ELSEVIER

Journal of Chromatography B, 691 (1997) 287–296

JOURNAL OF CHROMATOGRAPHY B

Isolation and quantitation of isotopically labeled amino acids from biological samples

Hans M.H. van Eijk[a], Dennis R. Rooyakkers[a], Anton J.M. Wagenmakers[b], Peter B. Soeters[a], Nicolaas E.P. Deutz[a,*]

[a]*Department of Surgery, Maastricht University, P.O. Box 616, NL-6200 MD Maastricht, Netherlands*
[b]*Department of Human Biology, Maastricht University, P.O. Box 616, NL-6200 MD Maastricht, Netherlands*

Received 29 August 1996; revised 3 October 1996; accepted 15 October 1996

Abstract

To face the problem of simultaneous isolation and quantitation of isotopically labeled amino acids in biological samples, two semi-preparative chromatographic methods were developed. One method was especially designed to isolate radioactively labeled amino acids for which we used derivatization with the fluorophore *o*-phthaldialdehyde (OPA), which is known to be easy and reliable. Isolation of amino acids labeled with stable isotopes required another approach as we wanted to use isotope ratio mass spectroscopy (IRMS), which can only be performed on pure, non-derivatized amino acids. Because the OPA probe cannot be removed after isolation of the derivative, we used 9-fluorenylmethylchloroformate (FMOC) instead. This probe is linked to an amino acid via a peptide bond which can easily be broken by gas-phase acid hydrolysis (103% recovery after 5 h at 150°C: S.D=3.5%, $n=14$). Run time (injection to injection) was 60 min for the OPA method and 75 min for the FMOC method. Both fluorescence and UV absorbance detection can be employed. The coefficient of variation (C.V.) for peak area measurement was below 2% for most OPA amino acids and below 3% for most FMOC amino acids. At maximum, a total of 1000 μl could be injected, representing approximately 200 μl of deproteinized plasma. The methods were linear up to injection of 0.5 μmol of all amino acids (OPA: $r^2=0.995$–0.999; FMOC: $r^2=0.992$–0.999). The C.V. of the IRMS measurement within the range which can be isolated maximally in one chromatographic run (50–500 nmol), was less than 3% above 100 nmol, indicating that chromatographic isolation fulfils the needs of the IRMS determination. The resulting methods are suitable for the isolation and quantitation of micromolar amounts of labeled amino acids from biological samples.

Keywords: Derivatization, LC; Amino acids

1. Introduction

A powerful tool in studying protein synthesis and/or degradation in laboratory animals is quantitation of the change in specific activity of selected radioactively labeled amino acids in blood or tissue. For this purpose, isolation of these amino acids and determination of their labeled and total amounts is required. This was formerly performed through ion-exchange chromatography [1]. However, as this

*Corresponding author.

PII S0378-4347(96)00475-6

technique requires several hours to separate all major plasma amino acids, short runs were optimized and used to isolate and quantitate only the isotopically labeled components. The remaining amino acids concentrations had to be determined in a separate analysis [2].

Alternatively, amino acids can be separated by reversed-phase chromatography. Although this technique may also be used to isolate non-derivatised amino acids [3,4], the selectivity of a reversed-phase system with regard to pure amino acids is mediocre. For analytical applications, selectivity and sensitivity were therefore commonly improved by derivatization with *o*-phthaldialdehyde (OPA) [2,5] or 9-fluorenylmethylchloroformiate (FMOC) [5–7]. Recently, OPA was also applied for a semi-preparative isolation of glutamine from plasma [8].

To study metabolism of slowly turning-over proteins in humans, the [1-^{13}C]leucine method is frequently used [9]. In this method purified proteins are hydrolyzed and the leucine fraction is isolated, mostly by preparative gas chromatography (GC) and then decarboxylated by heating with ninhydrin at low pH. The ^{13}C enrichment in the liberated CO_2 is then measured by isotope ratio mass spectroscopy (IRMS) [10]. Unfortunately, the amounts and purity of leucine, isolated by preparative GC were variable in our hands. We therefore investigated whether liquid chromatography would be a suitable alternative. We first used ion-exchange chromatography [1] as, in contrast to most reversed-phase techniques, it allows direct isolation of pure amino acids needed for the ninhydrin decarboxylation procedure which is required for the IRMS measurement. However, the separation of leucine from isoleucine is difficult and analysis times are long. An alternative possibility could be the isolation of FMOC-derived amino acids, from which the FMOC probe can be removed after isolation as it couples to the amino acid via a peptide bond that can easily be broken [11]. Unfortunately, the FMOC derivatization procedure is more difficult than the OPA method due to excess reagent interference and problems with the recovery of the derived product [12,13] and this application therefore needed further development.

Therefore, two new applications were designed. One, for the isolation of radioactive tracers using the OPA-probe and another for the isolation of stable isotope tracers using the FMOC-probe.

