L-[1-^{11}C]Leucine: Routine Synthesis by Enzymatic Resolution

J. R. Barrio, R. E. Keen, J. R. Ropchan, N. S. MacDonald, F. J. Baumgartner, H. C. Padgett, and M. E. Phelps

University of California, Los Angeles, Los Angeles, California

L-[1-^{11}C]leucine, suitable for the determination of cerebral protein synthesis rates in man using positron emission tomography, has been synthesized using a modified Bucherer-Strecker reaction sequence. The isolation of the pure L-amino acid isomer from the enantiomeric mixture, initially obtained using either an open or closed reaction vessel, was achieved using a D-amino acid oxidase/catalase enzyme complex immobilized on a Sepharose support. The O_2 required by the D-amino acid oxidase as the hydrogen acceptor was supplied by catalase. The L-[1-^{11}C]leucine was obtained with a radiochemical purity of >99% and with a radiochemical yield of 25%. Using a remote, semiautomated synthesis system, typical production time was 30–40 min after preparation of H^{11}CN. The use of immobilized enzymes for rapid and effective resolution of amino acid enantiomers eliminates the possibility of protein contamination and assures the production of a sterile, pyrogen-free product.

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Recent cerebral studies have used radiolabeled amino acids, together with tracer kinetic modeling and autoradiographic techniques, to investigate rates of cerebral protein synthesis (*1*), axonal transport (*2*) and sprouting (*3*) and development of the visual cortex (*4*). Their success has encouraged us to extend the application of these principles to in vivo studies in humans. The autoradiographic method for measuring local cerebral rates of protein synthesis with L-[1-^{14}C]leucine (*5*) has been adapted to man using L-[1-^{11}C]leucine in conjunction with positron emission tomography (PET) (*6,7*).

We now report the routine preparation, in multimillicurie amounts, of sterile, pyrogen-free L-[1-^{11}C]leucine from noncarrier-added hydrogen [^{11}C] cyanide by a modified Bucherer-Strecker reaction sequence, using either an open or a closed reaction vessel (*8*). Isolation of the pure L-amino acid stereoisomer from the initially produced racemic mixture was accomplished by an immobilized D-amino acid oxidase/catalase enzyme complex (Fig. 1). This process is rapid and efficient, and eliminates the possibility of protein contamination in the final product.

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For reprints contact: Dr. J. R. Barrio, Div. of Biophysics, Chemistry Sec., UCLA School of Medicine, Los Angeles, CA 90024.

MATERIALS AND METHODS

(I) Enzyme immobilization. Porcine kidney D-amino acid oxidase* (E.C.1.4.3.3) (340 units) and dog liver catalase* (E.C.1.11.1.6) (500 units) were immobilized on CNBr-activated Sepharose† (570 mg) in the presence of sodium pyrophosphate (30 m*M*, pH 8.3)/NaCl (0.5 μM)/flavin adenine dinucleotide (FAD, 1.0 μM). FAD is a required cofactor for enzymatic activity preventing dissociation of the holoenzyme that is the active form of D-amino acid oxidase (*9,10*). Immobilization in the active holoenzyme form, in the presence of substrates and other necessary cofactors, can increase the resulting enzymatic activity of the coupled enzyme by protecting active sites from covalent linkages to the Sepharose support (*11,12*). All procedures involving FAD and D-amino acid oxidase were performed with protection from light, due to the photosensitivity of FAD and of D-amino acid oxidase in the presence of FAD (*13*). The

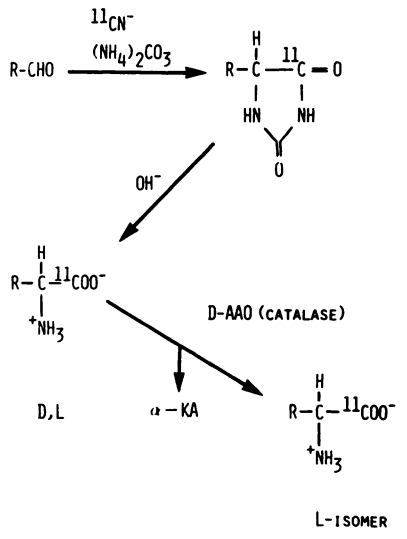

FIG. 1. Reaction to produce L-[1-^{11}C]leucine [R = $(CH_3)_2CH$—CH_2—]

