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DETERMINATION OF LEUCINE AND α-KETOISOCAPROIC ACID CONCENTRATIONS AND SPECIFIC ACTIVITY IN PLASMA AND LEUCINE SPECIFIC ACTIVITIES IN PROTEINS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Plasma concentrations and ^{3}H and ^{14}C specific activities (specific radioactivities) of leucine and α-ketoisocaproate (KIC) and leucine specific radioactivity in hydrolyzed tissue and plasma proteins were determined using an automated isocratic high-performance liquid chromatographic (HPLC) system Within-day variability of leucine and KIC specific radioactivity in plasma was $\simeq 1\%$, whereas that observed for leucine derived from protein hydrolysis was $\simeq 5\%$ Day-to-day variability of leucine and KIC specific radioactivity in plasma was $\simeq 5\%$ and in protein-derived leucine $\simeq 6\%$ In addition, an indirect method is described to measure low specific activities of [^{3}H]- and [^{14}C]leucine derived from the hydrolysis of in vivo labeled proteins with low turnover rates (skeletal muscle and diaphragm) In proteins with higher turnover rates (fibrin, kidney, liver, jejunum and heart muscle), this indirect method gave similar results to the direct HPLC method Using these methods, fractional protein synthetic rates in a variety of tissues can be accurately determined using radioisotopes of KIC and/or leucine

INTRODUCTION

In recent years, increased attention has been given to the role of branched-chain amino acids and their α-ketoacids in the regulation of protein metabolism A variety of techniques have been reported for the measurement of these compounds [1–9], however, many of these methods are only briefly described and, in some case, little information about the exact procedure or the precision

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and accuracy of the method is available We previously reported a method to measure simultaneously plasma concentrations and specific activities [dpm (disintegrations per minute)/nmol] of α-ketoisocaproate (KIC) and its parent amino acid, leucine (following the enzymatic conversion of leucine to KIC) by high-performance liquid chromatography (HPLC) [2,8] However, this method was only described for plasma and was accurate only for injected samples containing up to 50 nmol of KIC (or KIC derived from enzymatic conversion of leucine to KIC)

During infusion of labeled leucine (or KIC), radioactive leucine is incorporated into tissue and plasma proteins Recently, there has been renewed interest in the measurement of fractional rates of protein synthesis in a number of tissues and under a variety of experimental conditions [10–12]. In order to measure the specific activity of leucine in a given protein or whole tissue proteins for the purpose of estimating a fractional protein synthetic rate, a significantly larger mass of leucine with relatively lower radioactivity must be analyzed Thus, factors affecting the precision and accuracy of the measurement of leucine mass and/or the radioactivity must be carefully considered

When our previously reported assay [2,8] was applied to the measurement of leucine specific activity (spec radioact) in hydrolysates of tissue and plasma proteins or to samples with high concentrations of leucine, inconsistent results were obtained In the present report, we provide precision and accuracy data for the determination of the spec. radioact of leucine derived from a variety of tissue and plasma proteins, which is applicable to plasma leucine and KIC as well In addition we identify that the primary cause of these inaccuracies was the result of non-linearity of the KIC and α-ketocaproate (internal standard) standard curves

EXPERIMENTAL

Chemicals and biologic materials

HPLC-grade solvents (acetonitrile, UV cut-off 190 nm, methylene chloride, UV cut-off 235 nm, and methanol, UV cut-off 235 nm) were obtained from Fisher Scientific (Pittsburgh, U S A) Cation-exchange resin (AG50 W$\times$80, H^+ form, 200 mesh) was obtained from Bio-Rad (Richmond, CA, U S A.), washed with water five times, and stored as a 50% (v/v) slurry in water Norleucine, leucine, KIC and α-ketocaproate, L-amino acid oxidase (type I) and bovine liver catalase, and all other chemicals were obtained from Sigma (St Louis, MO, U S A) Aquassure® was purchased from NEN Research Products (Boston, MA, U S A) A 15-ml volume of ethanolamine (Kodak, Rochester, NY, U S A) dissolved in 285 ml of deionized water was added to 4 l of Aquassure to trap $^{14}CO_2$ generated from the spontaneous decarboxylation of [^{14}C]KIC during the collection of the HPLC peak of interest L-[1-^{14}C]Leucine and [4,5-^{3}H]leucine were obtained from Amersham (Arlington Heights, IL, U S A)

Plasma and tissue samples (kidney, liver, jejunum, heart muscle, diaphragm, and skeletal muscle) were derived from dogs infused with [^{3}H]leucine and [^{14}C]KIC [13].