2. Experimental

2.1. Equipment

Our HPLC system consisted of a Model 2248 reagent pump, a Model 2249 gradient pump controlling a Model 2248-201 low pressure ternary mixing panel and a Model 2256 solvent conditioner, all from Pharmacia (Woerden, Netherlands). The analytical column for the OPA application was a 250×10 mm I.D. column filled with Spherisorb ODS II 5 μm, while for the FMOC application the same column dimensions were used, but filled with Inertsil 5 μm (both columns: Chrompack, Bergen op Zoom, Netherlands). Both columns were placed in a programmable Mistral column oven (Spark, Emmen, Netherlands). The Spherisorb column was operated at 25°C and the Inertsil column at 30°C. For the automated sample processing a WISP Model 715 or Model 717 sample processor (Waters, Etten-Leur, Netherlands) was used, equipped with a cooled sample storage compartment and a 48 position sample tray. Amino acid fractions were collected, using a Helifrac fraction collector (Pharmacia) equipped with a 43 position tray for 18 ml scintillation vials. For a more efficient use, it was controlled externally by a Model 900 interface (Perkin-Elmer/Nelson, Gouda, Netherlands). The interface was programmed from a Tandon 486 personal computer, running Model 2700 TURBOCHROM software (version 3.2: Perkin-Elmer/Nelson) under Microsoft Windows 95. The interface was also used for the collection and processing of data produced by the fluorescence detector. Fluorescence was monitored with a Jasco Model 821FP detector (B & L systems, Maarssen, Netherlands) equipped with a xenon lamp and a 16 μl flow-cell. Measurements were made at the excitation wavelengths of 330 and 260 nm and at the emission wavelengths of 550 and 360 nm for OPA and FMOC, respectively. Radioactivity was determined using a Beckman Model LS 3801 Liquid scintillation counter. The ^{13}C enrichment of CO_2

was measured on a Model MAT-252 isotope ratio mass spectrometer (IRMS) (Finnigan, Veenendaal, Netherlands).

2.2. Sample preparation

2.2.1. Standards

Amino acid standards were prepared by dissolving pure amino acids in water to a final concentration of 250 μmol l^{-1} each, and calibrated against a physiological standard (Sigma, Amsterdam, Netherlands), using HPLC [2]. The calibrated standard was divided in 1 ml portions and stored at −80°C.

2.2.2. Plasma

Heparinized blood samples were obtained from laboratory animals and humans and collected on ice, followed by immediate centrifugation (5 min, 11 000 *g*, 4°C) in a Hereaus biofuge (Dijkstra, Amsterdam, Netherlands). Next, plasma was deproteinized with 5-sulfosalicylic acid (SSA), 8 mg/200 μl plasma, frozen immediately in liquid nitrogen and stored at −80°C. Before analysis, samples were thawed at 4°C, vortex-mixed vigorously and centrifuged as described before.

2.3. Reagents and solvents

All solutions were prepared with ultra-pure water, generated by a Super-Q system (Millipore/Waters). All chemicals used were of analytical grade (Brunschwig, Amsterdam, Netherlands), solvents of chromatographic grade (Across Chimica, 's-Hertogenbosch, Netherlands).

2.4. OPA system

2.4.1. OPA reagent

The OPA reagent was prepared daily by dissolving 10 mg of OPA (Fluoraldehyde, Pierce, Brunschwig, Amsterdam, Netherlands) in 0.50 ml of methanol, adding 3.00 ml of potassium borate buffer (1.0 mol/l, pH 10.4) and 12.5 μl of 3-mercaptopropionic acid (3-MPA).

2.4.2. Derivatization

The OPA-reagent (400 μl) was added by the WISP autosampler to a vial pre-filled with 200 μl of deproteinized plasma and mixed once. After 2 min, 55 μl of citric acid (1 *M*) was added to adjust the pH of the reaction mixture to the pH of the eluent. Next, 600 μl was injected into the chromatograph, corresponding to 183 μl of pure sample.

2.4.3. Solvents

Solvent A was 25 mmol l^{-1} citrate buffer, pH 6.8 containing 2% (v/v) tetrahydrofuran (THF). Solvent B was citrate buffer mixed with acetonitrile and THF (50:40:10, v/v/v). Gradient conditions are given in Table 1.

2.5. FMOC system

2.5.1. FMOC reagent

The FMOC solution was prepared daily by dissolving 20 mg of FMOC in 4.0 ml of acetonitrile. Potassium borate (0.75 *M*, pH 8.75) served as reagent buffer.

2.5.2. Derivatization

Deproteinized plasma (see above) (200 μl) was added to a 3 ml WISP vial, containing 400 μl of reagent buffer and mixed thoroughly. Samples were placed in the WISP sample processor and derivatization was initiated by the addition of 400 μl of FMOC reagent solution. After 90 s, 200 μl of a 50 m*M* 1-aminoadamantane (ADAM) in ethanol was added to neutralize the FMOC excess, after which 1000 μl was injected into the chromatograph, corresponding to about 183 μl of pure sample.

2.5.3. Solvents

Solvent A was a 50 mmol l^{-1} sodium acetate buffer, pH 4.2 acetonitrile solution (70:30, v/v). Solvent B was acetonitrile–water (75:25, v/v). Gradient conditions are given in Table 1.

