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High-performance liquid chromatographic technique for non-derivatized leucine purification: evidence for carbon isotope fractionation

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method, utilizing an ion-pairing agent with a reversed-phase column, isocratic elution and ultraviolet detection, was developed for the separation of non-derivatized leucine from a mixture of amino acids. Fractionation of leucine isotopes during HPLC separation was observed, creating potential problems for collection of leucine plasma or tissue protein hydrolyzates for subsequent isotopic determinations. It was demonstrated that [^{13}C]leucine and [^{12}C]leucine have different retention times ($p < 0.01$). It is concluded from this study that partial collection of leucine eluted from an HPLC column will result in erroneous estimation of the isotopic enrichment of leucine.

INTRODUCTION

Leucine is an essential amino acid which is well represented in all proteins in the body. Hence, *in vivo* leucine kinetics present a theoretically valid index of protein turnover. Consequently, isotopically labelled (^{2}H, ^{3}H, ^{13}C, ^{14}C or ^{15}N) leucine is one of the most commonly used tracers for the study of protein metabolism in humans and in animals [1–6]. For example, fractional skeletal muscle protein synthesis can be estimated in humans from the increment of [^{13}C]leucine in muscle protein, obtained by serial muscle biopsies during continuous intravenous infusion of L-[1-^{13}C]leucine [7–9].

Gas chromatography–mass spectrometry (GC–MS) has been extensively used to measure [^{13}C]leucine, [^{15}N]leucine and [^{2}H]leucine enrichment in plasma samples [2,10–13]. Since the incorporation of [^{13}C]leucine in tissue proteins is below the detectability threshold of quadrupole GC–MS, magnetic sector isotope ratio mass spectrometry (IRMS) must be used. Since only gas samples can be analyzed by IRMS, the labelled carboxyl group of leucine must be liberated as CO_2 for subsequent analysis by IRMS [14] which can be accomplished by means of the ninhydrin reaction [15]. The ninhydrin reaction depends on an intact amino group on leucine. It is, therefore, important that purified, intact leucine be collected without conversion to its keto acid or derivatization of the amino group.

One current high-performance liquid chromatographic (HPLC) method for the separation of amino acids involves separation by the classic ion-exchange method, with buffer delivered in a gradient and post-column derivatization to allow detection [16,17]. Non-derivatized amino acids have been separated on reversed-phase columns with addition of an ion-pairing agent to the mobile phase, followed by post-column derivatization [18,19]. A technique of HPLC separation

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and isolation of non-derivatized leucine from hydrolyzed muscle protein by ion exchange has been used, in which [^{14}C]leucine was used as a marker for leucine collection [20]. This technique has several advantages, provided there is no significant fractionation between different isotopes of carbon (*e.g.* ^{12}C *versus* ^{13}C *versus* ^{14}C) during HPLC separation as has been reported for separation by GC [21]. The work reported here examines whether complete separation of non-derivatized leucine from an amino acid mixture can be achieved by HPLC when an ion-pairing agent and UV detection are utilized, and whether isotopic fractionation occurs during the separation.

EXPERIMENTAL

Separation of amino acids

Mixtures of amino acids were separated using an HPLC system, consisting of a 500E system controller, a 484 tunable absorbance detector and a U6K manual injector, equipped with a Delta Pak C_{18}, 100 Å pore size, 5 μm particle size column (150 mm × 3.9 mm I.D.) from Millipore, Waters Chromatography Division (Bedford, MA, USA). Solvent (water–acetonitrile, 95:5, with 0.4% trifluoroacetic acid added, v/v; pH adjusted to 2.2 with ≈30% ammonium hydroxide) was delivered at 1.0 ml/min. Amino acids were detected by their absorbance at 200 nm. Aqueous solutions of amino acids (alanine, glycine, isoleucine, leucine, lysine and phenylalanine, from Sigma, St. Louis, MO, USA) were injected manually. The injections contained 34 μg of each amino acid (0.010 ml of 3.4 mg/ml).

