ELSEVIER

Journal of Chromatography B, 660 (1994) 251–257

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Automated simultaneous isolation and quantitation of labeled amino acid fractions from plasma and tissue by ion-exchange chromatography

Hans M.H. van Eijk, Marc P.L. Huinck, Dennis R. Rooyakkers, Nicolaas E.P. Deutz*

Department of Surgery, Biomedical Center, University of Limburg, P.O. Box 616, NL-6200 MD Maastricht, Netherlands

First received 26 April 1994; revised manuscript received 14 June 1994

Abstract

In order to trace metabolic pathways of amino acids in the body, a known labeled amount of an amino acid is infused. Dilution in the body pool is measured, using the specific activity and calculated by dividing the labeled amount of an amino acid (tracer) by its total pool (tracer + tracee). This paper describes a method, which combines fractionation and quantitation of multiple amino acids in one chromatographic run. To achieve this, we performed a classical amino acid ion-exchange separation on standard HPLC equipment. The column effluent was divided continuously into two solvent streams using a rapidly switching, pump controlled "split-valve". The main part (90%) was directed to a computer controlled fraction collector, while the remaining 10% was mixed with *o*-phthaldialdehyde reagent after which fluorescence was measured. Using this system, 10–1000 μl of deproteinized plasma, representing a maximum of 50 nmol of each amino acid, could be fractionated and quantitated in the same chromatographic run. In addition to optimal counting efficiency of an off-line radioactivity counter, it enabled easy measurement of the specific activity of multiple amino acid tracers.

1. Introduction

Labeled amino acids are widely used to determine protein synthesis or degradation rates of the body or of a specific organ. To enable this, it is necessary to isolate each amino acid and to measure the total and the labeled amounts in the collected fractions. For metabolic studies, the standard procedures for individual amino acids are too laborious and require too much sample and/or radioactivity [1–11]. Therefore, there is a need for a universal method for the isolation and quantitation of amino acids in one chromatographic run.

Usually, amino acids are quantitated by ion-exchange or reversed-phase chromatography. As we wanted to isolate large amounts of pure, underivatized amino acids, ion-exchange chromatography is the separation technique of choice for this application. However, detection of the separated amino acids requires a post-column reaction, thereby converting the isolated pure amino acids into a derivative, diluting the resulting fraction and complicating counting of the radioactive part, due to quenching and/or chemiluminescence.

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00297-I

To avoid these problems, we developed a split-flow technique which divides the column effluent dynamically into two liquid flows by a pump-controlled "split-valve". The main part (90%) is directed to a computer-controlled fraction-collector, while the remaining part (10%) is used for quantitation by mixing it on-line with *o*-phthaldialdehyde reagent and fluorescence monitoring. Because flow-rates, split-percentage and collection-windows are known, the amount of each collected amino acid can be calculated from the obtained chromatogram.

2. Experimental

2.1. Equipment

The HPLC system used (Fig. 1) 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

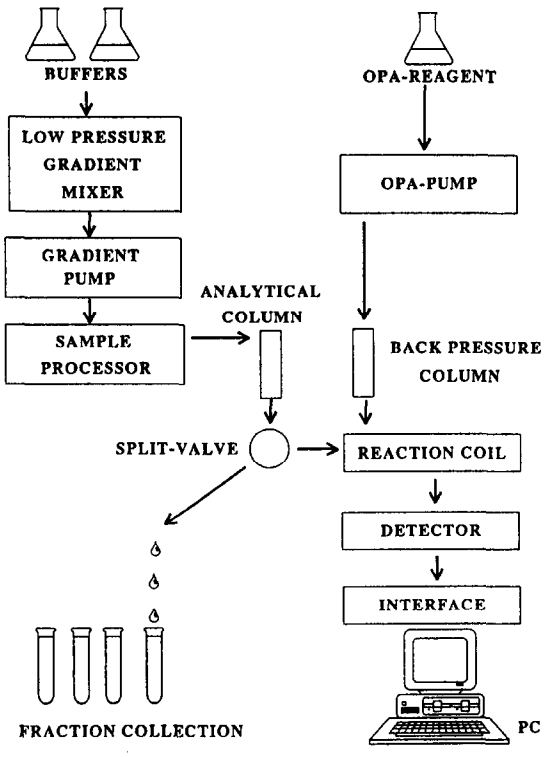

Fig. 1. Schematic system description.

solvent conditioner, all from Pharmacia (Woerden, Netherlands).

