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Note

Rapid high-performance liquid chromatographic method to measure plasma leucine: importance in the study of leucine kinetics in vivo

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A significant amount of useful data concerning amino acid and protein metabolism in mammals has been obtained with tracer methods using isotopes of leucine, an essential branched-chain amino acid (BCAA). With such methods, rates of appearance (R_a) and disappearance (R_d) of leucine molecules into and out of the plasma compartment can be estimated. A major assumption for the use of these methods is that the system is in a steady state condition, where leucine R_a equals R_d. Perturbants that induce non-steady-state conditions, changing the plasma concentrations, render the data from these tracer methods difficult to interpret. We hypothesized that minimizing the fluctuations in plasma leucine during metabolic perturbations by rapid measurement and variable exogenous leucine infusion would allow us to better estimate leucine flux.

We set out to develop a method to be used in human and animal experimentation, that would allow a constant circulating plasma leucine level to be maintained in the presence of various perturbations. A system is therefore required from which leucine concentrations can be reported in intervals of less than 15 min. Until recently, physiological amino acid determinations required long run times on expensive dedicated analyzers. Currently, high-performance liquid chromatographic (HPLC) methods are being developed to measure BCAAs and other amino acids more economically in terms of equipment, sample volume and time [1–5]. These methods, however, still involve multiple pumps and rather sophisticated controllers to accomplish the necessary gradients. Many of these methods require at least 1 ml of sample and at least 20 min analysis times. We describe here a sensitive, isocratic reversed-phase method using pre-column derivatization with *o*-phthalaldehyde (OPA) to quantitate the BCAAs, primarily leucine, with greater speed and efficiency.

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EXPERIMENTAL

Chromatographic equipment

The chromatographic equipment consisted of a U6K injector, a Model 510 pump, a Model 420 fluorescence detector equipped with a 338-nm excitation filter and a 425-nm emission filter and a Model 740 integrator, all from Waters Chromatography Division (Milford, MA, U.S.A.). Separation of the BCAAs was accomplished with either a 15 cm × 3.9 mm column of 4-μm spherical, end-capped C_{18} particles (Nova-Pak™, from Waters) or with a radial compression module and a Nova-Pak cartridge (Waters Chromatography Division). The chromatographic system was housed on a portable cart along with a Model 59A microcentrifuge, a vortex mixer and a standard lab timer (all from Fisher Scientific, Pittsburgh, PA, U.S.A.)

Mobile phase preparation

Phosphate buffer (0.005 *M*) was prepared using HPLC-grade potassium dihydrogenphosphate (Fisher Scientific) and HPLC-grade water. The pH was adjusted to 7.0 (Orion Model 501 pH meter, Fisher Scientific) with triethanolamine (Fisher Scientific) and the buffer was filtered using a Millipore solvent clarification kit with a 0.45-μm type HA filter (Millipore, Bedford, MA, U.S.A.). Mobile phase was prepared by mixing the above buffer with methanol and acetonitrile (both HPLC grade from Burdick and Jackson Labs., American Scientific Products (Stone Mountain, GA, U.S.A.)) at a ratio of 50:40:10 (v/v/v). The mobile phase was degassed prior to use by mixing under vacuum.

Standard preparation

Leucine, isoleucine, valine and methionine (Sigma, St. Louis, MO, U.S.A.) were weighed and dissolved in 3% sulfosalicylic acid (SSA) (Fisher Scientific). A single mixed standard containing 50 μmol/l of each of the above four amino acids was used for a typical study. Standard solutions were stable for at least six months at 4 °C.

Sample preparation

Plasma was deproteinized with 6% SSA (1:1), mixed, then centrifuged for approximately 1 min. The supernatant was used for analysis. Deproteinized samples were stable at 4 °C for at least one week.

Procedure

Standard and OPA solution (a potassium borate buffer solution of 0.8 mg/ml OPA and 2-mercaptoethanol available commercially as Fluoraldehyde™ solution from Pierce (Rockford, IL, U.S.A.)) were allowed to come to room temperature. Replicate injections of the standard solution were made prior to receiving the first sample in order to establish the response factor. Standards were repeated approximately every 2 h throughout the study and once at the completion of the study. As whole blood samples were received, they were centrifuged for less than 1 min at 11 000 *g* in the microfuge, and the plasma was added to tubes containing

6% SSA at a ratio of 1:1 (v/v). These tubes were then mixed and centrifuged once more for approximately 1 min. BCAA standard or deproteinized plasma was reacted at a ratio of 1:1 (v/v) with Fluoraldehyde solution, vortexed, and 2 min after the addition of Fluoraldehyde a 20-μl injection was made for HPLC analysis. Separation was isocratic with no wash between samples. At the end of the study day the system was washed with and stored in 30% methanol.

