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Measurement of amino acid isotope enrichment by liquid chromatography mass spectroscopy after derivatization with 9-fluorenylmethylchloroformate

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Abstract

An automated method is described to measure tracer-tracee-ratios (TTR) of plasma amino acids after their separation as 9-fluorenylmethylchloroformate derivatives. In a 45 min cycle, 5 μl plasma aliquots were derivatized, HPLC separated and subjected to electrospray ionization. By applying source collision induced dissociation, derivatives were dissociated at the peptide bond releasing the originating amino acids into the mass spectrometer. This approach enabled the determination of plasma amino acid TTRs with a standard deviation between 0.15 and 0.36%, which is sufficient to study the fate of infused tracers and their conversion products in an *in vivo* experiment in humans.

Keywords: Amino acid; Stable isotope; HPLC; Mass spectrometry

1. Introduction

Today, it is widely recognized that measurement of the dilution of administered amino acid stable isotope tracers in the body pool (expressed as the tracer-tracee-ratio: TTR) can provide valuable information about changes in protein synthesis and/or breakdown or specific metabolic pathways of interest. As it only requires the infusion of a tracer into the bloodstream and collection of blood samples before tracer infusion (for natural enrichment) and in the period thereafter, this approach is presently a common and well-tolerated method to study metabolism in the intact *in vivo* situation in experimental animals as well as in humans [1,2].

To determine TTRs of target amino acids, mass spectrometry is required [3]. In the early days, dedicated isotope ratio mass spectrometers with a very high precision were applied to collect the required data [4], while more recently the less expensive combination of gas chromatography and mass spectrometry (GC–MS) has been popularized [5,6]. Today however, atmospheric pressure ionization (API) techniques like electrospray enable the coupling of liquid chromatography with mass spectrometry (LC–MS). Especially for the analysis of polar compounds like amino acids, which are otherwise difficult to analyze using gas chromatography, this technique provides new opportunities for sample preparation, optimization of chromatographic separations and MS-analysis.

The method described herein is designed to exploit this new approach and provide an easy alternative to traditional GC–MS or gas chromatography–combustion–mass spectrometry (GC–C–IRMS) methods. Although we already demonstrated the power and applicability of LC–MS in a previous publication [7], the troublesome estimation of low enrichments in physiological samples due to low abundant co-eluting contaminants remained a problem.

In the present paper we have anticipated on this, using an alternative derivatizating reagent, 9-fluorenylmethylchloroformate (FMOC). Amino acids react with FMOC through the

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formation of a peptide bond, resulting in chemically stable fluorescent derivatives, which can be separated by reversed phase chromatography. Interestingly, the hydrolysis of protein peptide bonds using mass spectrometry has become a key tool in proteomics [8,9] and, considering the peptide bond between amino acids and FMOC is of the same nature, we exploited this feature to enable an additional level of selectivity (and thus precision) through the application of MS/MS techniques.

These modifications enabled us to enhance the precision of amino acid TTR measurements in blood, compared to our previously published approach.

2. Materials and methods

2.1. Solvents

All solutions were prepared with ultra-pure water, generated by a Super-Q system (Millipore, Amsterdam, The Netherlands). Solvent and reagents were of spectrophotometric grade or better and mostly obtained from Acros (s-Hertogenbosch, The Netherlands).

A high-pressure gradient was created through mixing of 1 mmol/l ammonium formate in water (Solvent A) and 1 mmol/l ammonium formate in acetonitrile:water, 95:5 (v/v) (Solvent B). Samples were loaded onto a trap column using 0.2% (v/v) formic acid in water (Solvent C), which was regenerated by flushing with 100% acetonitrile (Solvent D). Helium sparging was used to degas the solvents.

2.2. Reagents

The derivatization reagent was prepared by dissolving 3 mg 9-fluorenylmethylchloroformate (FMOC, Sigma–Aldrich, Zwijndrecht, The Netherlands) in 4500 μl acetonitrile. Potassium borate buffer (0.25 M, pH 10.4) was prepared by titration of 0.25 M boric acid with 6 N potassiumhydroxyde solution. Formic acid 0.6% (v/v) was used to terminate the reaction and to neutralize the reaction mix, enabling optimal trapping of the derivatives onto a "trap"-column.

2.3. Standards

Amino acid standards were prepared by dissolving analytical grade solid amino acids (Sigma) in water to a final concentration of 50 μmol/l each. Standards were divided in 1 ml portions and stored at −80 °C prior to use.

2.4. Sample preparation

Heparinized blood samples from human volunteers were collected on ice, immediately centrifuged at 8500 × *g* in a Model 5413 biofuge (Heraeus, Dijkstra Vereenigde, Lelystad, The Netherlands) for 10 min at 4 °C. Plasma supernatant was deproteinized through addition of 5-sulfosalicylicacid (SSA) to a final concentration of 6 mg/100 μl plasma, vortex mixed vigorously, frozen immediately in liquid nitrogen and stored at −80 °C. Before analysis, samples were thawed at 4 °C, vortex-mixed again and centrifuged at 50,000 × *g* in a Model biofuge Stratos (Heraeus) for 10 min at 4 °C [10].

