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Rapid measurement of leucine-specific activity in biological fluids by ion-exchange chromatography and post-column ninhydrin detection

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ABSTRACT

Commonly used methods for the measurement of leucine-specific activity use either high-performance liquid chromatography (HPLC) and pre-column derivatization or conventional ion-exchange chromatography. These are time-consuming, labor-intensive, relatively costly procedures, requiring high concentrations of radioactivity for accuracy. The present paper describes a method for the measurement of plasma leucine-specific activity using HPLC equipment, a large-bore ion-exchange column and post-column ninhydrin detection. With this method, determination of leucine concentration and leucine radioactivity was found to be linear ($r^2 > 0.999$) over physiological ranges for both standards and deproteinized plasma. The intra- and inter-assay coefficients of variation for leucine concentrations were 1.4 and 2.7%, respectively. The intra- and inter-assay coefficients of variation for leucine-specific activities were 1.5 and 1.9%, respectively. The automated method is relatively fast (injection to injection time $\approx$ 45 min), economical and capable of accurately assessing relatively small amounts of radioactivity.

INTRODUCTION

Leucine radioisotopes are widely used for the estimation of rates of amino acid kinetics and protein turnover *in vivo*. Leucine is an essential amino acid, and in the absence of food intake, the only source of blood leucine is from the breakdown of endogenous proteins. The avenues for leucine metabolism are limited either to incorporation into protein or to transamination to the keto-acid, α-ketoisocaproate (KIC). Decarboxylation of KIC is an irreversible event whereby the first carbon is lost to carbon dioxide; the rest of the carbon skeleton is ultimately, through various metabolic steps, transformed into acetyl-coezyme A (CA) and Co_2 [1]. Therefore, the use of L-[1-^{14}C]leucine as a tracer, either by bolus injection or by continuous infusion, leads to the distribution of ^{14}C into either the free leucine pool, the free KIC pool, the protein pool or the carbon dioxide pool. Knowledge of the specific activities of the labelled compounds in each of these pools allows mathematical modeling of leucine metabolism and thereby the estimation of the rates of leucine appearance into the plasma com-

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partment. Additionally, the incorporation of leucine into protein and its ultimate oxidation into carbon dioxide may be determined [2,3].

The commonly used methods for estimating leucine-specific activity in blood and various tissues are time-consuming, labor-intensive and relatively costly. Several methods utilize high-performance liquid chromatography (HPLC) in conjunction with pre-column derivatization [4,5]. While these methods are relatively fast and reliable, they have several disadvantages. First, these methods use columns which allow only small sample volumes (<200 μl) and hence require high concentrations of radioactivity to accurately determine specific activity. This limits the usefulness of such a method to either *in vitro* studies or animal studies in which large doses of radioisotopes are permitted. Furthermore, these methods are also complicated by increased risk of cross-reactivity of the derivatizing agents with other compounds present in biological samples, thus resulting in a large number of unresolved peaks in the chromatogram.

Another approach to the determination of amino acid-specific activities utilized separation of sample components on a conventional ion-exchange system. This is accomplished by diverting a portion of the post-column effluent to a fraction collector while the remaining flow is diverted to post-column derivatization with ninhydrin [6]. This method allows accurate and simultaneous measurements of both concentration and radioactivity. However, this technique is time-consuming, usually requiring more than 2 h per sample, and utilizes expensive and inflexible instrumentation not routinely available in analytical laboratories.

The present paper describes a rapid automated method for the measurement of plasma leucine-specific acitivity using HPLC equipment, a large-bore ion-exchange column and post-column ninhydrin detection. The method is reliable, economical and capable of accurately assessing relatively small amounts of radioactivity and a broad range of physiological concentrations.