The analytical column was a 200 × 4.6 mm I.D. ion-exchange column filled with Ultropac 8 (8 μm) (Pharmacia). The column was placed in a programmable Mistral column oven (Spark, Emmen, Netherlands). The column effluent was divided dynamically into two solvent streams using a 24 VDC three-way PTFE split-valve (Cole-Parmer, Applikon, Schiedam, Netherlands) controlled by the Pharmacia Model 2248 reagent pump. The switching time was set to 1400 ms and the switching volume to 1 μl at a split-percentage of 10%.

For the automated sample injection a WISP 715 sample processor (Millipore/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 76-position tray. 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 (Version 3.1). This 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 and L systems, Zoetermeer, Netherlands) equipped with a xenon lamp and a 12-μl flow-cell. Measurements were made at an excitation wavelength of 330 nm and an emission wavelength of 445 nm.

2.2. 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 (Merck, Amsterdam, Netherlands), solvents of chromatographic grade (Janssen Chimica, Amsterdam, Netherlands).

The derivatization reagent was prepared by dissolving 0.5 g of *o*-phthaldialdehyde (OPA) in

12.5 ml of methanol, adding 500 ml of potassium borate buffer (0.5 mol/l, pH 10.4) and 0.5 ml of 3-mercaptopropionic acid (3-MPA). This reagent was placed in a 500-ml PTFE-coated brown Duran glass bottle. The bottle was kept under 0.02 MPa helium pressure using a Pharmacia solvent degassing cap.

A "short" gradient profile with a binary solvent system was used to isolate isoleucine, leucine, tyrosine, phenylalanine and norleucine (as an internal standard). Solvent A was a 0.3 mol/l lithium citrate buffer pH 3.00, containing 15 ml iso-propanol, while solvent B was 0.3 mol/l lithiumhydroxide. Gradient conditions are described in Table 1.

Amino acid standards were prepared by dissolving pure amino acids in water to a final concentration of 25 μmol/l each, and calibrated against a physiological standard (Sigma, Amsterdam, Netherlands), using a standard HPLC technique [12]. The calibrated standard was divided in 1-ml portions and stored at −80°C.

2.3. Sample preparation

Heparinized arterial blood samples were obtained from rats and collected on ice, followed by immediate centrifugation at 11 000 *g* in a Hereaus biofuge (Dijkstra, Amsterdam, Netherlands) for 10 min at 4°C. Next, plasma was deproteinized with 5-sulfosalicylic acid (5-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. A 50-μl volume of the clear supernatant was then mixed with 50 μl of an ice-cold 0.2 mol/l lithium citrate "loading buffer" pH 2.20 (containing 60 μmol/l norleucine as internal standard) in a 300-μl insert and placed in a 4-ml WISP vial, and sealed with a PTFE seal.

Table 1
Gradient conditions

Time (min)	Solvent A (%)	Solvent B (%)	Temperature (°C)
0–35	100	0	45
35–50	100	0	60
50–55	0	100	60
55–80	100	0	45

3. Results

Three different three-way valves were tested to serve as a split-valve: the Lee LFYA 1 bar ministac valve, the Lee LFYA 4 bar ministac valve (both Dennis de Ploeg, Valkenburg, Netherlands) and the 24 VDC Cole Parmer Teflon PTFE solenoid valve (Applikon, Schiedam, Netherlands). From these, the Cole Parmer valve was the cheapest and most reliable valve. Although its internal volume was higher compared to the Lee-valves (32 μl versus 18 μl), this proved not to be a disadvantage in practice. Therefore, the Cole Parmer valve was used throughout our experiments.