2.5. Equipment

The HPLC system consisted of a Model 233 XL sample processor (Gilson, Rijswijk, The Netherlands), equipped with Peltier chilled sample/reagent trays and two Rheodyne 6 port high-pressure valves. On one valve a 50 μl stainless steel loop was mounted, while a "trap-column" (7.5 × 2.1 mm i.d., filled with Allsphere C8 5 μm, Grace, Breda, The Netherlands) was

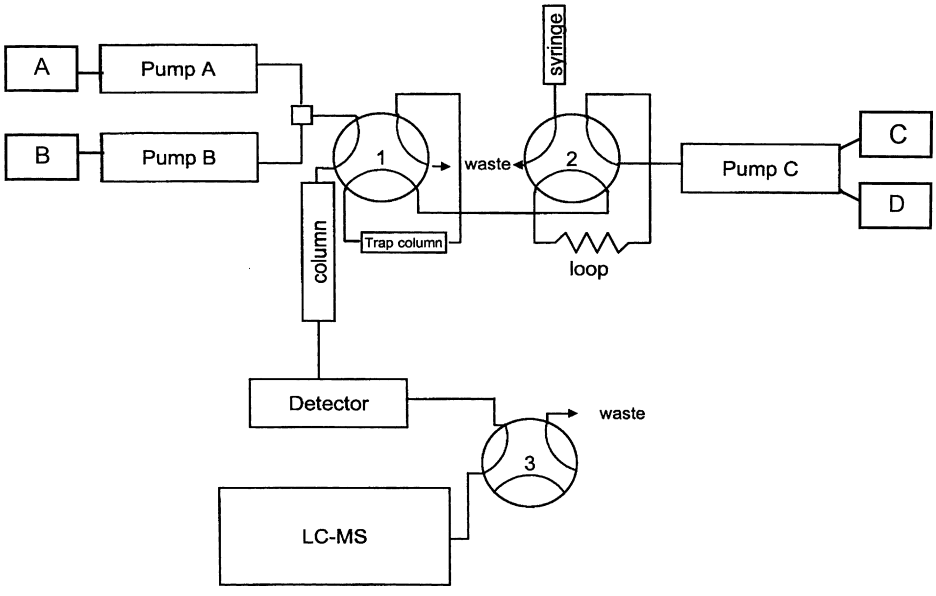

Fig. 1. Schematic overview of the flow-path (with permission from Elsevier adapted from Ref. [7]). Step 1: Load sample reaction mix to loop. Step 2: Pump C flushes the mix onto the trap-column, removing reagent-buffers-solvents. Step 3: Elution of trapped derivatives from trap column onto the analytical column and start of gradient. Meanwhile sample loop and valve are flushed with Solvents C and D to clean out.

attached to the second valve (Fig. 1, with permission of Elsevier [7]).

A high-pressure gradient between Solvents A and B was generated using two Model PU-1585 pumps (Jasco Benelux, Maarssen, The Netherlands). An additional Model PU 980 pump, equipped with a Model LG-1580-02 low-pressure gradient unit was used to pump sample and reagents onto the trap column using Solvents C and D. In between runs, this pumping system was used to flush the sample loop and its valve alternating with Solvents C and D to prevent sample carry-over.

The separation was performed on a 150 mm × 2.1 mm, 3 μm, Alltima HPLC-column, mounted in a Spark Mistral column oven (Separations, Hendrik Ido Ambacht, The Netherlands) kept at 25 °C.

Before entering the mass spectrometer, fluorescence of the eluting derivatives was monitored (λ_{ex} = 265 nm; λ_{em} = 310 nm) using a Model FP 1520 fluorescence detector (Jasco Benelux), from which the cuvette was exchanged for a quartz cylinder (6 mm outside diameter, 0.6 mm inside diameter) to withstand the pressure built-up (about 15–20 bars) from the coupled electrospray probe of the MS system. Fluorescence data were collected through a Total Chrom data acquisition system (Perkin-Elmer, Zoetermeer, The Netherlands).

The bench top mass spectrometer was a Model LCQ-Classic (Thermo Electron, Veenendaal, The Netherlands), equipped with an electrospray (ESI) probe. To obtain reliable ion statistics, the ion time was set to 1000 ms using 1 micro scan only. Maximal sensitivity was obtained with the heated capillary set to 210 °C and sheath and auxiliary gas set to 99 and 20 arbitrary units each. The tube lens offset was 25 V and the spray-voltage was 4.5 kV. To maximize the system's sensitivity for the lower concentrated isotopomeric peaks, the electron multiplier gain was increased (from −860 V to −1250 V). Due to the increased signal, the systems automatic gain control (AGC) limits the number of ions entering the trap, which is bad for ion statistics. By increasing the systems "target value" proportionally (from $2E^7$ to $5E^8$) a five times better signal to noise ratio was obtained with the same number of ions in the trap, thus prohibiting "space-charging" effects which otherwise might occur [7].