EXPERIMENTAL

Equipment (Fig. 1)

The chromatographic equipment (all from Waters Chromatography Division, Milford, MA, USA) consisted of a system controller (Model 680), two HPLC pump (Models M45 and 510), an auto-injector (Model 710B WISP) fitted with a 2-ml loop, a 320 mm × 9 mm I.D. jacketed glass column (Beckman, Fullerton, CA, USA) and an Exacal EX 100 (Neslab Instruments, Newington, NH, USA) heated circulating water bath connected to the column jacket with 7.9-mm Nalgene tubing (Nalgene, Rochester, NY, USA). PTFE tubing (1.5 mm × 0.5 mm I.D., Beckman) was used to connect both the injector and post-column apparatus (proportioning pump, reaction bath and colorimeter) to the column. The fraction collector was connected to the system immediately post-column via a size 18/3 tee (Small Parts, Miami, FL, USA).

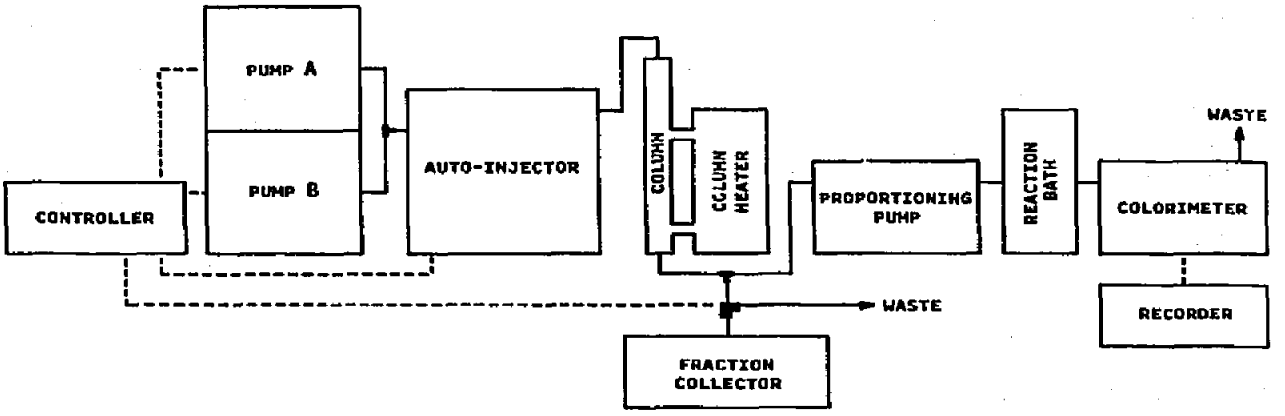

Fig. 1. Diagram of the system. Electronic signal flow and solvent flow are denoted by the dashed and solid lines, respectively. Pumps A and B deliver solvent through the auto-injector, where the sample is injected. After the effluent leaves the column, the flow is split with 80% going to a fraction collector or waste and 20% going to a reaction bath. In the reaction bath, the sample forms a color complex with ninhydrin which is measured by the colorimeter. Details are included in the Experimental section.

A peristaltic proportioning pump (Autoanalyzer II, Technicon, Terrytown, NY, USA) continuously diverted 20% of the effluent to the reaction bath using 0.030 mm I.D. Solvaflex tubing (Technicon Part No. 116-0533p07). Ninhydrin, hydrazine sulfate and nitrogen gas were delivered via the proportioning pump to a connector block (Technicon Part No. 117-B004-01) for mixing. The combined reagents were then introduced to the sample flow using an A-10 connector (Technicon Part No. 116-B034-01). A ten-turn delay coil (Technicon Part. No. 178-G196-02) completed the mixing before the flow entered the heated reaction bath. The reacted sample continued to the single-channel colorimeter (Technicon Part No. 119-A001-04L) fitted with a 570-nm filter (Technicon Part No. 170-B070P33) and a 15 mm × 1.5 mm flow cell (Technicon Part No. 119-B018-01). The signal was registerd with a chart recorder.

Immediately post-column, the remaining 80% of the effluent flowed through a 12-V DC valve (Part No. 983018, P. J. Cobert Assoc., St. Louis, MO, USA) routinely to waste. Upon being activated by the system controller, the valve diverted flow to a fraction collector (Gilson Model MFK), also managed by the system controller.