2.6. Liquid scintillation counting

Amino acid fractions (6 ml) were collected in 18 ml plastic scintillation vials and mixed with 12 ml Ultima Gold XR (Packard, Groningen, Netherlands).

Table 1

OPA method				FMOC method			
Time (min)	Flow (ml/min)	A (%)	B (%)	Time (min)	Flow (ml/min)	A (%)	B (%)
0	0	100	0	0	0	100	0
0.1	2.00	100	0	0.1	2.00	100	0
0.2	4.00	100	0	0.2	4.00	100	0
44.0	4.00	55	45	5.0	4.00	100	0
45.0	4.00	0	100	10.0	4.00	98	2
49.0	4.00	0	100	45.0	4.00	60	40
50.0	4.00	100	0	50.0	4.00	59	41
60.0	0	100	0	53.0	4.00	55	45
				55.0	4.00	0	100
				60.0	4.00	0	100
				65.0	4.00	100	0
				75.0	0.00	100	0

To eliminate chemoluminescence, samples were rested overnight at room temperature and ^{3}H and/or ^{14}C activity was determined using a Beckman Model LS 3801 liquid scintillation counter, preset to 20 min counting time.

2.7. Stable isotope determination

2.7.1. Hydrolysis

After isolation of the FMOC amino acid fractions in 6 ml glass scintillation vials, the samples were lyophilized after which they were put in a hydrolysis container of our own design (Fig. 1). The container consisted of a stainless steel outer vial and a Teflon inner vial and contained a support rack for seven 6 ml vials. A 20-ml portion of 6 *M* HCl was put on the bottom and the rack was filled with the dried samples. The container was closed and placed in an oven for 5 h at 150°C. Hereafter, samples were redissolved in 250 μl of water, transferred to a 2-ml crimp cap vial and lyophilized again.

2.7.2. Decarboxylation

A 20-mg portion of ninhydrin, together with 100 μl of helium-degassed 0.1 *M* citric acid solution was added to the lyophilized samples in a 2-ml vial, which was flushed with helium and closed with a crimp cap. Decarboxylation was initiated by heating to 100°C for 30 min. Next, a 500-μl portion of helium was injected into the vial using a 1-ml gastight syringe equipped with a gas-tight valve (SGE Bester, Amstelveen, Netherlands), then the water in the vial was frozen by holding the vial in solid carbon dioxide. ^{13}C enrichment of the liberated CO_2 was then measured by a head-space injection of 500 μl into the injector of a GC (Hewlett Packard, Amstelveen, Netherlands: containing a Poroplot column operated at room-temperature) which was connected on-line to the IRMS via a water trap.

3. Results

Our goal was to process 200 μl of deproteinized plasma or tissue homogenate, requiring an injection volume (of derivatization mix) of 600–800 μl. This volume was too large to apply to an analytical column. Therefore we used a semi-preparative column (250×10 mm I.D.), which was equipped with a 10×4.6 mm (I.D.) guard column, filled with the same packing material. For the OPA application we used Spherisorb ODS II 5 μm packing, while for the FMOC application we used Inertsil 5 μm packing. On both columns, a maximum of about 1000 μl could be injected without significant loss of resolution. The flow-rate for both methods was 4 ml/min, resulting in a back-pressure of 16 MPa at the start of the gradient.

3.1. OPA method

Derivatization with OPA is performed at high pH (10.5). As large sample volumes are applied to the column, the stability of the Spherisorb packing

material is challenged with each injection. This effect was amplified if peroxide is present in the tetrahydrofuran (THF), used for the solvents. Taking no precautions, these effects caused a rapid decrease in the carbon load of the column (12% to 10.5%, within 100 injections), resulting in a k' change of fluroanthene from 8.5 to 6.3 in a mixture of acetonitrile–water (70:30, v/v) (determined courteously by Chrompack, Middelburg, Netherlands). This degradation could be minimized by neutralization of the reaction-mixture prior to injection and the use of peroxide free THF (Across Chimica). The use of butylated hydroxytoluene (BHT) stabilized THF also slowed down the column degradation but introduced "ghost"-peaks. Neutralization of the reaction-mixture was performed by the addition of 55 μl 1 *M* citric acid, prior to injection by the autosampler. This addition resulted in the formation of a buffer with a pH of about 7, also compensating for small differences in the acidity of samples, due to differences in their protein content. A beneficial side effect of this method was that the retention times of especially the first eluting peaks were stabilized and baseline separation for most amino acids could be obtained (Fig. 2A and B). Column performance could thus be improved to about 500–1000 runs.

Although UV absorbance detection at 330 nm can also be used, we choose fluorescence detection to get a better baseline-stability. Response overflow was prohibited by selecting the optimal excitation wavelength (330 nm) but a sub-optimal emission wavelength (550 nm). Linearity of the method was checked in the range from 50 nmol to 0.5 μmol resulting in an r^2 of 0.992 or better for all amino acids. The coefficient of variation (C.V.) was determined for peak area measurement by injection of 10 standards. The C.V. for most peak areas was below 2% (Table 2).