2.6. Procedure

Five μl SSA deproteinized plasma supernatants together with 25 μl water were added to 4 ml WISP-style vials holding spring adjusted 300 μl glass inserts and queued in a Gilson peltier chilled sample tray set to 10 °C (Fig. 1, with permission from Elsevier, see also Ref. [7]). For each sample, the preprogrammed reaction sequence of the sample processor was initiated with the addition (and mixing) of 50 μl FMOC reagent, followed by 50 μl of 0.25 M potassium borate buffer. After a 60 s reaction period, 100 μl of the reaction mix was transferred to a 300 μl spring adjusted "mix vial" pre-filled with 30 μl 0.6% (v/v) formic acid in water. After a 60 s mixing period, 100 μl of this reaction mixture was injected into the sample loop (which is thus twice overloaded), mounted on the left valve. Next, this valve was switched and pump C flushed the sample-reaction mix onto the trap column mounted on the right valve using Solvent C. After a 6 min flush period to elute all buffer salts from the trap column to drain, valve 2 was switched, placing the trap column into the flow path of the analytical column system. Gradient elution (Table 1) was initiated and the column effluent was directed into the LCQ system by switching its bypass valve. Meanwhile, valve 1 and its mounted sample loop were flushed alternating with Solvents C and D to clean out reaction mix remainders and thus prevent sample carry-over. After elution of the last amino acid derivative, the analytical column was regenerated by pumping 100% Solvent B.

Table 1
Gradient conditions

Time (min)	A (%)
0	75
1	65
25	40
32	12
32.1	0
38	0
38.1	75

2.7. Determination of tracer-tracee-ratios (TTR)

Many elements have isotopes, some of which are stable. Carbon for instance, the backbone of biological molecules has a high natural abundance (1.1%) for its ^{13}C isotope. This feature determines that a given molecule will not just show up as a single peak at the calculated mass in a mass spectrum, but as a set of peaks, called the isotopomeric envelope. The theoretical intensities of these peaks relative to the intensity of the non-stable isotope containing "base-peak" can be calculated [3]. Here, we will refer to the isotopomeric peaks of a given molecule with mass M as $M+1$ (containing one heavy isotope), $M+2$ (containing two heavy isotopes) and so on. Today compounds like amino acids can be purchased, in which one or more atoms are exchanged for (a) stable isotope(s). These "tracer" molecules usually have the same physical properties as their natural counterparts (the "tracee"), but with the use of a mass spectrometer they can be distinguished from each other and their relative contribution can be quantified. If a known amount of these "tracer" molecules is supplied to an organism, a change in the ratio between the tracer and the tracee, known as the tracer-tracee-ratio (TTR), can provide information about ongoing physiological processes. Thus, the TTR is:

$$\mathrm{TTR}\,(\%) = \left(\frac{[\mathrm{tracer}]}{[\mathrm{tracee}]}\right) \times 100\%$$

To calculate a change in TTR following the infusion of an isotope, one has to subtract the natural abundance measured before infusion (see above) from the measured value following infusion. This approach not only allows comparing the measured with the theoretical TTR, validating analytical performance of the applied system, but also corrects for any reaction performed on the target metabolite (like derivatization). The increase (Δ) in TTR in time, resulting from the supplied tracer can now be

Table 2
Stable isotope infusion protocol in the human experiment

	Prime (μmol)	Continuous infusion (μmol/h)
L-[$^{2}H_{3}$-^{13}C]Citrulline	43.8	20.4
L-[guanidine-$^{15}N_{2}$]Arginine	410.6	394.9
L-[*ring*-$^{2}H_{2}$]Tyrosine	71.2	57.9
L-[*ring*-$^{2}H_{5}$]Phenylalanine	164.3	169.4
L-[1-^{13}C]Leucine	164.3	180.7

Prime was infused as a bolus of 45 ml; 2-h continuous infusion at 100 ml/h in all subjects ($n = 8$ healthy men). Amino acid tracer solutions were prepared from certified 98 + % purity chemicals, purchased from Cambridge Isotope Laboratories (CIL, Massachusetts, USA). Tracers were tested for isotopic and chemical purity and prepared solutions were submitted to standard heat sterilization and tested for sterility before application.

calculated according to:

$$\Delta\text{TTR}\,(\%) = \text{TTR}_{(\text{post infusion})}(\%) - \text{TTR}_{(\text{pre infusion})}(\%)$$

To measure the change in TTR we usually monitor target metabolites in a 10 AMU wide selected ion monitoring (SIM) scan window, allowing us to determine the target peak areas of the isotopomeric envelop $M - M + 5$, which is usually sufficient for the tracers we apply.

2.8. Human experimental protocol

Approval for the experimental protocol of this study was obtained from the local Medical Ethical Committee (MEC 04-021). Eight healthy male human volunteers aged between 50 and 70 years were recruited through advertisement, screened to exclude metabolic diseases, and written informed consent was obtained. After an overnight fast of at least 10 h, body weight and height were measured and whole body fat free mass (FFM) was determined by bioelectrical impedance analysis (BIA). Mean age was 64 ± 2 years, mean body mass index (BMI) was 25.8 ± 0.9 kg/m^2.