Reagent preparation

The elution buffer consisted of 0.085 *M* sodium citrate (Pierce, Rockford, IL, USA) prepared with deionized, filtered water to which 5 ml/l 30% Brij-35 solution (Pierce) had been added. The buffer was then brought to pH 3.30 using concentrated reagent-grade hydrochloric acid (Fisher, Pittsburgh, PA, USA). A solution of 0.2 *M* sodium hydroxide (Fisher) with 1% ethylenediaminetetraacetic acid (EDTA) in deionized water was used for column regeneration. Both the

buffer and hydroxide solutions were filtered and degassed using vacuum filtration before use (0.22 μm, Millipore, Bedford, MA, USA).

The post-column reaction used ninhydrin reagent prepared with a 1% solution of ninhydrin in a 3:1 (v/v) ratio of dimethylsulfoxide (DMSO) and 4 *M* lithium acetate buffer pH 5.2 (all from Pierce) [7]. A 0.002 *M* hydrazine sulfate (J.T. Baker, Phillipsburg, NJ, USA) solution was prepared using dionized water to which three drops of concentrated sulfuric acid had been added.

Preparation of standards, samples and controls

Initially, single amino acid (10 m*M*) stock standards of methionine, valine, isoleucine and leucine were prepared in 0.01 *M* hydrochloric acid (all from Sigma, St. Louis, MO, USA). Dilutions of the stock standards using 3% sulfosalicylic acid (SSA) were used to determine elution time and reproducibility. Leucine was quantified using pH-adjusted standard mixtures from Sigma.

Blood was collected in vacuum tubes containing 15 mg dry disodium EDTA per tube (Terumo Medical Corporation, Elkton, MD, USA), mixed and centrifuged at 4°C for 10 min at 2000 *g*. The supernatant was combined with cold 6% SSA (1:1, v/v), vortex-mixed and centrifuged again as described above. Studies in our laboratory indicated that the deproteinized plasma may be stored at 4°C for at least six months with no detectable change in leucine concentration. Dickinson *et al.* [8] demonstrated that amino acids in deproteinized plasma do not change within the limits of experimental error when stored at −68°C for eights months.

Controls were prepared by pooling deproteinized plasma and adding L-[^{14}C]leucine (DuPont, Boston, MA, USA). Aliquots were analyzed daily to assess reproducibility and recovery.

Column preparation

The 320 mm × 9 mm I.D. jacketed column was packed with 7–20 μm 7.25% cross-linked sulfonated polystyrene cation-exchange resin (BP-AN6, Benson, Reno, NV, USA) using a slurry technique (1:1, v/v; 0.2 *M* NaOH–resin mixture). The packing procedure was considered complete when a 2 ml/min flow-rate of 0.2 *M* NaOH produced a resin bed height of 13 ± 0.5 cm. Before analysis could begin, the column had to be conditioned by pumping 0.2 *M* NaOH at 2.0 ml/min for 3 min followed with sodium citrate buffer at 2.0 ml/min for 15 min. The column was heated and maintained at 53.5°C.

An inexpensive porous PTFE disk cut from 3 mm stock using a No. 5 cork bore (9 mm) was placed on the resin bed. This provided protection from particulate matter and allowed uniform dispersion of sample in square wave fashion onto the resin.

Procedure

The sample (750 μl) was injected by the auto-sampler into a 2.0 ml/min isocratic flow of sodium citrate. The exact elution times of radioactive leucine and KIC