3.2. FMOC method

The FMOC-method was primarily designed to isolate amino acids enriched with stable isotopes in

Table 2

Amino acid	OPA method			FMOC method		
	Area mean (μV*s)	Area S.D. (μV*s)	Area C.V. (%)	Area mean (μV*s)	Area S.D. (μV*s)	Area C.V. (%)
Asp	3 082 141	23 203	0.75	4 489 578	111 145	2.48
Glu	2 830 136	20 572	0.73	6 969 069	164 922	2.37
Asn	2 987 576	25 506	0.85	7 775 923	118 187	1.52
Ser	3 403 090	27 335	0.80	11 004 208	175 407	1.59
Gln	3 338 218	29 880	0.90	9 734 687	114 425	1.18
His	2 502 723	31 780	1.27	2 183 421	69 615	3.19
Gly	3 078 663	45 799	1.49	11 403 174	105 144	0.92
Thr	3 208 384	30 343	0.95	8 165 493	128 707	1.58
Cit	2 719 669	24 130	0.89	7 451 300	97 202	1.30
Ala	3 154 292	23 811	0.75	8 099 607	117 904	1.46
Arg	3 156 594	51 952	1.65	9 375 717	97 925	1.04
Tau	3 285 553	39 536	1.20	10 368 836	82 198	0.79
αab	3 968 403	78 705	1.98	9 014 150	102 358	1.14
Tyr	2 778 615	52 794	1.90	3 350 931	84 984	2.54
Val	3 393 233	27 681	0.82	9 605 071	104 577	1.09
Met	3 193 338	50 596	1.58	8 906 826	214 798	2.41
NVal	6 720 479	81 202	1.21	17 035 191	17 013	1.00
Ile	3 251 257	35 474	1.09	9 336 035	90 808	0.97
Phe	3 078 111	32 620	1.06	11 621 542	96 535	0.83
Trp	3 069 970	26 825	0.87			
Leu	3 239 852	29 086	0.90	8 810 612	93 213	1.06
Orn	778 829	14 574	1.87	3 071 869	137 232	4.47
Lys	960 095	18 056	1.88	7 283 754	293 197	4.03

general and focused more specifically on leucine. The method development regarded two aspects:

(1) Hydrolysis of the derivative to liberate the amino acid, enabling isotope ratio mass spectrometer analysis.
(2) Adaptation of reagent composition

3.2.1. Ad (1)

Two types of acid hydrolysis were studied, namely hydrolysis in glass vials sealed under vacuum at 110°C and gas-phase hydrolysis at 150°C in especially designed containers. Vacuum hydrolysis gave a maximal recovery of 86% after 34 h at 110°C (not shown). Shorter or longer hydrolysis times resulted in a decrease of recovery. Gas-phase hydrolysis required the development of our own hydrolysis vial as none of the commercially available containers we tried could withstand a leak-test at 150°C. Our design consisted of a stainless steel outer vial (for structural strength) and an inert Teflon inner vial, containing a Teflon rack to support the glass vials which contained the samples (Fig. 1). Using this vial, the mean hydrolysis recovery of leucine-FMOC was 103% (C.V.=3.5%, $n=14$), after 5–6 h at 150°C.

3.2.2. Ad (2)

For a preparative application, the commonly used reagent prescription [5–7] was not suitable, because only minimal amounts of amino acids could be derivatized. Instead of acetone, we therefore used acetonitrile, thus enabling the preparation of more concentrated FMOC solutions. For the analysis of deproteinized plasma, a 7.5–10.0 mmol l^{-1} FMOC reagent solution was sufficient. Protein hydrolysates, which are more concentrated (mmol of each amino acid), required a more concentrated 50 mmol l^{-1} FMOC solution, to guarantee a sufficient reagent excess. However, the solubility of FMOC derivatives