Equipment

HPLC was performed using a C_{18} reversed-phase 5 μm particle size column (25 mm × 4 6 cm, Ultrasphere® ODS, Beckman, Fullerton, CA, U S A), a Varian liquid chromatograph (Model 441) and a UV-5 selectable-wavelength detector (Varian Assoc , Walnut Creek, CA, U S A) set at 214 nm The HPLC mobile phase (1 4 ml/min) consisted of 0 2 *M* sodium phosphate, pH 7 0–acetonitrile (90 10, v/v) Between each sample the column was flushed for 0 7 min with water, then methanol, and then again with water After the washes the system was re-equilibrated for $\simeq$5 min with the running buffer All injections were made with an automatic sample injector (WISP, Waters Assoc , Milford, MA, U S A) The entire peak of interest containing the radioactivity was collected by an Isco Retriever III fraction collector (ISCO, Lincoln, NE, U S A) and measured by liquid scintillation spectrometry using a Beckman LS9800 Series liquid scintillation counter as described below

Standards

Three sets of stock standards were made. (i) A concentration standard to be used for the quantitative standard curve to determine leucine and KIC concentrations (ii) An internal standard to be added to samples of unknown concentration (iii) An absolute standard to be injected directly onto the HPLC column to quantitate the nanomole levels of KIC and α-ketocaproate observed in unknown samples The stock concentration standard [containing both leucine (450 μM) and KIC (75 μM)], the internal standard [containing both norleucine (3 2 mM) and α-ketocaproate (800 μM)], and the absolute standard [containing both KIC (2 mM) and α-ketocaproate (2 mM)] were made from dried and weighed commercially available chemicals dissolved in deionized water.

The leucine and KIC concentration standard curves were formulated in the following fashion For the KIC concentration standard curve, 80 nmol of α-ketocaproate (100 μl of 800 μM stock standard) were added to 2 5, 2.0, 1 5, 1.0, 0 5, and 0 25 ml of KIC stock concentration standard (75 μM) For the leucine concentration standard curve, 320 nmol of norleucine (100 μl of 3 2 μM stock standard) were added to 3 0, 2 0, 1 5, 1 0 and 0 5 ml of the leucine stock concentration standard (450 μM) This amino acid concentration standard curve was processed with plasma and tissue samples, and thus leucine and norleucine were converted to KIC and α-ketocaproate, respectively, before HPLC analysis as described below

Determination of KIC and leucine specific activity and concentrations

A 2 0-ml volume of plasma, which had been stored at −70 °C, was adjusted to pH <1 by adding 230 μl of 2 *M* HCl, and 100 μl of a mixed internal standard [containing 80 nmol of α-ketocaproate (for KIC analysis) and 320 nmol of norleucine (for leucine analysis)] were added to each sample Aliquots of standard solutions of KIC (12 5–62 5 μ*M*) and leucine (75–450 μ*M*) were processed along with each set of plasma samples KIC and leucine were initially separated by transferring the plasma to a 5 cm × 1 cm column (Isolab) to which had been previously added 3 ml of a 50% aqueous suspension of a cation-exchange resin (or 1.5 ml bed volume) Following sample application, the columns were washed four times with 1-ml aliquots of 0 01 *M* HCl and the effluent plus washings were collected in 150 mm × 25 mm glass screw-capped tubes for KIC analysis

The effluent from the columns containing KIC was extracted once in 35 ml of methylene chloride After centrifugation for 5 min at 800 *g* the aqueous supernatant layer was aspirated and discarded The methylene chloride infranatant was transferred to a clean 150 mm × 25 mm tube and back-extracted with 370 μl of 0 2 *M* sodium phosphate buffer pH 7 0 After centrifugation for an additional 5 min at 800 *g*, the aqueous layer was transferred to 350-μl centrifuge tubes (Chrom Tech, Apple Valley, MN, U S A) briefly centrifuged (Beckman Microfuge), and 200 μl of the aqueous solution were injected into the HPLC system