After insertion of a catheter in the right antecubital vein, a blood sample was taken for baseline measurements. Immediately thereafter, a primed-constant 2 h intravenous infusion of stable isotopes (Table 2: 100 ml/h) was started ($T = 0$ h) using a calibrated pump (IVAC Corporation, San Diego, USA). The arm was positioned approximately 10 cm above the level of the heart, so that the heart would receive a uniform dose of tracers throughout the experiment. A second catheter was inserted in a superficial dorsal vein of the hand of the contra-lateral arm, which was placed in a thermostatically controlled hot box (internal temperature: 60 °C), a technique to mimic direct arterial sampling. Arterialized venous blood was sampled at 60–90–105 and 120 min after start of the tracer infusion, to reassure a tracer steady state, usually occurring within 1 h. Blood samples were collected in pre-chilled, heparinized tubes (Becton Dickinson vacutainer system, Franklin Lakes, New Jersey, USA) and prepared as described above.

3. Results and discussion

3.1. General

An ion-trap mass spectrometer is a relatively cheap, but very versatile system. Although there are only a limited number of ions involved in each individual "scan", the cumulative measurements across an eluting chromatographic peak make it very well possible to use an ion-trap system to measure isotopic distributions with a surprising precision. We evaluated this capability, by comparing an ion-trap performance with some representative alternative mass spectrometers [11]. This evaluation revealed that an ion-trap system is very well capable to accurately estimating the theoretical abundance (TTR = 8.43%) of the arginine $M + 1$ isotopomer (TTR measured = 8.55%), although a beam instrument (triple quadrupole system) can provide these data more precisely as expressed by a lower relative standard deviation (R.S.D.) (0.04% compared with 0.09% obtained with the ion-trap). An even more complex (and expensive) hybrid system like a Q-TOF performs only equally to an ion-trap system (R.S.D. also 0.09%) for this specific application. Considering these facts, an ion-trap is perhaps an unexpected but nevertheless easy-to-use and sensitive alternative to study isotopic enrichments in clinical experiments. We confirmed this statement by measuring the isotopic enrichment of free amino acids in physiological fluids following the chromatographic separation of o-phthaldialdehyde (OPA) derivatizated amino acids [7]. Although this application proved to be very satisfactory to estimate the more concentrated $M + 1$ and $M + 2$ isotopomers, quantification of the higher isotopomers, especially in physiological fluids of patient samples appeared more difficult, mainly due to baseline noise and co-eluting (plasma) contaminants. So an additional level of specificity, especially for low concentrated or low enriched metabolites is of importance. Tandem MS (or MS/MS) would be a logical answer to this problem and so we first applied this on the OPA-amino acid derivatives of the above application [7]. Unfortunately, OPA binds to amino acids very tightly by reacting through a ring-closure. As a result we could not generate suitable fragment ions for all derivatives with the ion-trap system used in this application.

For this reason, and because OPA does not react with the also important "imino acids" proline and hydroxyproline, alternative reagents like fluorescamine [12], dansyl- [13], dabsylchloride [14] and 9-fluorenylmethyl-chloroformiate (FMOC) [15] were considered. From these, only FMOC reacted rapidly with both primary and secondary amino acids at room tem-

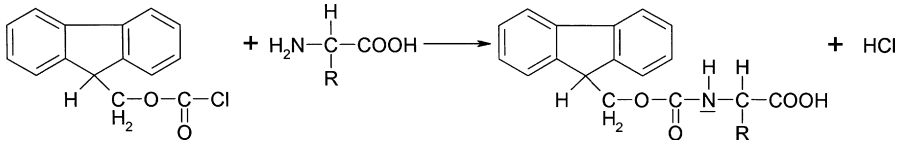

Fig. 2. Reaction of FMOC with amino acids.

perature, enabling an on-line automated derivatization (Fig. 2). Moreover, from a previous study [16] we already concluded that the peptide bond of amino acid FMOC derivatives could easily be hydrolyzed, thus providing the potency to accomplish the desired MS transition. Furthermore, FMOC had already been applied successfully in combination with LC–MS analysis [17] to the enhancement of sensitivity, confirming it was easily possible to create ions through electrospray ionization.

3.2. Method development

3.2.1. Chromatography

In spite of these favorable properties, the limited solubility of the FMOC-reagent conflicts with the goal of a chromatographer to minimize sample dilution, while the accurate MS measurement of lowly concentrated isotopomeric peaks also requires the injection of an as large as possible sample amount. To prevent reagent precipitation, sample derivatization requires the addition of a large volume of reagent mix at a low concentration, diluting the resulting FMOC-derivatives in a large volume with a high concentration of organic solvent. As the chromatographic separation of amino acid FMOC derivatives under ideal circumstances is already difficult (the bulky FMOC group minimizes existing differences in (a) polarity between amino acid residues), this dilution stresses separation even more. Additionally, the high concentration of organic solvent in the sample-reagent mix would cause the derivatives to pass the column un-retained. If the goal of the present study would have been an HPLC separation only, this problem could be addressed by the application of high salt solvents, buffering the sample-reagent mix, but this conflicts