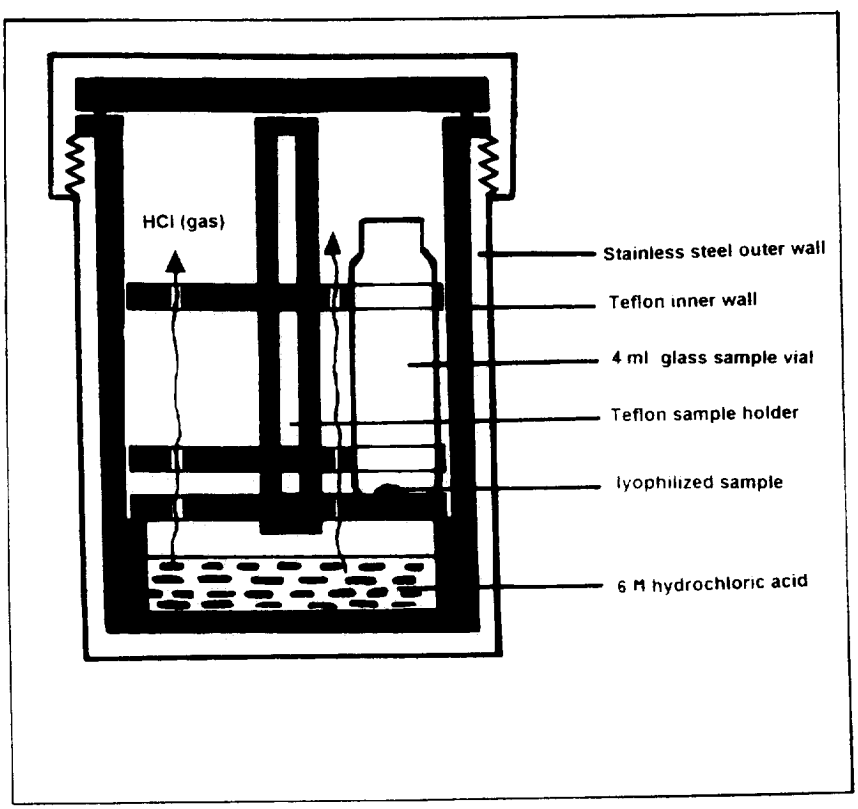

Fig. 1. Hydrolysis vial for gas-phase hydrolysis.

is limiting. Thus, a maximal total amount of 5–10 μmol of amino acid (250–500 nmol each amino acid) can be processed in one run. The reagent solution was stable for at least one week, if stored in the dark at 4°C in an amber 4 ml WISP vial, equipped with self-sealing Teflon stopper. After 90 s reaction time, the excess FMOC was neutralized with 200 μl 1-amino adamantane (ADAM, 50 mmol/l) dissolved in ethanol, thus preventing the formation of a large FMOC hydroxide peak.

A second factor influencing the response is the reaction pH. It is known that maximal fluorescence is obtained at pH 7.7 [7]. However, the pH range for maximal fluorescence is very narrow (pH 7.4–7.8), while above pH 8.0 most amino acids have a stable (although some 30% lower) response curve up to pH 10.4. We therefore increased the reaction pH to 8.75, thus also increasing the capacity of the borate buffer and ensuring better reproducibility. A three-fold dilution of acid deproteinized plasma with 0.75 *M* borate buffer (pH 8.75) was sufficient to maintain this reaction pH. As a result of these adaptations, the total process volume of a 200 μl deproteinized plasma sample (or 50–100 μl protein hydrolysate) was now 1200 μl. From this, we injected 1000 μl which enabled good chromatography (Fig. 2C and D).

The linearity and C.V. of the method was checked up to 0.5 μmol in the same way as for the OPA method (r^2=0.992 or higher). Above this, linearity was lost due to precipitation of derivatives. The C.V. for peak area measurement was below 3% for most amino acids (Table 2), which is comparable with the OPA method.

3.3. IRMS measurement

According to the literature [9,10] a minimum of 250 nmol of amino acid derived CO_2 is required to enable a good IRMS measurement. The present