Leucine was eluted from the washed AG50 column using four 1-ml aliquots of 25% ammonium hydroxide and collecting the effluent into 60 mm × 17 mm screw-capped vials (Kimble) The NH_4OH eluate was subsequently dried overnight using a Speedvac (Savant, Instruments, Farmingdale, NY, U S A.), and the dried samples containing the amino acids were kept at room temperature until analyzed A 1 0-ml volume of a solution containing 10 mg amino acid oxidase and 2 mg of catalase in 50 ml of 0 5 *M* Tris buffer, pH 7 6 was added to each sample of dry amino acids The sample was flushed for 10 s with oxygen, capped, and placed in a shaking water bath at 37 °C for 1 h A 130-μl aliquot of 2 *M* HCl was then added to make the pH <1, and the samples were subsequently processed exactly as described above for KIC except that only 6 5 ml of methylene chloride were added and only 100 μl of the aqueous solution were injected into the HPLC system In both assays described above, the entire peak of interest was collected for subsequent liquid scintillation spectrometry

The plasma concentrations of KIC and leucine were calculated using extracted KIC (12 5–62.5 μ*M*) and leucine (75–450 μ*M*) standards as follows:

$$[\text{leucine (KIC)}] = \frac{\text{CF} \times \text{PH}_{\text{LEU (KIC)}}}{\text{PH}_{\text{I S}}}, \text{ where } \text{CF} = \frac{[\text{STD}] \times \text{PH}_{\text{I S}}}{\text{PH}_{\text{LEU (or KIC)}}}$$

and where [STD] (μ*M*) is the concentration of the leucine or KIC concentra-

tion standard injected, PH is the chromatographic peak height of all components analyzed [internal standards (I S), KIC, or leucine (LEU)], and CF (μM) is the calculation factor required to convert PH of KIC or leucine to plasma concentrations For most analyses, the calculation factor is assumed to be constant over the concentration range of a given method This was observed for KIC (see results below and Fig 4, upper panel), however, for leucine (enzymatically converted to KIC before HPLC analysis) this was not the case over the wide range of leucine concentrations analyzed, the calculation factor was instead linearly related to the mass of leucine analyzed when the internal standard mass was held constant (see results below and Fig. 4, lower panel).

To determine the specific radioactivity of any compound, the absolute mass of unlabeled material and the radioactivity in the mass must be quantitatively determined. In the determination of the plasma KIC and leucine (the latter analyzed as KIC following its enzymatic conversion) specific activity, the absolute nanomoles of the injected unknown sample must be accurately quantitated A 0–50 nmol amount (absolute standard curve) of KIC was injected for the quantitation of the KIC derived from an unknown sample and 0–100 nmol of KIC for the absolute quantitation of the plasma leucine (analyzed as KIC following enzymatic conversion) derived from an unknown sample It is necessary to use KIC as the absolute standard for both the KIC and the leucine specific activity determinations, since leucine is enzymatically converted to KIC prior to the HPLC analysis The entire effluent of the peak of interest was collected ($\simeq$1 2 ml), 14 ml of Aquassure (containing ethanolamine) were added, and the radioactivity was determined by liquid scintillation spectrometry (see below) The ^{3}H and ^{14}C specific activities of KIC and leucine were calculated by dividing the radioactivity (dpm) by the absolute nanomole content of the injected sample

Determination of leucine specific activity in tissue proteins

Approximately 0 2–1 0 g of frozen tissue were powdered in liquid nitrogen in a 4°C walk-in refrigerator and added to pre-weighed vials containing 5% sulfosalicylic acid ($\simeq$1 ml per 100 mg powdered tissue) Tissue samples were homogenized (Polytron homogenizer, Kinematica, Kriens, Switzerland) in 5% sulfosalicylic acid on ice for 45 s and then centrifuged at 5000 *g* (Beckman J 6B centrifuge) for 40 min at 4°C The resultant supernatant was discarded, and the pellet was used to determine ^{3}H and/or ^{14}C specific activity of leucine in the tissue proteins sampled (see below) The protein pellet was resuspended in 10 ml of 5% sulfosalicylic acid with a Polytron homogenizer as described above Thereafter, the samples were centrifuged at 5000 *g* for 20 min at 4°C, and the supernatant was discarded This process was repeated twice using deionized water With each resuspension the tissue was homogenized for 15 s in order to eliminate any carry-over of trapped ^{3}H or ^{14}C from free leucine or KIC in the tissue protein pellet When samples were processed as described

above, less than 1% of ^{3}H and ^{14}C present in the initial supernatant was recovered in the final supernatant The final protein precipitate was placed in 6 ml of 6 *M* HCl, gassed with nitrogen to remove any dissolved oxygen and hydrolysed at 110°C in a sealed container for 72 h [14] Approximately 0 5 g of charcoal was subsequently added to this hydrolysate to remove colored material After the charcoal was settled, the sample was filtered through a 0.2-μm filter (Acrodisc, Gelman Science, Ann Arbor, MI, U S A)