Our primary goal was the isolation of phenylalanine and tyrosine. Therefore, a "short" isolation gradient was developed using norleucine as an internal standard. The resulting chromatograms (Fig. 2) show a baseline separation for these peaks.

The correct functioning of the valve was evaluated by programming a value of 8% split and measuring the resulting flow-rates. Considering the low back-pressure generated by the reagent flow-path and the helium over-pressure present on the OPA reservoir, we suspected this flow to be too high. Therefore, we measured the splitted solvent flow-rates with and without the influence of the reagent flow (direct and indirect method, Table 2). From the results, we concluded that the split-percentage was correct and stable, but the reagent flow was too high and irregular. In order to stabilize this reagent flow, the back-pressure had to be increased. Placement of a back-pressure valve in the reagent flow-path resulted in a pulsating reagent flow and consequently irregular baseline. Therefore, the reagent back-pressure was increased by placing a 150 × 3 mm I.D. column filled with the cation-exchange resin Durrum DC-6A in the reagent flow-path, enabling a stable reagent flow down to

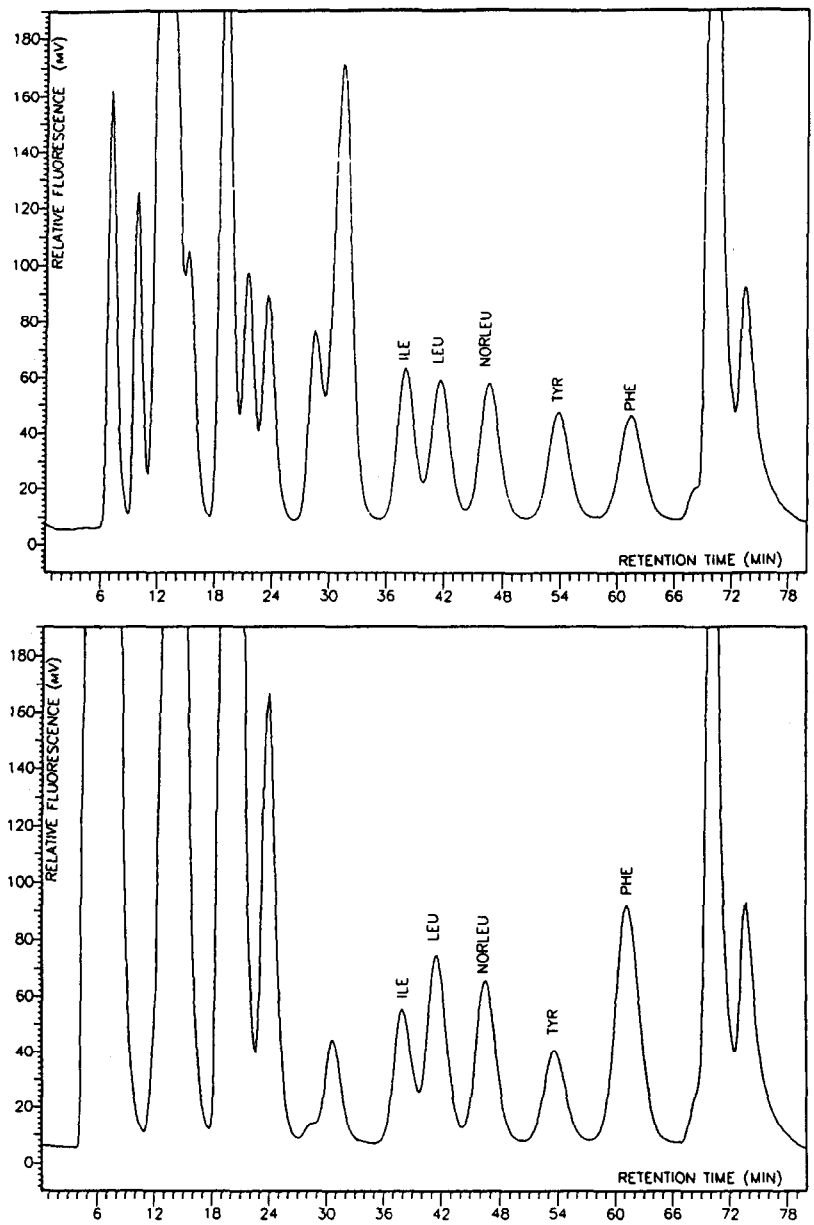

Fig. 2. Separation of tyrosine and phenylalanine in a 100 μmol/l physiological standard containing all major plasma amino acids (upper panel) and in plasma (lower panel). Injection volume was 75 μl for each sample.

0.035 ml/min (not shown) and resulting in a back-pressure of 0.2 MPa. Using this minimal OPA flow, an optimal chromatogram was obtained with a solvent flow of 0.050 ml/min. This limited the minimal applicable split-percentage in this application to 10% at a total eluent flow of 0.5 ml/min. So, 90% of the column effluent could be used for radioactivity counting.

As an optimal reaction time of amino acids with OPA reagent is reported to be 2–3 min [13], the present flow-rates required a reactioncoil of ca. 1.5 m × 0.3 mm I.D. The resulting calculated

Table 2
Determination of the variation in split-percentage by direct and indirect measurement

Measurement	Direct method (%)	Indirect method (%)
1	9.87	7.97
2	9.57	7.96