Tests for fractionation of isotopes

Radioactive isotopes. L-[3,4,5-^{3}H(N)]Leucine and L-[1-^{14}C]leucine were obtained from New England Nuclear Research Products (Boston, MA, USA). Samples (0.010 ml) of natural (99.5 atom% excess of ^{12}C) leucine standard (3.4 mg/ml) containing 0.2 μCi/ml [$^{3}H_3$]leucine and 0.2 μCi/ml [^{14}C]leucine were injected. The portion of the leucine peak which emerged first from the column (the "front") was collected separately from the portion of leucine which emerged last (the "back") (Fig. 1). Fractions were dried on a nitrogen evaporator then redissolved in 0.2 ml of water. Half of the sample was then reserved for subsequent determination of the leucine concentration by ion-exchange HPLC with post-column derivatization. The remaining half was counted in a liquid scintillation counter.

Stable isotope. Samples (0.010 ml) of leucine standard (3.5 mg/ml) mixed with L-[1-^{13}C]leucine (99 atom ^{13}C, obtained from MSD Isotopes, Rahway, NJ, USA) were injected and collected as described above for radioactive isotopes. Pooled collections were made to provide enough leucine for CO_2 production and IRMS analysis. Three different dilutions were injected and collected. Samples were lyophilized after collection, then prepared for IRMS analysis using a modified version of the method of Read *et al.* [22].

Ninhydrin reaction and IRMS analysis

Lyophilized samples were redissolved in 0.5 ml of methanol for transfer to 5-ml side-arm Pyrex reaction tubes which were acquired from Fayran Glass (Hemlock, NY, USA). Samples were evaporated to dryness at 70°C, under a continuous stream of nitrogen. The samples were then cooled to 4°C in ice water, and 100 μl of cold, diluted orthophosphoric acid (pH 2.5) and 200 μl of a cold, saturated solution of ninhydrin in dilute orthophosphoric acid (30 mg/ml) were added to each tube. The tubes were then attached to the sample manifold of a SIRA II IRMS (VG Isogas, Middlewich, UK), evacuated to $5 \cdot 10^{-10}$ mbar for 5 min, maintaining the temperature at 4°C,

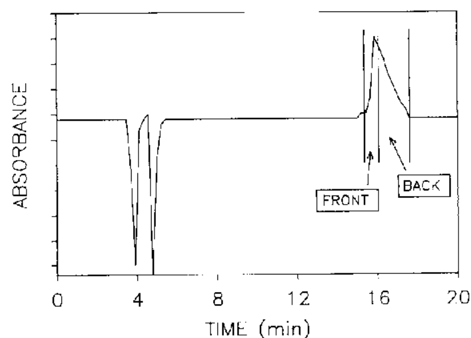

Fig. 1. Collection of fractions of leucine. Lines that appear on the figure denote starts and stops of collections. Front fractions contained the material under the curve which is located beween the first and second lines. Back fractions contained the material under the curve which is located between the second and third lines.

and sealed under vacuum. Following their removal from the IRMS, the tubes were heated in an aluminum block at 100°C for 30 min. After heating, the bottom 2.5 cm of each tube was submerged for 15 min in methanol cooled to < -70°C with dry ice. The tubes were removed from the methanol, frozen in liquid nitrogen for an additional minute, then returned to the methanol bath. The freezing procedure as described was found to be necessary prior to sample introduction into the IRMS system for the removal of water vapor that was present in quantities too great for the capacity of the water trap provided with the IRMS. The samples were introduced into the automated sample preparation area of the IRMS system where CO_2, liberated by the ninhydrin reaction, was automatically separated from other gases and concentrated by a cold finger for introduction into the mass spectrometer.

Determination of relative retention times of isotopes

Separate samples (0.010 ml) of standard leucine (3.4 mg/ml) and L-[1-^{13}C]leucine (99 atom% ^{13}C, 3.5 mg/ml) with norleucine (3.5 mg/ml, Sigma) added as an internal standard were injected into the HPLC system and separated by the described method. The retention times of the two leucine isotopes relative to the retention time of norleucine were determined.

Calculations and statistical analysis

Relative retention times were determined by division of the retention time of leucine (^{12}C or ^{13}C) by the retention time of norleucine. Comparison of mean relative retention times was by a two-tailed t-test. Mean counts in radioactive fractions were also compared using the t-test. Results were considered significant at $p < 0.05$.