Analytical procedure to determine leucine specific activity in tissue

Tissues with higher protein turnover rates (direct method) A 200-μl volume of the resultant clear filtrate (pH < 1) of hydrolysed protein (fibrin, heart muscle, liver, jejunum, kidney) was placed over a column containing a 1 5-ml bed volume of a cation-exchange resin The column was then rinsed four times with 1-ml aliquots of 0 01 *M* HCl The free amino acids were eluted from the column with four 1-ml aliquots of 25% NH_4OH. The NH_4OH eluate was subsequently dried overnight using a Speed Vac concentrator To each sample of dry amino acid, 1 0 ml of a solution containing 50 mg amino acid oxidase and 10 mg of catalase in 50 ml of 0 5 *M* Tris buffer, pH 7 6 was added (five times that described above) The samples were subsequently processed as described above for plasma leucine except that (i) only 50–150 μl (dependent on the anticipated mass of KIC to be derived from the original sample) of the final aqueous phosphate buffer solution was injected into the HPLC system and (ii) a 100–400 nmol KIC absolute standard curve was used

Determination of specific activity of leucine in tissue proteins with low turnover (indirect method) Using the above described direct HPLC method, we were unable to measure the specific activity in leucine derived from proteins with low turnover rates (skeletal muscle, diaphragm), because the leucine mass required to collect sufficient radioactivity in these low-specific-activity proteins exceeds the capacity of the HPLC column and/or the detector used Thus we developed an indirect method to measure the specific activity of leucine in low turnover proteins using in part the above described HPLC method

A 1 0-ml volume of the protein hydrolysate was processed as described above for tissue protein samples from high-turnover proteins, except that (i) a 3-ml bed volume of a cation-exchange resin was used, (ii) the column was first rinsed four times with 2 ml of 0.01 *M* HCl, and (iii) the amino acids were eluted four times with 2 ml of 25% NH_4OH The eluate was dried as described above, resuspended in 1 0 ml of distilled water, and sonicated (Bransonic 42, Shelton, CT, U S A) for 30 min to redissolve all of the leucine To 30 μl of this solution 320 nmol of norleucine were added, the resultant sample was then processed as described above for plasma leucine except that only the concentration of leucine was determined by HPLC To 0 9 ml of the remaining solution, 12 ml of the scintillation cocktail (Aquassure) were added to determine the radioactivity in leucine The specific activity of leucine in proteins of all tissues

investigated was then calculated using the dpm/ml measured by scintillation spectrometry and the nanomole content calculated from the concentration of leucine measured in the 30-μl aliquot of the original 1-ml sample

To validate this indirect method, proteins with high turnover rates (heart muscle, jejunum, kidney, liver) were analyzed using both the direct and indirect methods In addition, specific activities of leucine from proteins with slow turnover rates were measured using just the indirect method

Determination of leucine specific activity in plasma fibrin A 100-μl volume of 1 *M* $CaCl_2$ and thrombin (10 I U) were added to 2 ml of plasma to precipitate fibrin The sample was then incubated for 1 h at room temperature, after which the fibrin clot was physically removed from the remaining serum with a small wooden probe Thereafter, the fibrin was processed as described above for tissue protein sample, except that it was not precipitated with 5% sulfosalicylic acid

Determination of radioactivity

The ^3H and ^{14}C radioactivity were determined using a Beckman LS9800 Series liquid scintillation counter using a dual-channel counting mode, which correct the radioactivity for both quench and the spillover of ^{14}C radioactivity into the ^3H energy spectrum

Statistical analysis

All results are expressed as mean $\pm$S E M Two-tailed paired and unpaired Student's *t*-test were used for statistical analysis, where appropriate