3	9.13	7.97
4	9.26	7.96
5	8.35	7.92
6	9.01	7.92
Mean	9.20	7.95
S.D.	0.52	0.02
C.V. (%)	5.66	0.30

Both methods are discussed in the text.

difference in delay time of the amino acid fractions before arrival in the fraction collector or the detector was checked by measuring the fraction recoveries of isoleucine, leucine, norleucine, tyrosine and phenylalanine of 10 consecutive runs of a 25-μM physiological amino acid standard with the described standard HPLC technique [12]. A nearly 100% recovery was obtained, confirming the correctness of the settings (Table 3). The somewhat higher results may be explained by minor contaminations in buffers and reagents, playing a significant role at the low levels in the collected fractions,

Operation of the system with fluorescence detection, limitates the maximum amount which can be isolated and quantitated at the same time to 50 nmol per amino acid, above which detector overload occurs. Using the less sensitive UV-detection at 330 nm, a 10 times higher amount can be fractionated. Above this amount, the reagent concentration becomes limiting, while the resolution deteriorates due to column overload effects (not shown).

In order to determine the accuracy of the method, 51 rat plasma samples were fractionated and the concentrations obtained from the resulting chromatogram, were compared to those obtained with the standard technique [12]. The mean values found for isoleucine, leucine, tyrosine and phenylalanine were respectively 96%, 96%, 104% and 97% of the standard method. Considering the coefficients of variation, which are about 3% for both methods, no significant difference at the 95% confidence level could be established when these results were compared using the method of Bland and Altman [14] (Fig. 3).

4. Discussion

Until now it was laborious to perform metabolic studies with multiple amino acid tracers as numerous specialized techniques were required to isolate and quantitate these tracers and their tracees [11]. An universal technique for the isolation and quantitation of all amino acids was therefore desired. To enable this, we combined the reliable chromatographic separation of an amino acid analyzer with modern HPLC techniques.