Sepharose-enzyme mixture (total volume = 15 ml) was gently rotated, end over end, and permitted to react over a period of 2 hr at room temperature. Prior to this process, D-amino acid oxidase and catalase were dialyzed against sodium pyrophosphate (30 m*M*, pH 8.3)/FAD (1.0 μM), at 4°C. After immobilization, the Sepharose-enzyme mixture was poured into a glass column (0.7 by 30 cm) covered with a red filter (cutoff <550 nm). Excess active groups on the Sepharose support were hydrolyzed by passage of Tris-HCl (30 m*M*, pH 8.0)/FAD (10 μM) through the column for 2 hr at room temperature. Successive washing of the column with sodium phosphate (30 m*M*)/FAD (10 μM), buffered at pH 8.3 and 4.0, respectively, removed any protein ionically bound to the Sepharose support. The column was stored at 4°C in sodium phosphate (30 m*M*, pH 7.5)/KCl (2.0 *M*)/FAD (10 μM) and protected from light. Before use the column was warmed to room temperature and washed with sodium pyrophosphate (30 m*M*, pH 8.3)/FAD (10 μM). Under these conditions the enzymes are stable and columns could be reused for up to three months.

(II) Formation of 5-(2-methylpropyl)-[4-^{14}C] hydantoin: Open-vessel reaction. Carbon-14-labeled NaCN (10 μCi) was introduced into a two-necked, cone-shaped flask assembled with a reflux condenser and a magnetic stirring bar and containing a solution (1.5 ml) of $(NH_4)_2CO_3$ (0.75 mmole), NH_4Cl (0.125 mmole), isovaleraldehyde (0.5 mmole) and NaOH (1.0 μmole). The top of the condenser supported a soda-lime trap for monitoring $H^{14}CN$ losses during the reaction. Before submerging the flask into the oil bath (100°C), an aliquot was removed from the flask (time = 0). The solution was refluxed for 20 min and aliquots were removed at intervals. These aliquots were cooled, neutralized (pH 8–9) and analyzed by high-pressure liquid chromatography (HPLC) by using the *o*-phthaldialdehyde (OPT) precolumn fluorescence derivatization procedure (*14,15*). The fractions collected were counted by liquid scintillation and the activity plotted. The plots indicated that 3 min was the optimum time for the formation of the radiolabeled hydantoin.

(III) Alkaline hydrolysis of 5-(2-methylpropyl-[4-^{14}C]-hydantoin to DL-[1-^{14}C]leucine: Open vessel reaction. This experiment was conducted in the same manner as II above, except that NaOH (6.25 *N*, 1.0 ml) was added after 3 min of refluxing, and aliquots were removed over a 20-min period after the addition of the NaOH solution. The plots showed that 15 min was the time necessary to hydrolyze completely the hydantoin to the DL-amino acid.

(IV) Open-vessel synthesis of L-[1-^{11}C]leucine. Following the method of Banfi et al. (*8,16*) the requisite hydrogen [^{11}C]cyanide (800–1,000 mCi) was prepared, via the $^{14}N(p,\alpha)^{11}C$ reaction, by the bombardment of nitrogen gas with 22-MeV protons; it was bubbled into a solution (1.5 ml) containing $(NH_4)_2CO_3$ (0.75 mmole), NH_4Cl (0.125 mmole) and NaOH (1 μmole). The solution was then transferred to a two-necked, cone-shaped flask (15 ml) assembled with a reflux condenser and a magnetic stirring bar. The top of the condenser was sealed with a silicone stopper that had been fitted with inlet and outlet tubings and a soda-lime trap. The flask was submerged in an oil bath (100°C) and isovaleraldehyde (0.5 mmole) was introduced. After 3 min, NaOH (6.25 *N*, 1.0 ml) was added via the inlet tube. Heating continued for an additional 15 min; then the solution was transferred (via the outlet tube) to the remainder of the semiautomated processing system. [See Fig. 2, and Sections (V) and (VII)].

