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Note

Determination of plasma concentrations and ^{3}H-specific activity of phenylalanine in plasma using high-performance liquid chromatography

LOUISE LECAVALIER[a], FRITZ F. HORBER and MOREY W. HAYMOND*

Departments of Pediatrics and Internal Medicine, Endocrine Research Unit, Mayo Clinic and Foundation, 5-154 W. Joseph, Rochester, MN 55905 (U.S A)

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Isotope dilution techniques employing radioactive and stable tracers have been widely utilized over the past ten years to investigate the factors which regulate protein and amino acid metabolism in vivo. Isotopes of the essential amino acid leucine and its α-keto acid, α-ketoisocaproate (KIC), have been used in both in vivo and in vitro studies [1–11]. We previously developed a high-performance liquid chromatographic (HPLC) method for the simultaneous measurement of the plasma concentration and specific activity of both leucine and KIC [12,13]. Since leucine is transaminated in virtually all tissues in mammals, the specific activity for the transaminated product of the infused tracer (e.g. [^{3}H]KIC during infusion of [^{3}H]leucine of [^{14}C]leucine during infusion of [^{14}C]KIC [14]) may provide a more accurate indicator of the intracellular specific activity of leucine than the specific activity of the infused tracer [1,6]. The transamination of leucine and KIC in tissues and the intracellular dilution and metabolism of leucine and KIC traces, however, make it nearly impossible to study quantitatively the metabolism of leucine by using mass and isotope balance studies across a tissue bed.

In contrast to leucine and other branched-chain amino acids, the essential amino acid phenylalanine cannot be metabolized by peripheral tissues such as muscle and is only degraded in the liver; therefore, it can only be released from

[a]Author deceased.

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or incorporated into proteins in virtually every tissue except liver. Thus, labeled phenylalanine may serve as an ideal tracer to investigate the hormonal and substrate factors which regulate protein metabolism in non-hepatic tissue such as the forearm [15,16].

The purpose of this paper is to describe a method for the simultaneous measurement of the concentration and specific activity of plasma phenylalanine. This method permits the specific, sensitive and accurate measurement of both concentrations and specific activities of leucine and phenylalanine during the simultaneous infusion of ^{3}H and/or ^{14}C tracers of leucine [12,13] and phenylalanine.

EXPERIMENTAL

Chemicals and biologic materials

HPLC-grade solvents acetonitrile (UV cutoff 190 nm), methylene chloride (UV cutoff 235 nm) and methanol (UV cutoff 235 nm) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Cation-exchange resin (AG50W-X80, 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. Phenylalanine, norleucine, leucine, phenylpyruvate, α-ketoisocaproate, α-ketocaproate, L-amino acid oxidase (type I), bovine liver catalase and all other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Safety Solve® was obtained from Research Products International (Mount Prospect, IL, U.S.A.). L-[2,6-^{3}H]Phenylalanine was obtained from Amersham (Arlington Heights, IL, U.S.A.). The [^{3}H]phenylalanine was >95% pure as measured by an amino acid analyzer (Beckman 119CL, Beckman Instruments, Irvine, CA, U.S.A.) and contained only 1.5% tritiated water. In selected experiments, a known amount of [^{3}H]phenylalanine was added to both standards and plasma samples. Plasma were derived from humans infused with [^{3}H]phenylalanine [14].

Equipment

HPLC was performed using a 5-μm Ultrasphere® ODS column (25 cm × 4.6 mm I.D.) (Beckman, Fullerton, CA, U.S.A.), a Model 441 Varian liquid chromatograph 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) and 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 approximately 5 min with the running buffer. The entire analysis time was 11 min. All injections were made with an automatic sample injector (WISP, Waters Assoc., Milford, MA, U.S.A.). The phenylpyruvate peak, containing the radioactivity originally present in phenylalanine, was collected by an Isco Retriever III fraction collector (ISCO, Lincoln, NE, U.S.A.) and was

measured by liquid scintillation spectrometry using a Beckman LS9800 Series liquid scintillation counter as described below. Plasma phenylalanine concentrations were independently determined using a Beckman 119 CL amino acid analyzer [17].

Standards

Three sets of stock standards were made: (1) a concentration standard to be used for the quantitative standard curve to determine leucine and phenylalanine concentrations; (2) an internal standard to be added to samples of unknown concentration; and (3) an absolute standard to be injected directly onto the HPLC column to quantify the nanomoles of phenylpyruvate and α-ketocaproate observed in unknown samples. The stock concentration standard [containing both leucine (150 μ*M*) and phenylalanine (50 μ*M*)], the internal standard [norleucine (3.2. m*M*)] and the absolute standard [containing KIC (2 m*M*), α-ketocaproate (2 m*M*) and phenylpyruvate (2 m*M*)] were made from dried and weighed commercially available chemicals dissolved in deionized water.