RESULTS AND DISCUSSION

KIC and α-ketocaproate standard curves

In the 0–40 nmol range, the peak-height response of KIC and α-ketocaproate was linear, although the slopes of the curves were different for each component [$Y=(3\ 49x+0\ 64)10^3$, $r=0\ 9999$, $y=(1\ 37x-0\ 24)10^3$, $r=0\ 9999$, respectively, Fig 1, upper panel] In contrast to KIC, the peak-height response of α-ketocaproate remained linear up to 120 nmol α-ketocaproate [$y=(1\ 38x+0\ 33)10^3$, $r=0\ 9999$, Fig 1, middle panel] The peak-height response of KIC is blunted above 50 nmol Between 50 and 200 nmol the KIC response can be described with another linear function [$y=(2\ 06x+61)10^3$, $r=0\ 9997$] Using peak-area response rather than peak-height response, a linear relationship exists for α-ketocaproate from 0 to 400 nmol, whereas for KIC this linear relationship is only valid from o to 240 nmol (Fig 1, lower panel) Because of the close relationship between the KIC and α-ketocaproate peaks (Fig 2), peak height rather than peak area should be used to quantitate KIC

From these data, we cannot determine the cause(s) of the non-linear (or discontinuous linear) nature of these two standard curves Several explana-

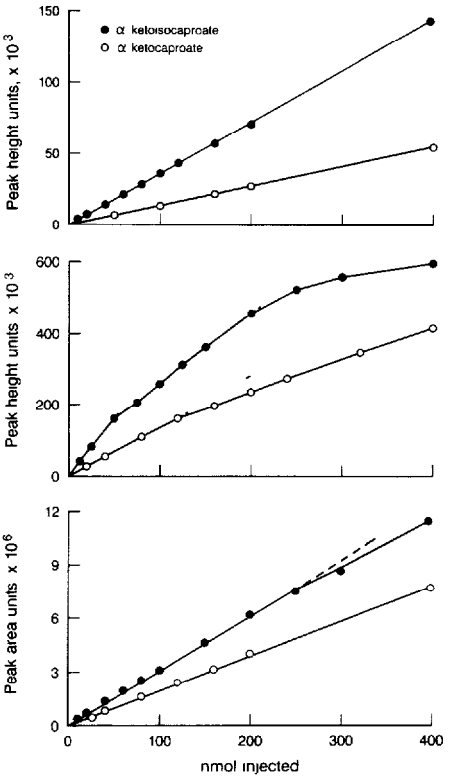

Fig 1 (Upper panel) Peak-height response of α-ketoisocaproate (●) and α-ketocaproate (○) from 0–40 nmol injected Note the linear relationship but different slopes of the two compounds (Middle-panel) Peak-height response of α-ketoisocaproate (●) and α-ketocaproate (○) from 0–400 nmol injected Note the discontinuous linear nature of the α-ketoisocaproate standard curve above 50 nmol injected and above 120 nmol for α-ketocaproate (Lower panel) Peak-area response of α-ketoisocaproate (●) and α-ketocaproate (○) from 0–400 nmol injected Note the change in slope of the α-ketoisocaproate standard curve above 240 nmol injected

tions might account for these findings At the larger injected mass of KIC and α-ketocaproate, saturation of the column binding sites is possible This is unlikely over the range of substrate injected in these studies, since the recovery of [^{14}C]KIC added to the standard was nearly 100% regardless of the mass of unlabeled KIC injected (Fig 3, upper panel) Thus it would appear, that the primary explanation for these findings relates to the detector employed (its optics or electronics) These hypothesis is supported by the observation (unpublished results) that changes in the a u f s (absorbance units full scale) on a given detector affects the linearity ranges of the standard curves Recognizing the importance of detector linearity (or non-linearity) and that changes in detector linearity occur with changes in the a u f s are of importance to any investigator attempting to establish a quantitative HPLC assay

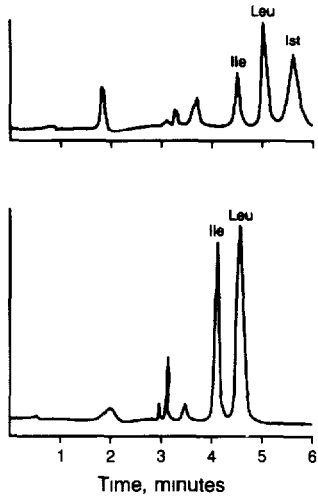

Fig 2 HPLC profiles of the α-ketoacids of leucine (Leu, KIC), isoleucine (Ile, α-ketoisovalerate) and norleucine (α-ketocaproate, internal standard, Ist) in plasma (upper panel) and of a protein hydrolysate of fibrin (lower panel, see Experimental section for details)