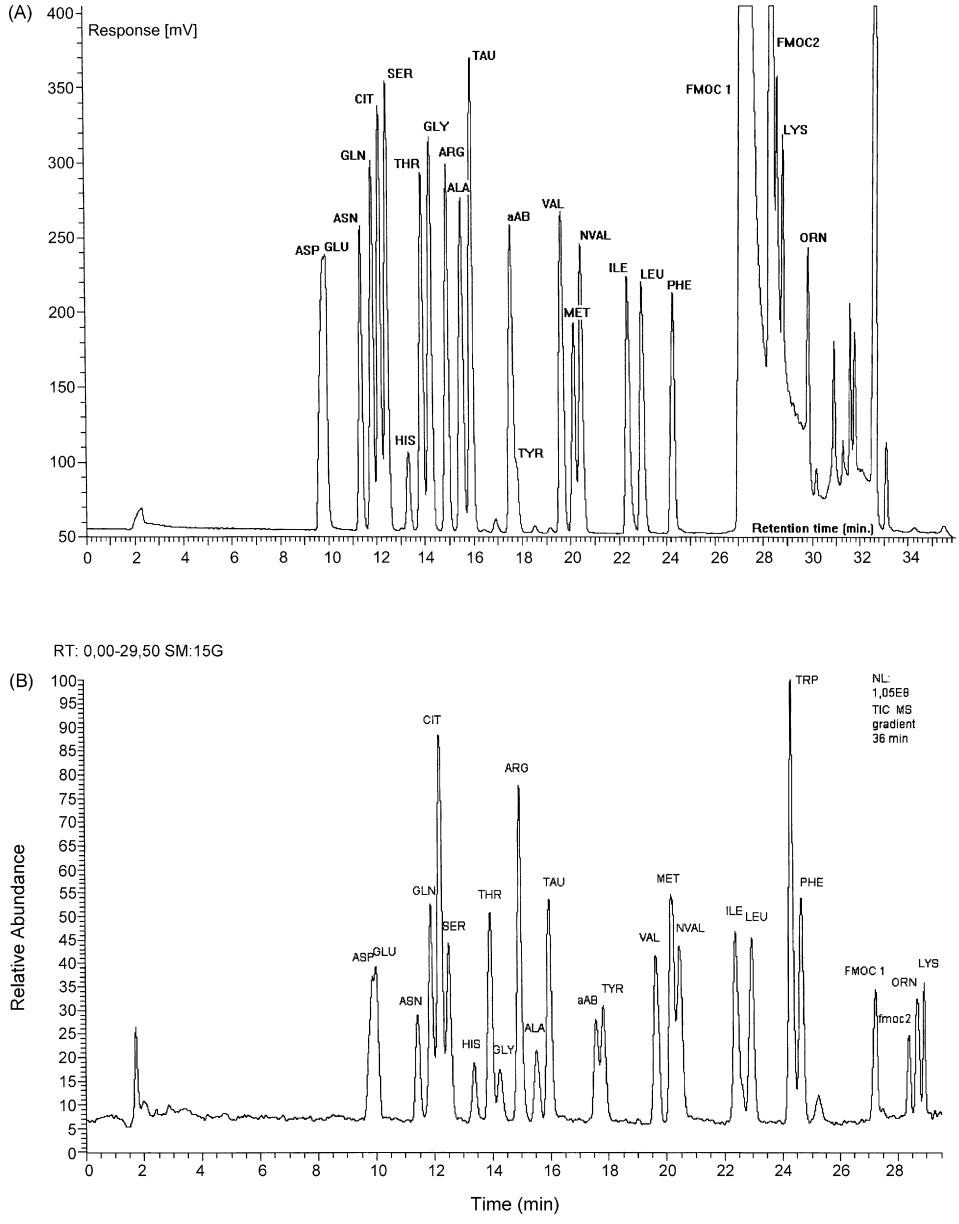

Fig. 3. Panel A: Fluorescent trace of a chromatographic separation of an FMOC derivatized 50 μmol/l amino acid standard. Panel B: MS Total Ion Current (TIC) trace of a chromatographic separation of an FMOC derivatized 50 μmol/l amino acid standard.

with the desired LC–MS approach which requires low solvent salt concentrations, to minimize chemical noise and prohibit blocking of the MS entrance.

One way to solve this problem is an off-line extraction, but this would enhance the workload. Therefore, we alternatively applied column switching (Fig. 1, with permission from Elsevier, see also Ref. [7]). Basically, the sample reagent mix is first injected into a sample loop, but instead of taking up this mixture directly into the flow path of the analytical column, a third pump is used to elute the mixture onto a "trap-column" retaining the target derivatives, but allowing to elute the reagent buffer to drain. Next, the "trap-column" is switched into the flow path of the analytical column and the gradient is started. To compensate for the band broadening occurring due to the migration of the target components over the trap column, an allsphere trap column was used which has a lower carbon load as compared to the alltima analytical column (both Alltech, Breda, The Netherlands). This combination causes a focusing on top of the analytical column prior to the start of the chromatographic separation.