Table 3
Determination of recovery percentages and reproducibility

Amino acid	Recovery (%)			On-line chromatography					
				Area (mV min)			t_R (min)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
ILE	102.2	3.31	3.24	169.2	7.28	4.30	70.93	0.20	0.28
LEU	107.3	6.80	6.34	170.6	4.73	2.77	74.90	0.12	0.16
NORLEU	94.8	2.64	2.78	166.6	2.57	1.54	80.22	0.10	0.13
TYR	107.3	3.20	2.99	147.6	4.10	2.78	87.97	0.22	0.25
PHE	105.8	5.91	5.59	165.2	4.27	2.59	96.73	0.17	0.18

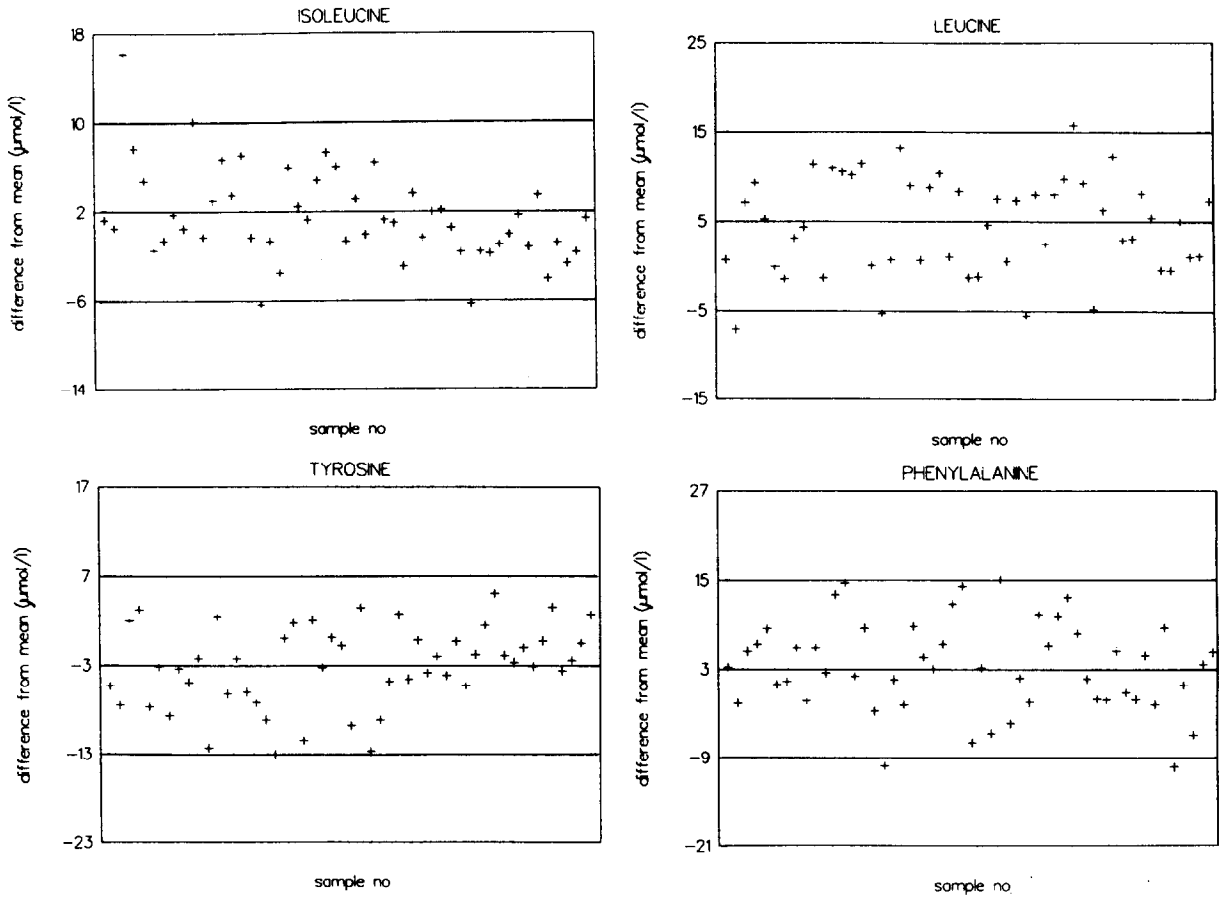

Fig. 3. Comparison of results obtained by the standard HPLC technique and the present isolation technique using the method of Bland and Altman [14]. The results (μmol/l) are plotted as the difference from the mean (center line) with the 2 S.D. levels at 95% confidence level (upper and lower lines).