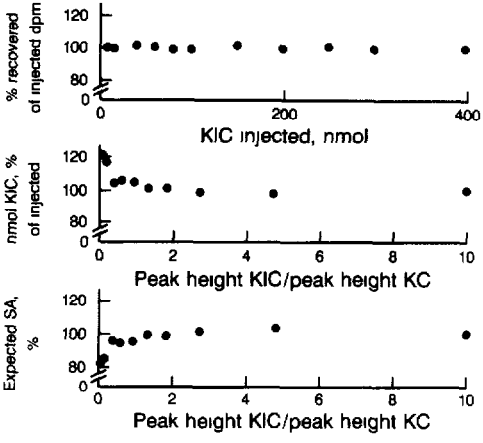

Fig 3 Effect of α-ketoisocaproate (KIC)/α-ketocaproate (KC) peak-height ratio on recovery of dpm and nmol using HPLC The upper panel depicts the percentage recovery dpm of [^{14}C]KIC when injected with 0–400 nmol of unlabeled KIC The middle panel depicts the nmol of unlabeled KIC expressed as percentage injected, when plotted against the peak-height ratio of KIC/α-ketocaproate (internal standard) Note that below a ratio of 1 0 the calculated nmol of KIC are overestimated and thus (lower panel) the specific activity of [^{14}C]KIC is underestimated

Effect of KIC/α-ketocaproate peak-height ratio on dpm and nmol determinations

Because of the close relationship between the α-ketocaproate and KIC peaks (Fig 2), it was important to determine the effect of the amount of internal standard α-ketocaproate on the nanomole content of KIC recovered when both

compounds were injected together Fig 3 (upper panel) shows that 99 1 ± 0 7% of the radioactivity injected as [^{14}C]KIC was recovered after HPLC analysis and was independent of the amount of unlabeled KIC injected In contrast, when the molar ratio of KIC to α-ketocaproate was less than 1, the nanomoles of KIC recovered were overestimated and, therefore, the specific activity of KIC was underestimated (Fig 3, middle and lower panels) Thus, the α-ketocaproate peak should optimally be smaller than the KIC peak

Factors used in the measurement of plasma KIC and leucine concentrations

To calculate the concentration of either leucine or KIC in a sample using the internal standard method, a factor must be calculated to convert the peak-height ratio of the internal standard (α-ketocaproate or norleucine) and parent compound (KIC or leucine) to the desired units (μM) In the physiological range of KIC (0–50 μM), the calculation factor for conversion of peak height to concentration was constant (Fig 4, upper panel) as a result of the linearity of the absolute KIC standard curve over this concentration range (Fig 1, upper panel) In contrast, when leucine concentrations were between 75 and 450 μM, the calculation factor was not constant and was best described as a linear function of the plasma leucine concentration ($y=0\ 22x+72\ 12$) (Fig 4, lower panel) The non-constant nature of the calculation factor is a direct result of the different, non-linear characteristics of the KIC and α-ketocaproate standard curves (Fig. 1, middle panel) As a result, it is of importance to analyze

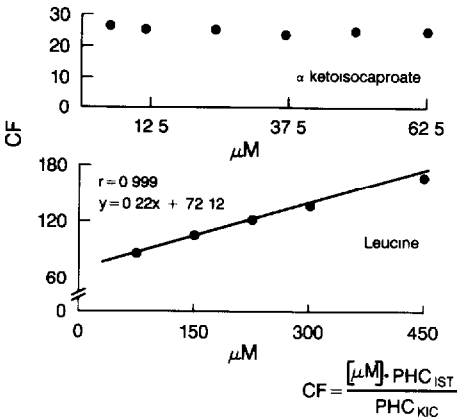

Fig 4 Calculation factor used in the measurement of plasma KIC and leucine concentrations using HPLC (Upper panel) To calculate the concentration of either leucine or KIC in a sample using the internal standard method, a factor must be calculated to convert the peak-height ratio of the internal standard (α-ketocaproate or norleucine) and parent compound (KIC or leucine) to the desired units (μM) In the physiological range of KIC (0–50 μM), the calculation factor for conversion of peak height to concentration was constant (Lower panel) In contrast, when leucine concentrations were between 75 and 450 μM, the calculation factor was not constant and was best described as a linear function of the plasma leucine concentration

complete leucine (and KIC) concentration standard curves with any analysis of unknowns to be able to determine accurately the appropriate calculation factor