RESULTS

Fig. 2 shows the chromatogram of a mixture of amino acids, demonstrating a clear separation of leucine from other amino acids, particularly isoleucine, achieved by the technique. Table I shows the results of the experiment in which radioactive isotope fractionation was examined. The specific radioactivity (cpm/nmol leucine) of [$^3H_3$]leucine was significantly ($p < 0.01$) higher (77.5%) in the front (18.45 ± 4.9) than in the back (4.15 ± 0.3) fractions. In the case of [^{14}C]leucine, specific radioactivity was significantly ($p < 0.01$) higher (19%) in the back (29.07 ± 1.1) than in the front (23.57 ± 2.3) fractions.

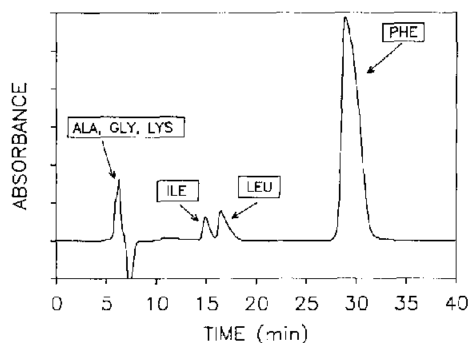

Fig. 2. Separation of an aqueous mixture of non-derivatized alanine, glycine, isoleucine, leucine, lysine and phenylalanine (concentration of each amino acid in the mixture is 3.4 mg/ml) on a reversed-phase (C_{18}) HPLC column (see text for description of method). Isoleucine and leucine are well resolved from one another as well as from the other amino acids in the solution.

The enrichments of the fractions of stable isotopically labelled leucine collected are displayed in Table II. Enrichment values obtained for the backs are higher than those obtained for the

TABLE I

FRACTIONATION OF RADIOACTIVE LABEL COLLECTED FROM FRONTS AND BACKS OF LEUCINE PEAKS FROM FOUR INJECTIONS

Injection No.	[$^3H_3$]Leucine (cpm/nmol of leucine)	[^{14}C]Leucine (cpm/nmol of leucine)
Front		
1	12.2	24.6
2	23.9	26.1
3	20.1	20.7
4	17.6	22.9
Mean ± S.D.	18.45 ± 4.9	23.57 ± 2.3
Back		
1	3.7	28.0
2	4.5	29.7
3	4.2	28.3
4	4.2	30.3
Mean ± S.D.	4.15 ± 0.3	29.07 ± 1.1
p	<0.01	<0.01

TABLE II

MEAN ATOM% EXCESS OF [^{13}C]LEUCINE IN WHOLE HPLC PEAKS AND PEAK FRACTIONS FOR EACH OF THREE SAMPLE DILUTIONS

Sample No.	Mean atom% excess (n = 2)		
	Whole peak	Front	Back
1	0.5319	0.4253	0.5454
2	0.0855	0.0317	0.1038
3	0.0281	−0.0169	0.0519

whole peaks. Values obtained for the backs are also consistently higher than the values obtained for the corresponding fronts. The dissimilarity of enrichments found in the front and back fractions and those found in the whole peak is more pronounced as the atom% excess of the whole peak decreases, as can be seen when the ratios of front and back to whole peaks are examined, as shown in Table III.

Retention times, relative to the internal standard norleucine, were determined for L-[1-^{12}C]leucine (leucine standard) and for L-[1-^{13}C]leucine, after ten injections each. The mean relative retention time for [^{12}C]leucine was 0.9076 ± 0.0016, and for [^{13}C]leucine was 0.9109 ± 0.0015 (Table IV). The difference between the two relative retention times was significantly different when compared by the t-test ($p < 0.02$).

DISCUSSION

A technique for separating non-derivatized leucine from an amino acid mixture was developed. This technique was used to collect leucine and measure isotopic enrichment in an IRMS system, following liberation of the carboxyl carbon of leucine by the ninhydrin reaction.