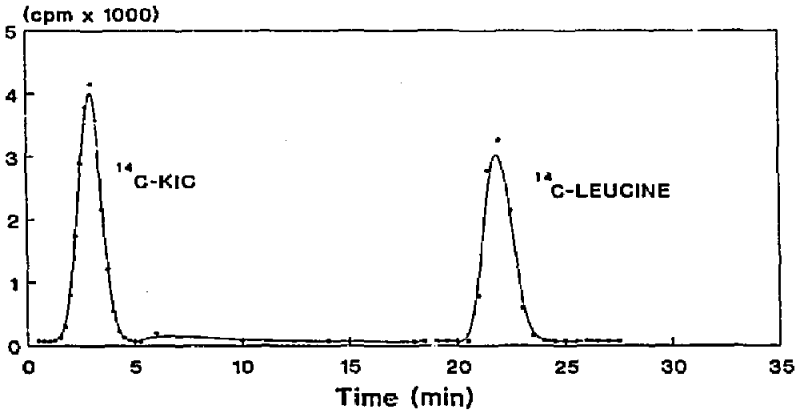

Fig. 2. Separation of KIC and leucine determined from 1-min collections taken throughout test runs of deproteinized plasma to which L-[1-^{14}C]leucine and [1-^{14}C]KIC have been added.

were determined from 1-min collections taken throughout test runs of standards and plasma to which L-[1-^{14}C]leucine and [1-^{14}C]KIC (Amersham, Arlington Heights, IL, USA) had been added (Fig. 2). The collection times were then set through the system controller which was programmed to signal both the valve and fraction collector at predetermined times. Column regeneration between runs was also accomplished via the system controller with a step-wise switch to NaOH immediately after leucine elution. Fractions were transferred to 20-ml glass scintillation vials containing 10 ml of Ecolite^{+} (ICN Biomedicals, Irvine, CA, USA) mixed and counted using standard β-counting techniques.

Addition of nitrogen bubbles, dispensed by the peristaltic pump at regular intervals, allowed segmented continuous flow of the portion of the eluent diverted for ninhydrin reaction. The mixing process was facilitated by an eight-turn glass mixing coil. The reacting mixture continued to the 95°C reaction bath followed by the colorimeter signaling the recorder. Total time delay from immediately post-column to recorder detection was approximately 11 min. Plasma leucine concentrations were determined using plasma sample peak heights relative to standards. Fig. 3a and b show representative chromatograms obtained for a standard solution and for a plasma sample.

The column was stored in 0.2 *M* NaOH with the inlet and outlet of the column plugged for storage (P.J. Cobert Assoc.). The chromatographic components, excluding the column, were then washed with deaerated, distilled water, followed wth 20% methanol for storage.

RESULTS

Separation of leucine was accomplished isocratically. Retention times were optimal for leucine with 0.085 *M* sodium citrate buffer, pH 3.30, flow-rate 2.0 ml/min, column temperature 53.5°C and resin bed height 13 ± 0.5 cm. Typical run times were less than 26 min from injection to fraction collection. At 2.0

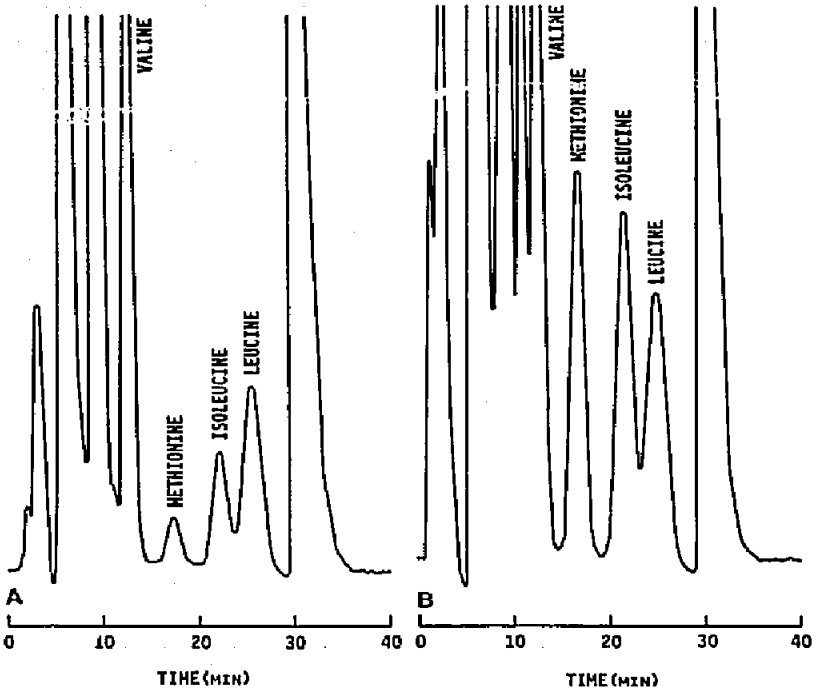

Fig. 3. Typical chromatogram for the analysis of standards (B) and deproteinized plasma (A) with leucine, isoleucine, valine and methionine labelled.