RESULTS

With a 15-cm column and a flow-rate of 1.2 ml/min, leucine was retained for slightly over 3 min. Isoleucine preceded leucine as a distinct peak. Valine and methionine preceded isoleucine as a single peak with the 15-cm column and with the 5-mm Nova-Pak cartridge (Fig. 1A). The use of an 8-cm Nova-Pak cartridge allowed the separation and quantification of all the BCAAs, tryptophan, methionine and phenylalanine with only slightly longer analysis times (Fig. 1C). Plasma samples produced no interfering peaks (Fig. 1B). However, late eluting peaks in plasma samples necessitated an interval of at least 7 min between injections, still allowing plasma determinations with ease at intervals of less than 10 min using the stainless-steel column or 5-mm cartridge.

Quantification of leucine in 3% SSA was linear throughout physiological concentrations, with a recovery of 96%, and paralleled plasma to which standards had been added, indicating quantitative recovery of leucine from plasma. Correlation coefficients for standard curves made up in SSA and in plasma were both greater than 0.999. The coefficient of variation of replicate analyses of leucine were 1.97% for standards prepared in acid, and 2.57% for samples of deproteinized plasma. Normal basal human leucine levels obtained using this method ranged from 93 to 133 μmol/l with a mean of 115 μmol/l ($n=15$). This is in agreement with previously reported values [6–9].

The method compares well with other methods used in our laboratory. Leucine values obtained using the rapid HPLC method are shown in Fig. 2A in comparison with values obtained using a Beckman amino acid analyzer. Close agreement also exists between the HPLC method and an ion-exchange method optimized for the BCAAs previously developed and used in our laboratory [10], as shown in Fig. 2B.

With the availability of rapid leucine determinations we were able to develop an insulin–leucine clamp, analogous to the insulin–euglycemic clamp [11] widely used in in vivo studies. Fig. 3 shows data from a study in which a dog with indwelling femoral arterial catheters was studied after an overnight (18 h) fast on two occasions. The dog was subjected to varying infusions of insulin, with euglycemia maintained. On one occasion, plasma leucine was allowed to fall freely. On the other occasion, plasma leucine was fixed basally using the rapid HPLC method every 10 min and a varying exogenous infusion of L-leucine as shown in Fig. 3. From the amount of leucine that must be infused to maintain constant plasma levels, one can obtain an index of tissue sensitivity to leucine metabolism and its response to varying doses of insulin.

An additional aliquot of plasma was collected in order to determine the specific

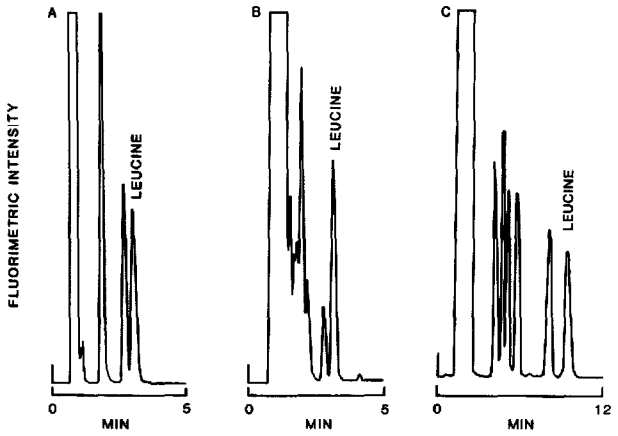

Fig. 1. Tracings of chromatographic recordings where the ordinate represents fluorimetric intensity from recorder baseline to full scale. Concentrations are derived from either peak area or height relative to that of the standard solution. (A) Chromatogram of a standard containing leucine (100 μM), isoleucine (100 μM), valine (100 μM) and methionine (100 μM). Leucine is preceded by a combined peak of valine and methionine and immediately preceded by isoleucine using a 10 cm×5 mm Nova-Pak cartridge and a flow-rate of 1.3 ml/min. (B) Sample of deproteinized plasma analyzed under conditions identical to those in (A). (C) Elution profile of a standard containing, in order of elution, tryptophan, methionine, valine, phenylalanine, isoleucine and leucine using a 10 cm×8 mm Nova-Pak cartridge with a flow-rate of 2.0 ml/min.