The phenylalanine concentration standard curve was formulated in the following fashion: 320 nmol of norleucine (100 μl of 3.2 m*M* stock standard) was added to 3.0, 2.0, 1.5, 1.0 and 0.5 ml of the phenylalanine stock concentration standard (50 μ*M*). This amino acid concentration standard curve was processed with plasma and tissue samples, and thus phenylalanine and norleucine were converted to phenylpyruvate and α-ketocaproate, respectively, before HPLC analysis as described below.

Determination of phenylalanine specific activity and concentrations

Plasma (2 ml), which had been stored at $-70°C$, was adjusted to $pH < 1$ by adding 230 μl of 2 *M* hydrochloric acid, and 100 μl of internal standard (containing 320 nmol of norleucine) were added to each sample. Aliquots of standard solutions of phenylalanine (12.5–150 μ*M*) were processed along with each set of plasma samples. Possible phenylpyruvate in plasma was initially separated by transferring the plasma to a 5×1 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* hydrochloric acid and the effluent was discarded.

Phenylalanine 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 ammonium hydroxide eluent 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-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 volume of 2 *M* hydrochloric acid was then added to make to pH < 1. After vortexing the samples (5 s), 6.5 ml of methylene chloride were added and vortexed for another 30 s. After centrifugation for 5 min at 550 *g* (Beckman J 6B centrifuge), the aqueous supernatant layer was aspirated and discarded. To the methylene chloride infranatant, 370 μl of 0.2 *M* sodium phosphate buffer (pH 7) were added and the sample was vortex-mixed for another 20 s. After centrifugation for an additional 5 min at 550 *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. The entire peak of interest was collected for subsequent determination of the radioactivity eluted in the peak(s) of interest by liquid scintillation spectrometry.

Calculation of plasma phenylalanine concentration and specific activity

The plasma concentration of phenylalanine was calculated using extracted phenylalanine concentration (12.5–150 μ*M*) standards as follows:

$$[\text{phenylalanine}] = \frac{\text{CF} \times \text{PH}_{\text{PHE}}}{\text{PH}_{\text{I S}}}$$

$$\text{where CF} = \frac{[\text{STD}] \times \text{PH}_{\text{I S}}}{\text{PH}_{\text{PHE}}}$$

and where [STD] is the concentration of the phenylalanine standard injected (in μ*M*), PH is the chromatographic peak height of all components analyzed (I.S. is internal standard and PHE is phenylalanine) and CF (μ*M*) is the calculation factor required to convert PH of phenylalanine plasma concentrations.

In the determination of the plasma phenylalanine (analyzed as phenylpyruvate following its enzymatic conversion) specific activity, the absolute nanomoles of the injected unknown sample must be accurately quantitated. A 0–200 nmol amount (absolute standard curve) of phenylpyruvate and α-ketocaproate was injected for the quantitation of the phenylalanine derived from an unknown sample. It is necessary to use phenylpyruvate as the absolute standard for phenylalanine specific activity determinations, since phenylalanine is enzymatically converted to phenylpyruvate prior to the HPLC analysis. The entire effluent of the peak of interest was collected (≃ 2.8 ml), 15 ml of Safety Solve were added, and the radioactivity was determined by liquid scintillation spectrometry (see below). The ^{3}H-specific activity of phenylalanine was calculated by dividing the collected radioactivity (dpm) by the absolute nanomole content of the injected sample.

Determination of radioactivity

The ^{3}H radioactivity was determined using a Beckman LS9800 Series liquid scintillation counter.

Statistical analysis

All results are expressed as mean ±standard error of the mean (S.E.M.). Two-tailed paired and unpaired Student *t*-tests were used for statistical analysis, where appropriate.

RESULTS AND DISCUSSION

Fig. 1 depicts an HPLC profile of the α-keto acids derived from a standard solution containing isoleucine, leucine, norleucine and phenylalanine and from a plasma sample to which standard solution was added. The retention time of these four α-keto acids (α-ketomethylvalerate, α-ketoisocaproate, α-ketocaproate and phenylpyruvate) were 4.7, 5.2, 5.7 and 9.2 min, respectively. All peaks were clearly separated from each other. When a known amount of [^{3}H]phenylpyruvate was injected, 98.0±1.2% ($n=3$) of the radioactivity was recovered in the 2-min collection period of the phenylpyruvate peak. The absolute recovery of both labeled and unlabeled phenylalanine from both plasma and standard in the phenylpyruvate HPLC peak was 30%.