ml/min column regeneration was accomplished with 3 min of NaOH wash followed by sodium citrate re-equilibration for an additional 15 min. Therefore, the total injection to injection run time was less 45 min. Typical chromatograms of standards and deproteinized plasma are shown in Fig. 3.

Serial dilutions of the stock standard in both 3% SSA and deproteinized plasma established the linearity of the assay for the determination of leucine concentration. Leucine quantification was linear throughout physiological concentrations (5–500 μmol/l) with correlation coefficients greater than 0.999 for both standards (slope = 1.0; y-intercept = −0.1) and deproteinized plasma (slope = 1.0; *y*-intercept = 6.43).

L-[1-^{14}C]Leucine was added to a stock solution of deproteinized plasma and serially diluted with 3% SSA to determine the linearity of radioisotope recovery. Determination of radioactive leucine was also linear with a correlation coefficient greater than 0.999. Typical radioactive recovery for leucine was 93%. Separation of leucine from KIC was excellent with less than 1% crossover of counts (Fig. 2).

The intra- and inter-assay coefficients of variation for leucine concentrations were 1.4 and 2.7%, respectively. The intra- and inter-assay coefficients of variation for leucine-specific activities were 1.5 and 1.9%, respectively.

DISCUSSION

The method described above offers several advantages over previously published methods for the determination of leucine-specific activity. First, it enables investigators to reliably determine specific activities similar to those routinely found in human studies where limited amounts of radioactivity are permitted. This is possible because of the system's capacity for large sample volumes. The methods described earlier require extensive counting times and often result in counts that are not sufficiently above background. In addition, with this method leucine is easily and completely separated from its metabolite, KIC.

Similar to typical HPLC methods, this method utilizes non-specialized and versatile instrumentation. Although our system was composed of a rather sophisticated auto-injector and HPLC pumps, less expensive components would also be satisfactory. Special care must be employed, however, to avoid delivery of excess pressure to the glass column. HPLC pumps deliver smooth, relatively pulseless flow and can easily be set to shut off in the event of excess system pressure. This makes them ideal for this application. The use of this standard HPLC technology also allows for near-continuous operation with little operator intervention.

The present method relies on standard, well established ninhydrin chemistry. It is both sensitive and linear over a wide range of physiological concentrations. Additionally, because no pre-column derivatization or extraction tech ˙ques are required, sample preparation is simple, quick and obviates the need for internal standards.

Typical reversed-phase stainless-steel columns rely on a column guard to protect the relatively expensive column from suspended particulate matter; hence, depending on the severity of contamination, the samples used may require precleaning by filtration. The method we describe requires no column guard or sample filtration, but uses an inexpensive porous PTFE disk to protect the resin bed.

When the efficiency of a reversed-phase system decreases below minimum acceptable values, HPLC columns traditionally are replaced. Using the method presently described, the ion-exchange resin can be removed from the column, cleaned and re-packed extending the service life far beyond that of normal reversed-phase columns.

It follows then that this method is relatively economical. No expensive premixed buffers, HPLC columns, guard columns or extraction reagents are required. In our hands, the total supply costs per sample are minimal.

In conclusion, the method described offers advantages of simplicity and economy over currently utilized methods for specific activity determinations in physiological samples. Although the approach described here is optimized for the analysis of leucine-specific activity, the methodology can be easily modified for similar analyses of other amino acids. Our laboratory is currently developing automated methods for the analysis of the specific activities of other amino acids important in the study of protein metabolism.

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