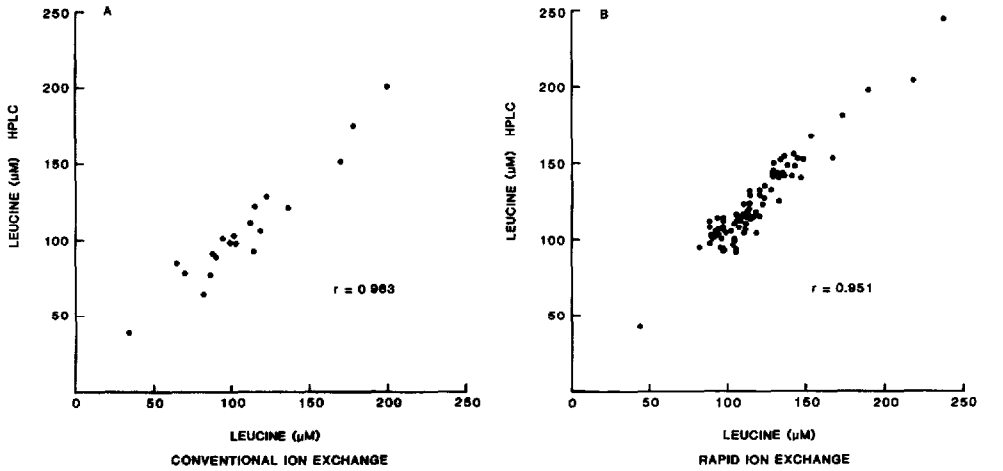

Fig. 2. Linear regression analysis of leucine concentration determined by various techniques plotted against leucine levels determined by HPLC as described in the text. (A) Values plotted against plasma leucine determined by conventional ion-exchange analysis (Beckman 190L amino acid analyzer); (B) values plotted against plasma leucine determined by a rapid ion-exchange analysis as described in ref. 10.

radioactivity of leucine and its keto analogue, α-ketoisocaproic acid (KIC). Plasma (2–3 ml) was deproteinized with 6% SSA (1:1, v/v). The supernatant was applied to cation-exchange resins (AG 50W-X8, 200–400 mesh, hydrogen form, from Bio-Rad Labs., Richmond, CA, U.S.A.) and separated with 0.01 M

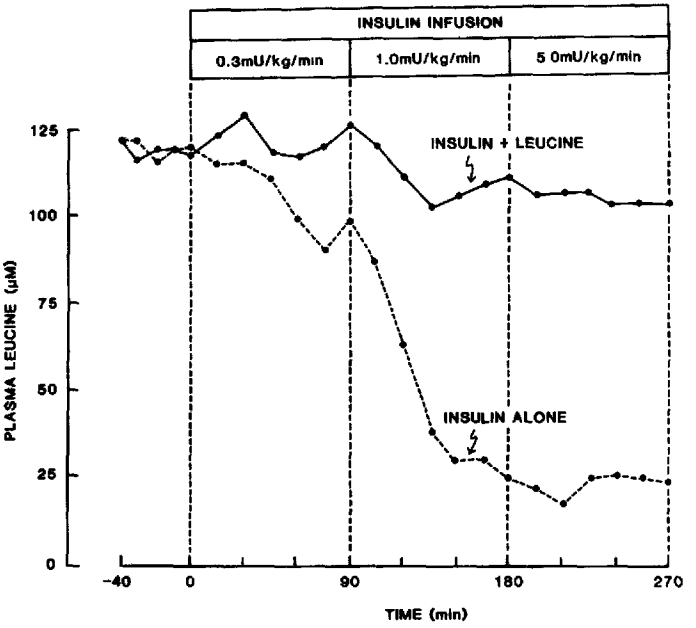

Fig. 3. Plasma leucine concentrations are shown from a study in which insulin was infused (shown by the broken line) and from an identical study in which basal leucine levels were maintained during insulin infusion using the method described in the text (shown by the solid line).

hydrochloric acid and 25% ammonium hydroxide washes. The ammonia wash was counted for radioactive leucine. The acid wash was divided into two aliquots: one was counted for radioactive KIC and the other was assayed for KIC concentration by the method of Nissen et al. [12]. The data were then used to calculate the R_a of leucine into the plasma compartment as well as the R_d and clearance of this amino acid from the plasma pool.

DISCUSSION

The advantages of this method are its speed and simplicity. Because minimal HPLC equipment is involved, the system is essentially portable and can be used at the experimental site or patient's bed side. This, coupled with the short analysis time, allows samples to be processed and reported in intervals of 10 min, enabling investigators to monitor and control circulating leucine levels in the presence of metabolic perturbations, and thereby better examine the effects of various physiologic and pathophysiologic parameters on leucine and BCAA kinetics.

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