Within-day and day-to-day variability of concentrations and specific activities of KIC and leucine in plasma and the specific activity of leucine incorporated into proteins

Nine to ten pooled plasma samples and samples containing leucine from proteins were processed and analyzed together on the same day Plasma samples exhibited a within-day coefficient of variation for replicate analysis of less than 1 1% for concentration and specific activity of leucine (Table I), whereas the within-day variability of leucine specific activity derived from proteins was higher (4.4 and 5 2%, Table I) The within-day coefficient of variation of KIC (ten plasma samples) concentrations and ^{3}H and ^{14}C specific activities were 1 2, 1 3 and 0 8%, respectively

Eight to nine aliquots of a pooled sample were prepared and analyzed with independently prepared standard curves on eight or nine different days The day-to-day coefficients of variation for replicate analysis for leucine and KIC concentrations and specific activities were 4 7 and 2 5% and 4 4 and 4 8%, respectively (Table II) The day-to-day variability of the specific activity of leucine in fibrin was 6%

To determine the effects of freeze-thawing and prolonged exposure to room temperature on leucine concentrations and specific activities, multiple plasma samples and samples containing leucine from different proteins were analyzed on two different days. For the first analysis, samples were thawed and then kept at room temperature for about 8 h After analysis, they were again frozen at $-20\,^{\circ}C$ for another 48 h After re-thawing, samples were analyzed a second

TABLE I

WITHIN-DAY VARIABILITY OF LEUCINE CONCENTRATIONS IN PLASMA AND OF LEUCINE SPECIFIC ACTIVITIES IN PLASMA AND PROTEINS

Sample	n^a	Concentration (mean ± S E M) (μM)	Specific activity (dpm/nmol)	Coefficient of variation (%)
Leucine in plasma	10	160 0 ± 0 9		0 6
[^{3}H]Leucine in plasma	10		91 8 ± 0 9	1 1
[^{14}C]Leucine in plasma	10		19 5 ± 0 3	1 0
[^{3}H]Leucine in fibrin	10		4 20 ± 0 22	5 2
[^{14}C]Leucine in liver	9		1 78 ± 0 09	4 4

[a]Nine or ten pooled plasma samples and samples containing leucine from hydrolysates of proteins were processed and analyzed together on the same day

TABLE II

DAY-TO-DAY VARIABILITY OF LEUCINE CONCENTRATIONS IN PLASMA AND OF LEUCINE SPECIFIC ACTIVITIES IN FREE PLASMA LEUCINE AND LEUCINE INCORPORATED IN PLASMA PROTEINS

Sample	Day-to-day sample analysis[a]			Day-to-day effects of temperature change[b]			
	n[c]	Mean ± S E M	C V (%)	n	Mean ± S E M		C V (%)
					Day 1	Day 2	
Concentration (M)							
Leucine	9	168 9 ± 2 7	4 7	56	163 0 ± 4 6	154 8 ± 4 0	3 9
Specific activity (dpm/nmol)							
[^{3}H]Leucine in plasma	8	88 9 ± 7 4	4 4	56	113 4 ± 4 0	110 4 ± 3 8	3 3
[^{14}C]Leucine in plasma	-	-	-	56	23 5 ± 0 7	23 4 ± 0 7	1 2
[^{3}H]Leucine in fibrin	9	3 95 ± 0 08	6 0	24	4 63 ± 0 10	4 54 ± 0 09	5 3
[^{14}C]Leucine in fibrin	-	-	-	24	1 65 ± 0 03	1 62 ± 0 03	5 8

[a]Eight or nine aliquots of a pooled sample were prepared and analyzed with independently prepared standard curves on eight or nine different days

[b]To determine the effects of freeze-thawing and prolonged exposure to room temperature on leucine concentrations and specific activities, multiple plasma samples and fibrin samples containing labeled leucine were analyzed on two different days For the first analysis, samples were thawed and then kept at room temperature for about 8 h After analysis, they were again frozen at −20 °C for another 48 h