The absolute standard curves of phenylpyruvate and α-ketocaproate are depicted in Fig. 2. These curves were linear only through 50 nmol and were best described by a second-degree polynomial equation. From these data, we cannot determine the cause(s) of the non-linear nature of these two standard curves. Several explanations might account for these findings. At the larger injected

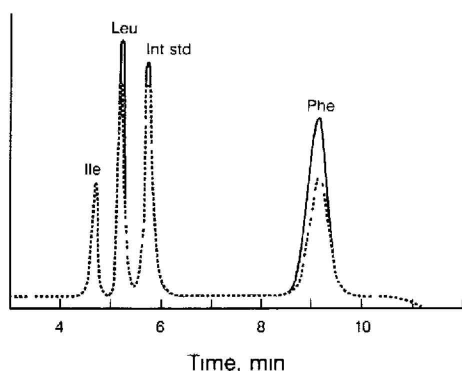

Fig. 1. HPLC profile of the α-keto acids (---) from a standard solution containing isoleucine (Ile), leucine (Leu), norleucine (Int std) and phenylalanine (Phe) and (——) from a plasma sample to which the standard solution was added. Note the excellent separation of phenylalanine. The ordinate is an arbitrary absorbance unit at 214 nmol.

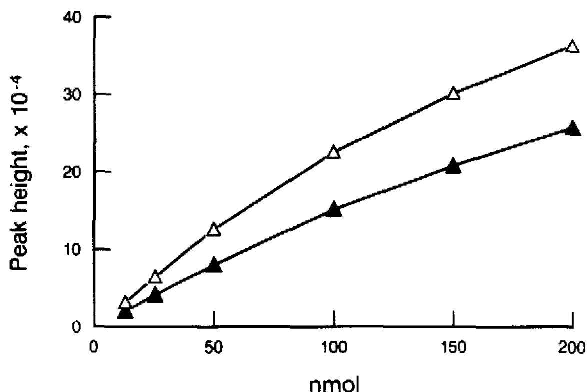

Fig. 2. Peak-height response of α-ketocaproate (▲) and phenylpyruvate (△) from 0 to 200 nmol injected. Note the polynome nature, but different slopes of the two standard curves.

mass of phenylpyruvate 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 [^{3}H]phenylpyruvate added to the standards was nearly 100%, regardless of the mass of unlabeled phenylpyruvate added (see above). Thus, the primary explanation for these findings relates to the detector employed (its optics or electronics). This conclusion is supported by the observation (unpublished results) that changes in the a.u.f.s. (absorbance units full scale) on a given detector affect 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. The slope of the theoretical concentration from weighed standards (12.5–150 μM) when plotted against those obtained by the HPLC method described above was 0.92 (data not shown) with a correlation coefficient of 0.997 ($p<0.001$); this indicates a high accuracy of the described method.

To assess the accuracy of the recovery of phenylalanine from plasma, varying amounts of phenylalanine standards (25–150 nmol) were added to triplicate 2.0-ml aliquots of pooled plasma, and both the standard and the plasma samples were analyzed by HPLC and amino acid analysis. These results are depicted in Fig. 3. The results obtained with HPLC and the amino acid analysis were similar (correlation coefficient of each method 0.997). When analyzed by the amino acid analyzer, the slope of the observed versus the theoretical values was 1.06 for both the standards and the plasma sample plus standard. When analyzed by HPLC, the slope of the standards was 1.03 and the slope of the plasma sample plus standard curve was 0.96. Thus, the dose–response curves of the standards and plasma plus standards were essentially parallel.

The intra-day coefficient of variation (C.V.) of replicate analysis ($n=10$)

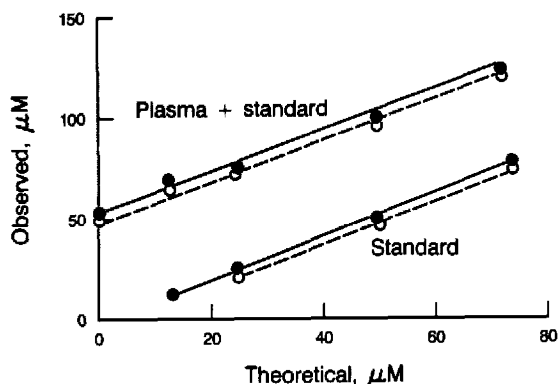

Fig. 3. Phenylalanine concentration as measured by HPLC (●) and amino acid analysis (○) before (standard) and after adding known amounts of phenylalanine to plasma (plasma+standard).

for the concentration determinations from standards and plasma samples was 2.5 and 4.5%, respectively, and for specific activity, 2.3 and 3.6%, respectively. The inter-day C.V. ($n=10$) for plasma concentration and specific activity was 7.1 and 7.5%, respectively.

In summary, we developed a new technique to measure accurately the concentration and specific activity of phenylalanine by converting phenylalanine to phenylpyruvate and injecting this last compound into the HPLC system. The major advantage of this new technique is that we can measure simultaneously plasma concentration and specific activity of phenylalanine and leucine [12,13] in the same 11-min analysis. In addition, this is a method which might be used in the rapid and accurate quantitation of phenylalanine or its α-keto acid phenylpyruvate in the diagnosis, monitoring and regulation of patients with phenylketonuria.

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