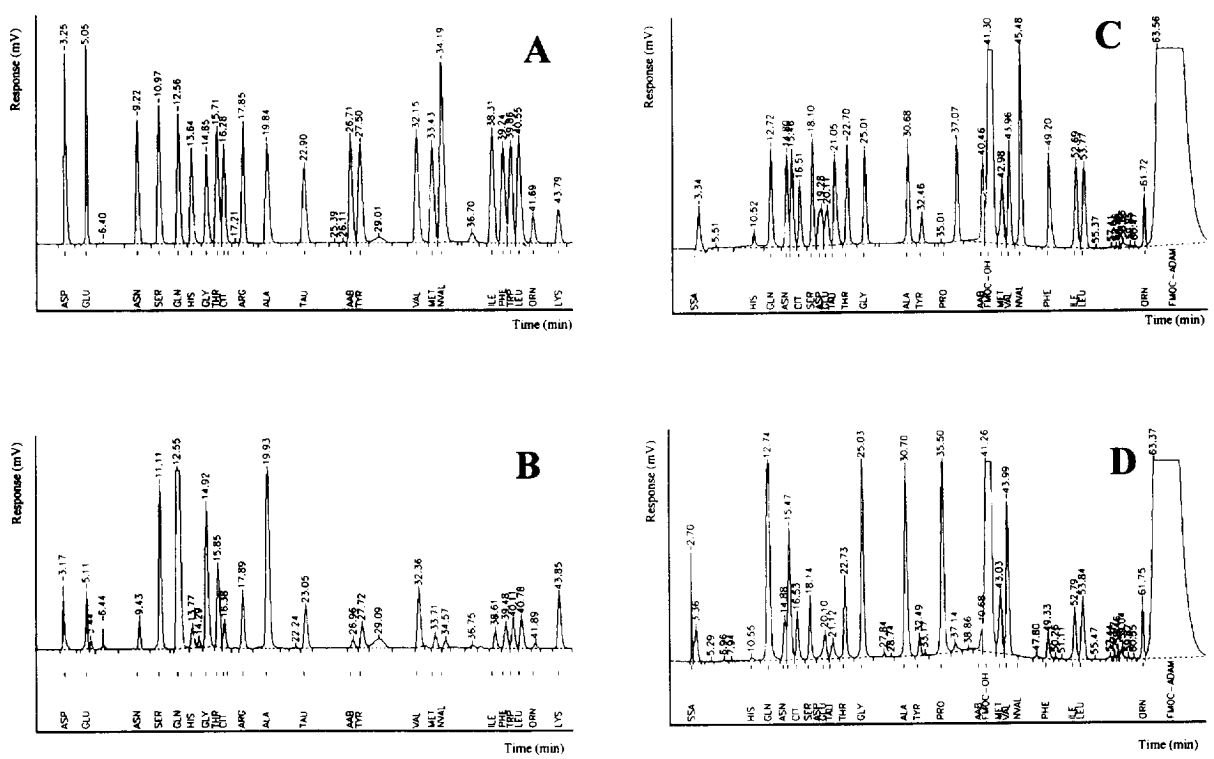

Fig. 2. Panel A: Separation of an OPA-amino acid standard (50 nmol injected each amino acid). Panel B: Separation an OPA derived rat plasma sample (200 μl plasma injected); Panel C: Separation of an FMOC-amino acid standard (50 nmol injected each amino acid). Panel D: Separation of an FMOC derived pig plasma sample (200 μl plasma injected).

FMOC method allows isolation of maximal 250–500 nmol of each amino acid. To check if these amounts allow accurate measurements on our IRMS system, we determined CO_2 enrichment of naturally enriched leucine in the range 50–500 nmol ($n=4$). Mean enrichment (expressed as the delta value [9]) was found to be constant, but the C.V. increased rapidly below 50 nmol (Fig. 3). We therefore concluded that the described chromatographic procedure allows the isolation of amounts of amino acid large enough to enable good IRMS measurement.

3.4. Determination of the enrichment or specific activity of isolated amino acids

The chromatographic separation of amino acids may result in isotope fractionation of the eluting peak [14], so we also examined this possibility for our reversed-phase methods. Therefore, 2 μmol of leucine enriched with [1-^{14}C] was injected (OPA-method, using a linear gradient to 100% in 60 min) and the eluting peak was collected in 0.1 min fractions. The recorded area of the isolated fractions and their radioactivity were determined. A definite shift in the retention of the heavier isotope (Fig. 4) was observed. As a consequence, for stable isotope measurements it is thus necessary that the peak of interest elutes freely from surrounding peaks and is collected completely. To check for this, we injected a mixture of isoleucine and leucine (both 5 mmol/l). Isoleucine and leucine fractions were collected in six-fold and in each fraction, concentrations of both amino acids were determined [2] (after hydrolysis of the FMOC derivatives). We found a leucine contamination ranging from 0.83% to 1.77% in the isoleucine fractions (mean=1.28%) and an isoleucine contamination ranging from 0.82% to 1.00% in the leucine fractions (mean=0.86%). This contamination was too low to interfere with the interpretation of the leucine enrichment measurement.

The radioactivity measurement efficiency is strongly influenced by the scintillation liquid used.