The next problem to solve was how to create a suitable separation of the amino acid derivatives within the solvent limitations set by the MS requirements. The choice was to either develop the separation at neutral pH, using either mixtures of water and organic solvent only or with the addition of low concentrated (up to 10 mmol/l) volatile ammonium acetate or formate buffers, or to apply acidic (acetic or formic acid) solvents.

The use of water-organic solvent combinations only, prohibited essential separations, but the addition of a few milli-mols ammonium acetate or formate improved these separations drastically (Fig. 3). Although both acetic and formic acid also enabled a satisfactory separation, they required at minimum a concentration of 50 mmol/l to obtain satisfactory peak symmetry, but these high concentrations degraded the MS signal.

3.2.2. Mass spectrometry

Either atmospheric pressure chemical ionization (APCI) or electrospray (ESI) can be applied to introduce the separated derivatives into the LC–MS system. Considering the more a-

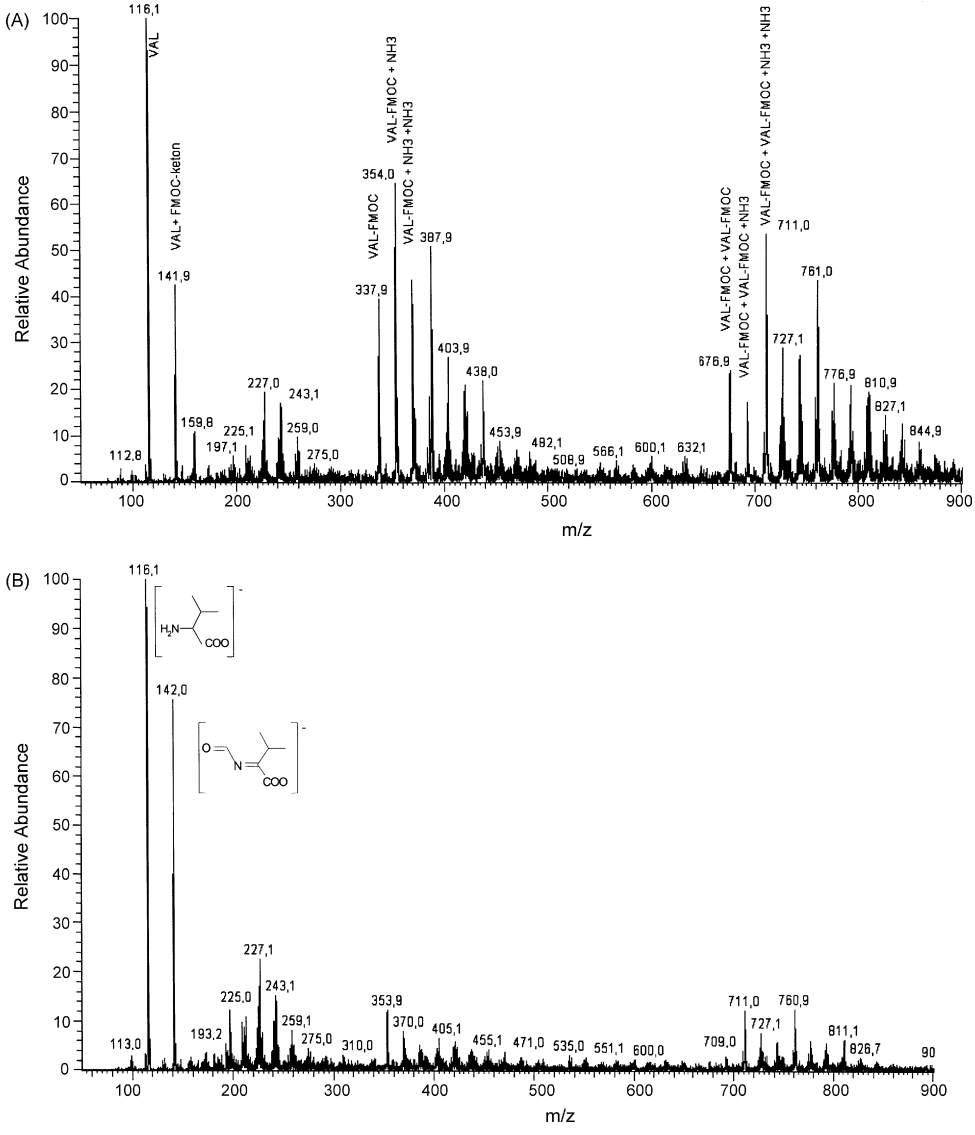

Fig. 4. Panel A: Normal MS spectrum of fmoc derivatized FMOC Valine. Panel B: Application of 20% source CID on FMOC-Valine.

polar nature of FMOC-amino acid derivatives we first tried APCI. Unfortunately, one of the key components in our research, the most basic amino acid arginine, could not enter the MS-system as long as the corona discharge was on. Unfortunately, arginine co-eluted with other important amino acids. To measure them all, the corona would have to be switched on and off in alternating scans, but this would stress the system electronics too much. Therefore, APCI was abandoned and we turned to use ESI only.

