L-phenylalanine and L-tyrosine. The plasma samples were from critically ill patients diagnosed with a septic shock. The TTR reached his maximum for L-[*ring*-$^2H_5$]-phenylalanine after 90 min infusion and was stable at a value of $3.33 \pm 0.19\%$ until the end of the tracer infusion. The enrichment for L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine also reached the maximum TTR between 90 and 120 min after the start of the L-[*ring*-$^2H_5$]-phenylalanine and L-[ring-$^2H_2$]-tyrosine infusion at a TTR of $2.40 \pm 0.43\%$ (corrected for the natural tyrosine ($m+2$) enrichment) and $0.29 \pm 0.07\%$ for L-[*ring*-$^2H_2$]-tyrosine and L-[*ring*-$^2H_4$]-tyrosine, respectively. The enrichment of the amino acids in time is illustrated by the TTR curves in Fig. 5. Whole-body protein breakdown (WbPB: 138.3 ± 10.3 μmol kg^{-1} h^{-1}), whole-body protein synthesis rates (WbPS: 114.6 ± 8.5 μmol kg^{-1} h^{-1}) and net protein loss (23.7 ± 1.8 μmol kg^{-1} h^{-1}) were calculated as described by Engelen et al. [20]. These protein kinetics are about 3 times higher than in age-matched healthy controls [20].

4. Conclusion

The development of a method for the measurement of very low stable isotope enrichments using the Phenomenex EZ:faast amino acid LC–MS free (physiological) amino acid analysis kit used for plasma sample preparation led to a newly selective and sensi-

tive robust LC–MS/MS method that enables the determination of very low tracer/trace ratios (min 0.01%) for the deuterated amino acids L-phenylalanine and L-tyrosine. The method was successfully applied to plasma samples from clinical studies studying protein metabolism. The method is compared with other analytical methods less laborious and time consumptive and more sensitive [3,5].

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References

[1] G.N. Thompson, P.J. Pacy, H. Merritt, G.C. Ford, M.A. Read, K.N. Cheng, D. Halliday, Am. J. Physiol. 256 (1989) E631.
[2] R.R. Wolfe, D.L. Chinkes, Isotope Tracers in Metabolic Research: Principles and Practise of Kinetic Analysis, Wiley, New York, 2004.
[3] H. Schweer, B. Watzer, H.W. Seyberth, A. Steinmetz, J.R. Schaefer, J. Mass Spectrom. 31 (1996) 727.
[4] B.W. Patterson, F. Carraro, R.R. Wolfe, Biol. Mass Spectrom. 22 (1993) 518.
[5] R.J. Meesters, H.M. van Eijk, G.A. ten Have, A.A. de Graaf, K. Venema, B.E. van Rossum, N.E. Deutz, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 854 (2007) 57.
[6] M.B. Goshe, V.E. Anderson, Anal. Biochem. 231 (1995) 387.
[7] J.B. Laurens, X.Y. Mbianda, J.B. Ubbink, W.J. Vermaak, J. Chromatogr. B Biomed. Sci. Appl. 762 (2001) 127.
[8] M.G. Zampolli, G. Basaglia, F. Dondi, R. Sternberg, C. Szopa, M.C. Pietrogrande, J. Chromatogr. A 1150 (2007) 162.
[9] C.D. Marquez, S.T. Weintraub, P.C. Smith, J. Chromatogr. B Biomed. Appl. 658 (1994) 213.
[10] T. Mohabbat, B. Drew, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 862 (2008) 86.
[11] A.A. Badawy, C.J. Morgan, J.A. Turner, Amino Acids 34 (2008) 587.
[12] H.M. van Eijk, D.R. Rooyakkers, P.B. Soeters, N.E. Deutz, Anal. Biochem. 271 (1999) 8.
[13] D.H. Chace, T.A. Kalas, E.W. Naylor, Annu. Rev. Genomics Hum. Genet. 3 (2002) 17.
[14] D. Fekkes, J. Chromatogr. B Biomed. Appl. 682 (1996) 3.
[15] E.L. Schwarz, W.L. Roberts, M. Pasquali, Clin. Chim. Acta 354 (2005) 83.
[16] P. Araujo, G.F. Wassermann, K. Tallini, V. Furlanetto, C.R. Vargas, C.M. Wannmacher, C.S. Dutra-Filho, A.T. Wyse, M. Wajner, Neurochem. Int. 38 (2001) 529.
[17] A.N. Fonteh, R.J. Harrington, M.G. Harrington, Amino Acids 32 (2007) 203.
[18] D. Sun, A. Ferrando, R. Wolfe, T. Farkas, LC–GC N. Am. (2005) 22.
[19] M.P. Engelen, N.E. Deutz, R. Mostert, E.F. Wouters, A.M. Schols, Am. J. Clin. Nutr. 77 (2003) 868.
[20] M.P. Engelen, N.E. Deutz, E.F. Wouters, A.M. Schols, Am. J. Respir. Crit. Care Med. 162 (2000) 1488.
[21] A. Del Roso, M. Bombara, V. Fierabracci, P.V. Fosella, Z. Gori, M. Masini, P. Masiello, M. Pollera, E. Bergamini, Acta Biol. Hung. 42 (1991) 87.
[22] R.A. Bank, E.J. Jansen, B. Beekman, J.M. te Koppele, Anal. Biochem. 240 (1996) 167.
[23] D.L. Pavia, G.M. Lampman, G.S. Kriz, Introduction to Spectroscopy: A Guide for Students of Organic Chemistry, Saunders Golden Sunburst Series, Harcourt College Pub, Washington, 1979.
[24] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375.