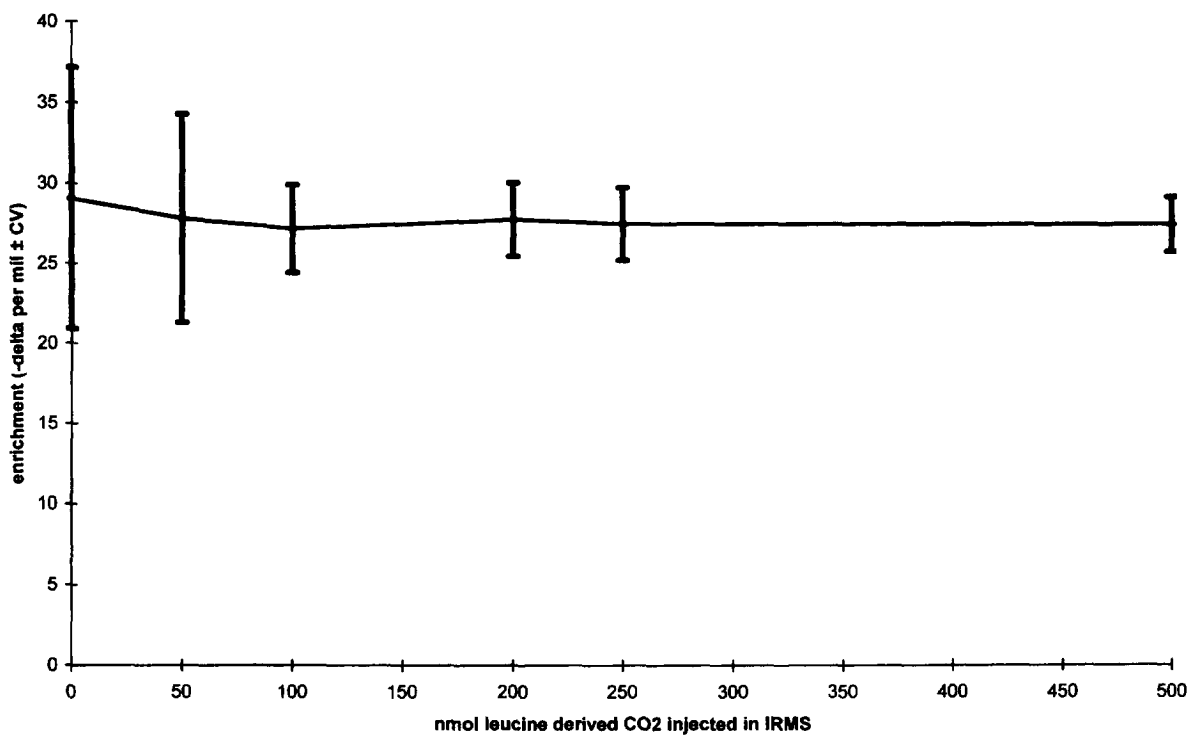

Fig. 3. Precision of IRMS measurement. Relation between the amount of CO_2 derived from decarboxylated leucine and its enrichment ($n=4$).

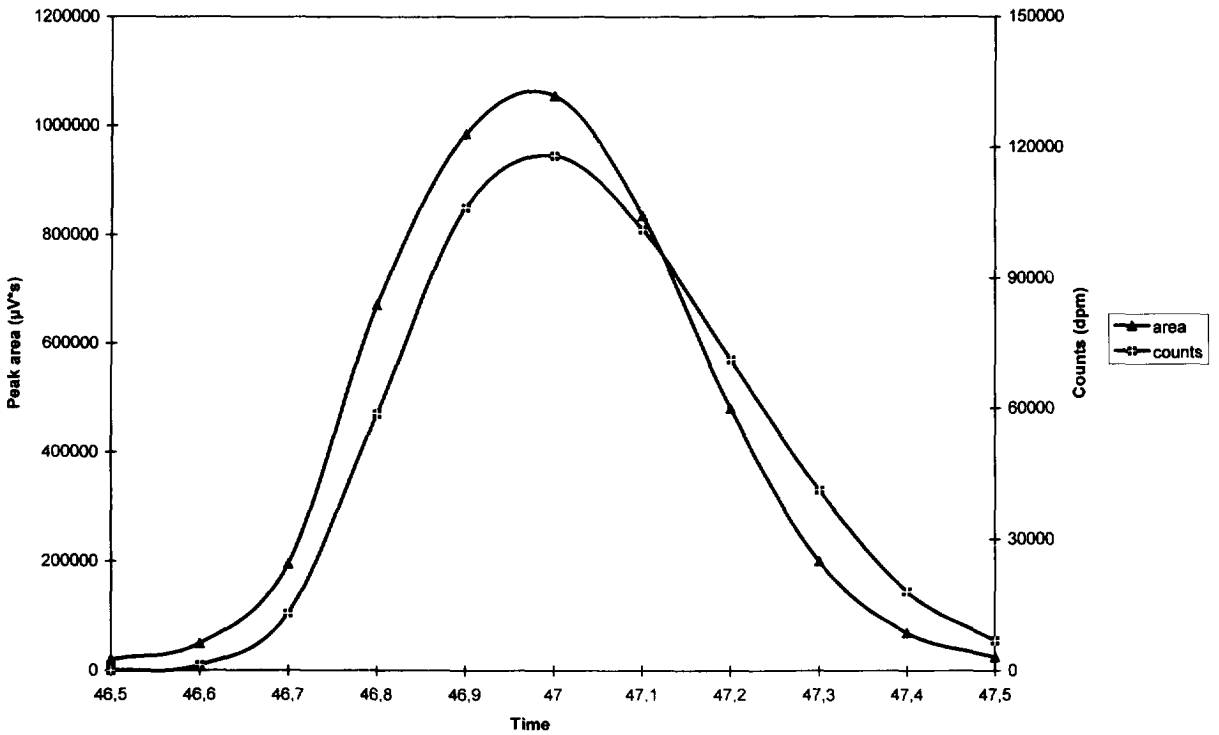

Fig. 4. Influence of isotope incorporation on retention behavior of ^{14}C-labeled leucine: evidence for isotope fractionation.

High solvent salt concentrations may cause precipitation and thus underestimation of activity, while an organic solvent on the other hand, may cause chemiluminescence and thus overestimation of activity. In search for an optimal cocktail, 5 different products were tested for solubility of the sample, counting efficiency and chemiluminescence activity. Best results were obtained if the 4–6 ml fractions were diluted at least twice with Optima Gold (not shown).