In electrospray mode, the MS noise level increased drastically when formic or acetic acid was applied at concentrations above 5 mmol/l. To obtain satisfactory separations however, the acid concentration should preferably be above 50 mmol/l (see previous section) and thus acidic solvents were abandoned too. Alternatively, low concentrations of ammonium-formate provided a satisfactory separation in combination with a low MS noise level. Unfortunately, the mass spectra of eluting derivatives appeared to be very complicated. The major ions observed were not the molecular ions of the derivatives as expected, but cluster-ions, ammonia adducts, unknown adducts and even the free amino acids were instead most dominant (Fig. 4, panel A). In addition, with changing amino acid (derivative) concentrations the ratio between these peaks shifted and thus no linear calibration curve could be constructed between the concentration of any amino acid and either one of its derivative ions.

Considering the above, we were faced with a dilemma. It seemed impossible to obtain a satisfactory signal in MS mode in combination with a satisfactory chromatographic separation. Therefore, we switched from standard MS mode to apply source collision induced dissociation (CID) in combination with an ammonium formate solvent system. In this way a satisfactory chromatographic separation and acceptable MS noise level was assured (Fig. 3). Source CID works by the application of an additional potential difference between the entrance of the MS system (in the applied ion-trap system: the heated capillary) and the first lens. As a result, the incoming ions are accelerated in a pre-vacuum region where they thus collide with considerable amounts of air and solvent molecules, causing fragmentation. In the present situation, however, source CID application provides a major benefit compared to regular MS/MS. In contrast to regular MS/MS, it not only hydrolyses the peptide bond between the amino acids and the FMOC group (for review see [9]), but it does so also with adducts of amino acid-FMOC derivatives. As a result, the complex mass spectrum of an eluting FMOC-valine peak obtained without CID (Fig. 4, panel A) is simplified to mainly the free amino acid (in Fig. 4, panel B the m/z 116^- ion of valine) and another product-ion of FMOC-valine obtained through a breake-up of the bond between the carbonyl carbon and the oxygen of the FMOC molecule, resulting in the plus 26 (m/z 142) ion. In contrast to regular MS/MS, where just one derivative (adduct) each time can be selected, now all derivative adducts were fragmented in the same process, resulting in maximal sensitivity. In addition, this approach adds a level of selectivity as the focus is now on the masses of the free amino acids instead of the amino acid FMOC derivatives. The interfering unknowns usually cannot make this transition and thus do not interfere anymore.

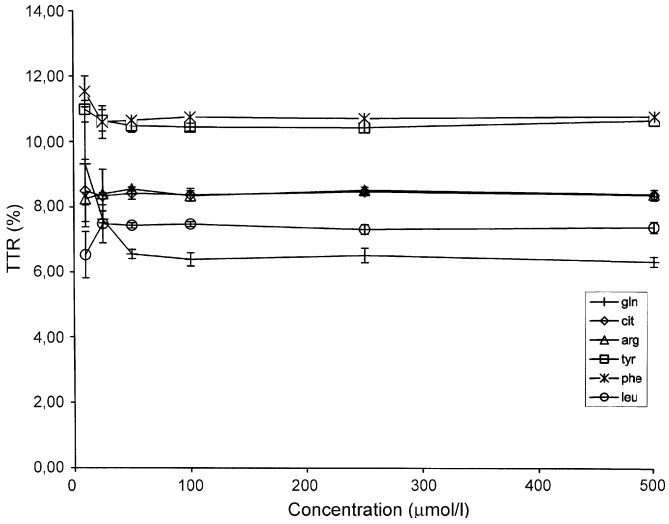

Fig. 5. Relation between TTR and concentration.

3.3. Application

3.3.1. Linearity, sensitivity and precision

Using the above described approach the linearity was investigated. Herefore, calibration curves were constructed using aqueous standards in the range from 10 to 190 μmol/l. A linear calibration curve was obtained with on average an R^2 of at least 0.99. In addition, we measured the $M+1/M+0$ TTR of most of the physiological important amino acids at a concentration of 50 μmol/l in tenfold. This is demonstrated in Table 3 and indicates that the standard deviation varies between 0.15 and 0.36%. Using our previous approach with the o-phthaldialdehyde (OPA) label (7), we could reach this precision only at 5 times higher concentrations (250 μmol/l).

To determine the precision of the TTR measurement in relation to the concentration, the natural abundances $M+1/M+0$ of six typical amino acids were determined in the range from 10 to 500 μmol/l. Fig. 5 shows that below the 50 μmol/l level the stan-

Table 3
System precision at 50 μmol/l level ($N=10$)

	GLN	3MH	GLU	VAL	GLY	CIT	ARG	TAU	TYR	PHE	LEU
Natural	6.56	9.12	6.22	6.17	2.74	8.09	8.43	3.60	10.66	10.62	7.31
Mean	6.74	9.43	6.01	6.53	3.05	8.32	8.46	4.74	10.51	10.66	7.43
S.D.	0.15	0.18	0.36	0.28	0.24	0.24	0.20	0.35	0.28	0.21	0.29
VC (%)	0.02	0.02	0.06	0.04	0.08	0.03	0.02	0.07	0.03	0.02	0.04