(V) Synthesis of L-[1-^{11}C]leucine in closed stainless steel vessel. Hydrogen [^{11}C] cyanide was prepared and bubbled into the reactants solution (1.5 ml) as in Section IV and transferred to a stainless steel reaction vessel. Isovaleraldehyde (0.5 mmole) was added, and the vessel was closed and heated at 220°C for 3 min to form the 5-alkylhydantoin. The vessel was cooled, and NaOH (6.25 *N*, 1.0 ml) was added; it was then resealed and heated at 220°C for 4 min to effect the hydrolysis of the hydantoin to the DL-amino acid. The reaction vessel was again cooled and the solution transferred to the next unit of the processing system (flask B, Fig. 2).

The content of the reaction vessel, after addition of glacial acetic acid (0.55 ml, final pH 9.5) was transferred to a column (C) (1.5 by 17 cm) containing ion-retardation resin (AG-11A8). The DL-leucine was eluted with deionized water while salts and unreacted $H^{11}CN$ were retained. The effluent fraction that contained the radioactivity (7.0 ml) was collected and H_2O_2 (50 m*M*, 0.1 ml) and a buffer solution (1.0 ml) containing sodium pyrophosphate (225 m*M*, pH 8.3) and FAD (75 μM) were added. The solution was transferred to a light-

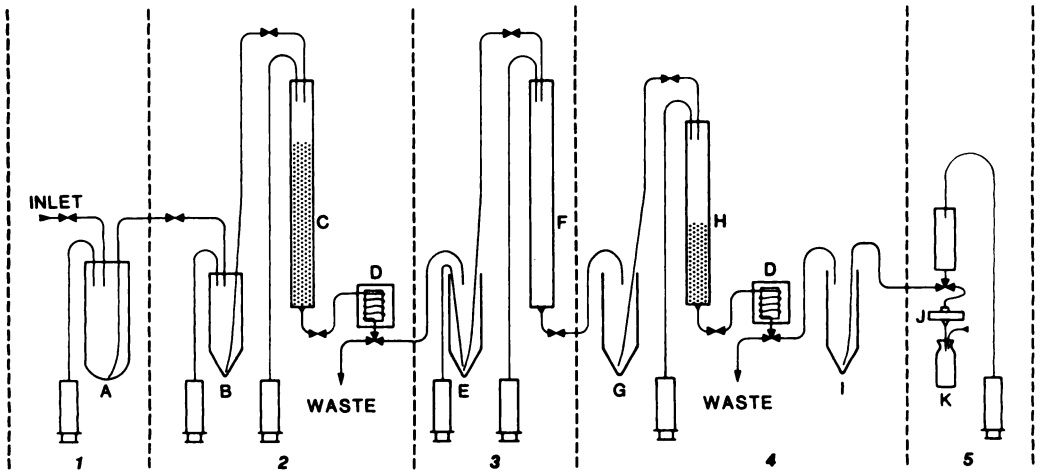

FIG. 2. Remote-controlled system for production of L-[1-^{11}C]leucine. It is conceptually divided into five operational units: (a) DL-amino acid synthesis; (b) deionization; (c) enzymatic resolution; (d) purification; and (e) sterilization. Specific components are: A = reaction vessel; B,E,G, and I = mixing vessels; C = ion-retardation resin column; D = flow-through gamma detector; F = enzyme column; H = ion-exchange column; J = 0.22-μm-pore membrane filter; K = injection vial.