[c]Same samples analyzed twice

time The coefficient of variation of the two analysis was less than 6%. These data demonstrate the stability of the processed samples and the reproducibility of the analytical equipment employed

Comparison of a direct and indirect method to determine leucine specific activity in proteins

Skeletal muscle contains about 20% of whole body protein Since skeletal muscle proteins have a very low turnover rate, we were unable to measure leucine specific activity accurately in proteins derived from hydrolysates of skeletal muscle and diaphragm by the direct HPLC method Therefore the indirect method described above was used to determine the amount of radioactivity incorporated in vivo into leucine in mixed skeletal muscle proteins To validate this indirect method, leucine specific activity in proteins with high turnover rates were determined by both the direct and the indirect method

Table III depicts the comparison of the indirect and direct method using tissue proteins with relatively higher rates of protein turnover (liver, kidney, jejunum) and one with a lower turnover rate (heart muscle) No significant difference could be detected between the two methods in all tissue proteins

TABLE III

DIRECT (HPLC) AND INDIRECT (AG50-ELUTED) DETERMINATION OF LEUCINE SPECIFIC ACTIVITIES IN PROTEINS FROM TISSUES WITH HIGH RATES OF PROTEIN TURNOVER

For detailed explanation of the direct and indirect analysis procedures see Experimental section
N S = not significant

Sample	Specific activity (mean ± S E M, $n=12$) (dpm/nmol)				P value AG50 versus HPLC data	
	^3H		^{14}C			
	AG50	HPLC	AG50	HPLC	^3H	^{14}C
Kidney, left	2 83 ± 0 12	3 00 ± 0 24	0 90 ± 0 06	0 92 ± 0 06	N S	N S
Kidney, right	3 04 ± 0 26	3 14 ± 0 12	0 91 ± 0 08	0 93 ± 9 09	N S	N S
Jejunum	4 44 ± 0 55	4 57 ± 0 53	1 71 ± 0 22	1 71 ± 0 22	N S	N S
Liver	2 45 ± 0 22	2 49 ± 0 17	0 85 ± 0 07	0 86 ± 0 06	N S	N S
Heart	0 71 ± 0 05	0 80 ± 0 05	0 28 ± 0 02	0 31 ± 0 02	N S	N S

TABLE IV

SPECIFIC ACTIVITY OF LEUCINE (USING THE INDIRECT METHOD) IN PROTEINS WITH LOW TURNOVER RATES

Sample	Specific activity (mean ± S E M, $n=8$) (dpm/nmol)	
	[^{3}H]Leucine	[^{14}C]Leucine
Quadriceps muscle, left	0 151 ± 0 015	0 058 ± 0 005
Quadriceps muscle, right	0 148 ± 0 013	0 057 ± 0 004
Rectus muscle	0 152 ± 0 014	0 058 ± 0 005
Diaphragm	0 246 ± 0 017	0 095 ± 0 004

investigated, indicating that this indirect method may be used to measure leucine specific activity in skeletal muscle and diaphragm

Using the indirect method alone, the specific activities of [^{3}H]- and [^{14}C]leucine derived from hydrolysates of low-turnover proteins (skeletal muscle, diaphragm) were lower by more than 50% than in heart muscle (Tables III and IV) In addition, identical results were obtained in samples derived from quadriceps muscle right and left, indicating a good reproducibility of the indirect method

With increasing attention being drawn to factors which regulate rates of tissue and/or specific protein synthesis in vivo [10], precise methodologies must be available to measure accurately the specific activity of an amino acid in both the product and precursor pools. Recent studies have demonstrated

that the specific activity of KIC during the infusion of labeled leucine may more accurately reflect the specific activity of the intracellular leucine pool [15], from which the t-RNA for protein synthesis are most likely charged [16], thus, the plasma KIC specific activity may provide a more accurate indicator of the precursor pool specific activity for protein synthesis than the plasma leucine specific activity This report describes for the first time a single methodology for the measurement of the plasma leucine and KIC specific activity and the specificity activity of leucine derived from hydrolysates of labeled proteins with high turnover rates In addition, using components of this same assay methodology, an indirect method for the determination of leucine specific activity in tissue protein with low turnover rates is described and validated The use of a single methodology in the determination of the product and precursor pool specific activity will minimize the potential for systematic analytical errors introduced by the use of different methods and thus increase the precision and accuracy of data derived from such studies

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