Table 4
Underestimation of abundance measurement due to noise

Theoretical abundance (%)					
8.43	0.72	0.04	0.00	0.00	
Conc (μmol/l)	Abundance after subtraction of noise level				S.D. (%)
	m1/m0	m2/m0	m3/m0	m4/m0	m5/m0	
10	8.12	0.41	−0.27	−0.31	−0.31	1.04
25	8.25	0.53	−0.14	−0.18	−0.18	0.82
50	8.36	0.65	−0.03	−0.07	−0.07	0.26
100	8.39	0.67	0.00	−0.04	−0.04	0.27
250	8.41	0.70	0.02	−0.02	−0.02	0.14
500	8.42	0.71	0.03	−0.01	−0.01	0.08

dard deviation increased and the accuracy of the measurement decreased rapidly. Furthermore, at low concentrations, baseline noise interferes increasingly. Especially for the measurement of the heavier isotopomers, possessing a low natural abundance, the isotopomer signal does not exceed baseline noise threshold. In Table 4 we determined for a representative amino acid (arginine) the average noise level at a range of concentrations for the individual isotopomers to determine the minimal TTR required to rise above the noise. From this table we concluded that given an arginine concentration of 10 μmol/l, the ΔTTR of for instance the $M+4$ tracer should be at least 0.31% to cross the noise threshold. To check the reliability of these calculations, we constructed enrichment curves for $M+2$ enriched arginine, $M+3$ enriched leucine and $M+5$ enriched phenylalanine in the range from 0 to 5% enrichment, at the 50 μmol/l level. A linear calibration curve for all tracers was obtained (Fig. 6), but while the $M+2$ arginine tracer could be measured down to natural abundance (0% enrichment), the $M+3$ leucine tracer required a minimum of 0.1% enrichment to be picked up and the $M+5$ tracer required a minimum of 0.25% enrichment to rise above background noise. Thus, realizing also standard deviations increase with decreasing concentrations, these data support the above conclusion.

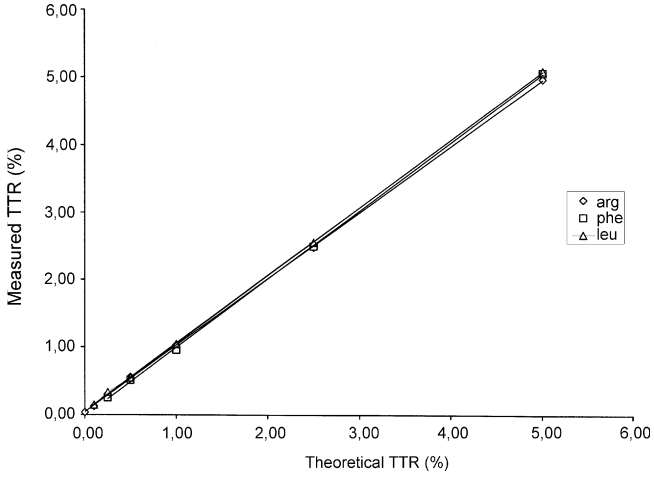

Fig. 6. Enrichment curves of selected amino acids.

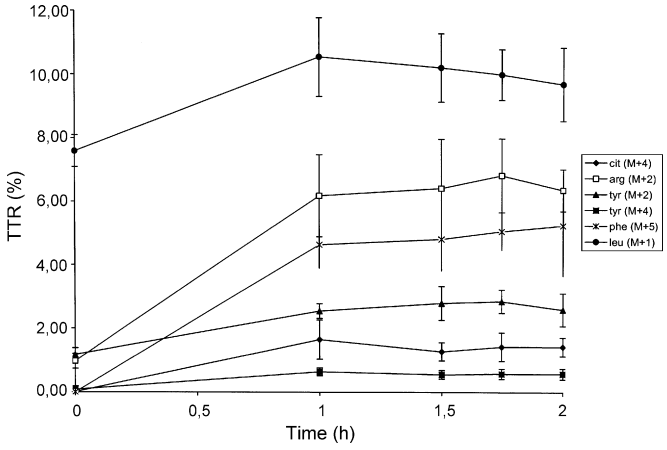

Fig. 7. Patient enrichment in time.

3.3.2. Application of human blood samples

After development of the method by using amino acid isotope standards at different concentrations and enrichments, our final goal was to improve the accuracy of the determination of multiple enriched amino acids in human and especially in patient's blood samples. In the previous OPA method, we often experienced a very enhanced sample related bias. Using the present approach, the application of FMOC derivatives and source collision induced dissociation resulted in an additional level of specificity which removed much of this bias. To test the efficiency of the present approach, we analyzed samples derived from the human experimental protocol. The clean spectra obtained by the MS system enabled us to simply and correctly determine the isotopic ratios of the infused tracers as well as the TTRs of tyrosine ($M+4$), which is derived from hydroxylation of phenylalanine ($M+5$) tracer (Fig. 7). In addition, the figure indicates that an isotopically steady state was reached after 1 h of tracer infusion.

4. Conclusion

Taken together, the here presented new method allows an improved measurement of amino acid TTR measurements in patient blood samples, requiring an automated run cycle time of 45 min and utilizes only 5 μl of (deproteinized) plasma.

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