It is evident from the results of this study that there is isotope fractionation of leucine isolated by HPLC. The fractionation becomes more apparent when smaller amounts of [^{13}C]leucine are present. An explanation of this phenomenon may be that the degree of overlap of the eluting bands of differently labelled leucine increases as a larger amount of a labelled species is present in the leucine mix. Fig. 3 presents a diagrammatic representation of this hypothesis.

The data (Table IV) show that [^{13}C]leucine and [^{12}C]leucine have significantly different retention times, when injected alone. One explanation for this may be a mass effect, resulting in the "heavier", labelled leucine eluting later than the non-labelled leucine. However, this explanation seems less likely given the observation that leucine labelled with tritium was found in greater abundance in the front of the peaks than in the back, whereas [^{14}C]leucine was present in greater amounts in the backs than in the fronts. If the differences in retention times could be explained by a mass effect, one would expect that the [$^{3}H_3$]leucine would elute even later than the [^{14}C]leucine, since it is two mass units greater than the [^{14}C]leucine.

Due to the demonstrated fractionation of leu-

TABLE III

ENRICHMENT (ATOM% EXCESS) OF [^{13}C]LEUCINE IN PEAK FRACTIONS FROM HPLC RELATIVE TO ENRICHMENT (ATOM% EXCESS) OF [^{13}C]LEUCINE IN WHOLE PEAKS

Sample No.	Front/whole	Back/whole
1	0.799	1.025
2	0.371	1.214
3	−0.601	1.847

TABLE IV

RELATIVE RETENTION TIMES OF TEN SEPARATE INJECTIONS OF [^{13}C]LEUCINE AND [^{12}C]LEUCINE

Injection No.	Relative retention time	
	[^{12}C]Leucine	[^{13}C]Leucine
1	0.9067	0.9116
2	0.9085	0.9130
3	0.9085	0.9125
4	0.9111	0.9109
5	0.9072	0.9084
6	0.9063	0.9095
7	0.9082	0.9121
8	0.9054	0.9089
9	0.9072	0.9110
10	0.9065	0.9108
Mean	0.9076	0.9109
S.D.	±0.0016	±0.0015

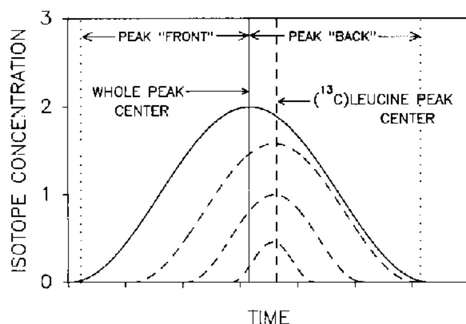

Fig. 3. Diagrammatic representation of possible explanation for differences in degree of fractionation seen in the study. A small difference in the retention time of [^{13}C]leucine from that of [^{12}C]leucine allows the two isotopes to appear to coelute when observed visually. However, if the isotopic abundance is analyzed in fractions of the peak, the relative abundance of [^{13}C]leucine in the fractions will vary, depending on from where the fractions are collected, and the total quantity of [^{13}C]leucine in the solution. This is due to differences in the degree of overlap of the [^{13}C]leucine peak and the observed peak which is usually closely represented by the [^{12}C]leucine peak, since it is the isotope in the greater quantity.

cine label shown, it is apparent that the use of labelled leucine as a marker for leucine collection for subsequent stable isotope analysis could lead to erroneous results. Detection of leucine by radioactive label can bias results since overlap of the label used for detection and total leucine in a sample is not complete. The same problem exists when using visual detection of total leucine, but the discrepancies in overlap are most pronounced when a particular isotopic species is present in very small amounts, as is the case when using a radioactive tracer for detection. These results indicate that this is not a problem for the levels at which metabolic studies are performed.

In summary, it was demonstrated that different isotopes of leucine have different retention times when separated by an HPLC method. Partial collection of the leucine peak can result in an erroneous estimation of the isotopic enrichment of leucine, so it is important that the entire leucine peak be collected in order to obtain a sample truly representative of the label distribution within a biological sample. Besides the obvious decrease in total leucine yield, the collection of partial peaks to avoid contamination by a closely eluting neighboring amino acid also introduces a label bias. Because of the different retention times, use of [^{14}C]leucine as a marker for the collection of leucine leads to partial collection of the leucine peak.

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