The present method was designed to obtain maximal recovery of radioactivity with a minimal amount of sample. We therefore chose to determine radioactivity off-line and not on-line. On-line counting requires at least 10–15 times more activity, because the solvent flow passes through the flow cell of the detector in a short time, resulting in a lower signal-to-noise ratio and thus a high background. Using an off-line determination, the counting time can be much longer. For tritium, this reduces the required activity for each desired amino acid to only 50–100 dpm for an accurate measurement. Because each amino acid can be isolated within the same chromatographic run, the total sample amount and the total amount of activity can be minimal.

Fluorescence detection was chosen for the same reason. The maximum amount which can be quantitated fluorimetrically is 50 nmol. Above this value the detector signal goes off-scale. This amount represents a maximum of 100–1000 μl of deproteinized plasma, depending on the amino acid in question. These volumes are well within the range of amounts that can be obtained from small laboratory animals. Also, it is within the maximum volume range that can be injected directly onto the column without a negative effect on the separation.

However, column overload effects appear between 200 and 1000 nmol per amino acid, a much higher level. Therefore, more concentrated samples can be fractionated, or larger plasma amounts after lyophilization and resuspension in a smaller volume. Quantitation may then be realized by applying the less sensitive UV-detection instead of fluorescence detection, enabling the measurement of even lower specific activities.

In conclusion, the present method offers a powerful tool for measuring the specific activity of several amino acids in one sample and in one chromatographic run on standard HPLC equipment. In combination with off-line radioactivity counting, minimal amounts of sample and radioactivity are required.

Acknowledgement

The authors wish to thank Mr. R.H.P. Oderkerk for his excellent analytical support.

References

[1] R.R. Wolfe, *Radioactive and Stable Isotope Tracers in Biomedicine*, Wiley-Liss, New York, NY, 1992, p. 357.
[2] E.P. Donahue, L.L. Brown, P.J. Flakoll and N.N. Abumrad, *J. Chromatogr.*, 571 (1991) 29.
[3] N. Vaidyanath, R. Birkhahn, J.R. Border, R. McMenamy, G. Oswald, G. Trietley and T.F. Yuan, *Anal. Biochem.*, 70 (1976) 479.
[4] P. Tessari, S. Inchiostro, M. Vettore, L. Sabadin and G. Biolo, *Clin. Biochem.*, 24 (1991) 425.
[5] F.F. Horber, J. Kahl, L. Lecavalier, B. Krom and M.W. Haymond, *J. Chromatogr.*, 495 (1989) 81.
[6] L. Lecavalier, F.F. Horber and M.W. Haymond, *J. Chromatogr.*, 491 (1989) 410.
[7] K. Bartlett and A.G. Causey, *Methods Enzymol.*, 166 (1988) 79.
[8] D.R. Reeve and A. Crozier, *J. Chromatogr.*, 137 (1977) 271.
[9] J.A. Grunau and J.M. Swiader, *J. Chromatogr.*, 594 (1992) 165.
[10] S.E. Møller, *J. Chromatogr.*, 613 (1993) 223.
[11] E. Newman, M.J. Heslin, R.F. Wolf, P.W.T. Pisters and M.F. Brennan, *Metabolism*, 43 (1994) 70.
[12] H.M.H. van Eijk, D.R. Rooyakkers and N.E.P. Deutz, *J. Chromatogr.*, 620 (1993) 143.
[13] H. Godel, T. Graser, P. Foldi, P. Pfaeder and P. Furst, *J. Chromatogr.*, 297 (1984) 49.
[14] J.M. Bland and D.G. Altman, *Lancet*, 8 (1986) 307.