4. Discussion

We have described a complex set of techniques and procedures with the purpose of measuring the enrichment of stable isotopes or the specific activity of radioactive amino acids. Our first goal was the isolation of radioactively labeled amino acids and to study the chromatographic limitations of a semi-preparative column. Pilot experiments had revealed that an injection volume of >250 µl was required to obtain sufficient material for radioactivity determination of biological samples obtained from laboratory animals. This injection volume is too large to enable good chromatography on an analytical column. Considering the maximal flow-rate which could be delivered by our pumping system, and the required injection volume, we expected a semi-prep column (250×10 mm I.D.) to be appropriate. For derivatization, we chose the OPA label as this derivatization reaction can easily be automated and as we have many years experience with it in an analytical approach [2]. The resulting separation enabled simultaneous isolation and quantitation of each major plasma amino acid from 100–200 µl quantities, which was sufficient to allow counting of its radioactivity.

Unfortunately, the method could not be employed for an IRMS measurement of the enrichment of [1-^{13}C]leucine, because the OPA-probe cannot be removed from the isolated derivatives. Using radioactive tracers, this is of no concern, but to enable IRMS measurements of amino acids with a low degree of ^{13}C-enrichment in the carboxylic

group, the enriched carbon is released from the amino acid through reaction with ninhydrin [10]. Because this reaction requires the presence of a free amino group, isolated OPA-amino acid derivatives are not suitable as the amino group is coupled irreversibly to the fluorescent label.

For these reasons, another probe was required for stable isotope measurements and FMOC was considered to be a good possibility, as the peptide-bond can easily be broken using acid hydrolysis [8]. Indeed, we were able to achieve a 100% recovery of free amino acid using gas-phase hydrolysis.

Next, the chemistry of the derivatization reaction had to be adapted to enable a semi-preparative application. A major problem in the use of FMOC has always been the interference of the unreacted product and control of the reaction pH, especially when processing acid deproteinized samples [8,10]. This may be why FMOC has not become as popular as OPA, despite the capability of the derivatization of imino acids also. We were able to solve most of these problems by using acetonitrile to dissolve the FMOC and by adjusting the pH of the reagent buffer, thus increasing its buffer capacity. As a result, we are now able to derivatise 200 μl plasma to a final volume of 1200 μl.

The resulting FMOC method can in principle also be used to isolate radioactive amino acids. However, the OPA method has several advantages. First, the final volume after derivatization of 200 μl plasma is 655 μl using the OPA method, compared to 1200 μl for the FMOC method. This enables easier chromatography. Also, the analysis time of the OPA-method is substantially lower than the FMOC-method (60 min compared to 75 min). This enables a more efficient sample throughput. Therefore, we use the FMOC method only for stable isotope measurements and the OPA-method to isolate radioactive amino acids.

These new methods open possibilities for multiple tracer studies, because relatively large amounts of amino acid can be isolated and quantitated in one run, thereby requiring infusion of only low amounts of tracer necessary to measure their specific activity (minimum: 100 dpm/10 nmol amino acid) or [1-^{13}C] enrichment (minimum: 100 nmol amino acid), thus reducing costs of these studies.

Acknowledgments

The authors wish to thank Mr. F. van de Vegt and Mr. B. Ciocchi for the stable isotope measurements and Mrs. S. Schrijen for her analytical support.

References

[1] H.M.H. van Eijk, M.P.L. Huinck, D.R. Rooyakkers and N.E.P. Deutz, J. Chromatogr. B, 660 (1994) 251.
[2] H.M.H. van Eijk, D.R. Rooyakkers and N.E.P. Deutz, J. Chromatogr., 620 (1993) 143.
[3] L. Lecavalier, F.F. Horber and M.W. Haymond, J. Chromatogr., 491 (1989) 410.
[4] P. Tessari, S. Inchiostro, M. Vettore, L. Sabadin and G. Biolo, Clin. Biochem., 24 (1991) 425.
[5] P. Fürst, L. Pollack, T.A. Gräser, H. Godel and P.Stehle, J. Chromatogr., 499 (1990) 557.
[6] S. Einarsson, B. Josefsson and S. Lagerkvist, J. Chromatogr., 282 (1983) 609.
[7] H. Godel, in Bestimmung von freien Aminosäuren in biologischenm material mittels HPLC (Thesis), Universität Hohenheim, Germany, 1986.
[8] T. Jenssen, N. Nurjhan, G. Perriello, A. Bucci, I. Toft and J. Gerich, J. Liq. Chromatogr., 17 (1994) 1337.
[9] R.R. Wolfe, Radioactive and Stable Isotope Tracers in Biomedicine, Wiley–Liss, New York, NY, 1992, p. 247.
[10] C.M. Scrimgeour, K. Smith and M.J. Rennie, Biomed. Environ. Mass Spectrom., 15 (1988) 369.
[11] L.A. Carpino and G.Y. Han, J. Org. Chem., 37 (1972) 3404.
[12] T. Näsholm, G. Sandberg and A. Ericsson, J. Chromatogr., 396 (1987) 225.
[13] S. Einarsson, B. Josefsson and S. Laferkvist, J. Chromatogr., 282 (1983) 609.
[14] P.Q. Baumann, D.B. Ebenstein, B.D. O'Rourke and K.S. Nair, J. Chromatogr., 573 (1992) 11.