protected (<550 nm) enzyme column (F) (5.0 by 0.7 cm) containing immobilized D-amino acid oxidase (340 units) and catalase (500 units). The enzyme column was washed with sodium pyrophosphate buffer (30 m*M*, pH 8.3)/FAD (10 μM) (3.0 ml) and the radioactive eluate from it was collected in a tube (G) containing 0.05 ml glacial acetic acid, which adjusts the pH of the solution to about 5.0. The solution was next passed through an anion-exchange resin (AG 1-X4) column (H) (1.0 by 5.0 cm) equilibrated with sodium phosphate (30 m*M*, pH 7.4), which retained the [1-^{11}C] alpha-ketoisocaproic acid. The column was washed with sodium phosphate (30 m*M*, pH 5.6, 6.0 ml) and the radioactive solution containing L-[1-^{11}C]leucine (pH 7.0) was made isotonic and then sterile by passage through a 0.22-μm pore filter (J) into a sterile vial. The final product yields were between 45–50 mCi, with a radiochemical purity of >99%, a specific activity of 3–10 Ci/mmole at the end of synthesis, and a synthesis time of 30 min. Pyrogenicity testing using the *Limulus* test as well as rabbit assay verified all samples pyrogen free.

(VI) Verification of radiochemical properties. The radiochemical purity of the L-[1-^{11}C]leucine produced was verified by reversed-phase HPLC using a Cu^{2+}/proline buffer system (*17*), which separates D- and L-leucine, as well as any remaining [1-^{11}C]alpha-ketoisocaproic acid. An aliquot of the final product was allowed to complex with cupric acetate (28.8 mg) and L-proline (35.1 mg) in a solution of sodium acetate (30 m*M*, pH 5.5, final volume = 0.3 ml) for two minutes; it was then injected into the chromatograph‡ (4.6- by 250-mm column†; solvent, 90% 8 m*M* cupric acetate, 17 m*M* L-proline, 30 m*M* sodium acetate, pH 5.0, and 10% MeOH; flow rate 1.0 ml/min; radioactivity detector); retention times: for L-[1-^{11}C]leucine = 12.3 min, and D-[1-^{11}C]leucine = 6.9 min. The specific activity of the L-[1-^{11}C]leucine was determined using the OPT precolumn fluorescence derivatization procedure (*14,15*).

(VII) The remote semiautomated synthesis system. The synthesis system used in the isolation and purification of L-[1-^{11}C]leucine, shown in Fig. 2, consists of five units corresponding to the five basic steps of the production process. These steps are: (a) DL-amino acid synthesis (hydantoin formation and subsequent hydrolysis); (b) deionization; (c) enzymatic resolution; (d) purification; and (e) sterilization.

The basic components of the system are five flasks, three columns, two flow-through gamma detectors and a sterilization unit, all connected through eleven two- and three-way Teflon solenoid valves using appropriate low-pressure Teflon tubing and fittings. The flasks and columns were mounted on an aluminum-rod (1.25 cm) lattice using standard equipment clamps. The solenoid valves were secured to an aluminum plate and attached to the support rod. External access tubings terminating in syringe connections allow the addition of reagents and solvents; they facilitate the transfer of the amino acid solution from one unit of the system to the next by application of syringe-created vacuum or pressure in conjunction with appropriate opening and closing of the solenoid valves. A highly detailed description of the construction and function of a similar but more complex synthesis system for the preparation of 2-deoxy-D-[1-^{11}C]glucose has been published (*18*).

RESULTS AND DISCUSSION

Since we are involved in the production of amino acids—and in particular are interested in the routine production of L-[1-^{11}C]leucine for reasons stated previously (*7*)—we decided to explore the feasibility of

producing L-[1-^{11}C]leucine in an open vessel within an acceptable time period. This achievement would make the synthesis more attractive from the standpoint of its compatibility with a simple, remote, semiautomated system, would in turn reduce the exposure to the chemist throughout the entire procedure, and would provide an alternative method of preparing radiolabeled DL-amino acids.

It has been shown by Bucherer (*19–22*) and others (*23*) that such a synthesis is not improbable, since 5-alkylhydantoins can be formed in an open vessel at temperatures from 25°C to 60–70°C in nearly quantitative yields with times varying from 30 min to several hours, depending on the starting materials and conditions. With these facts in mind, we set out to optimize the conditions by systematically conducting a series of experiments using sodium [^{14}C] cyanide, as outlined in the experimental section.

In the first set of experiments no alkali was added to the reaction vessel. Fractions were removed from the reaction flask over a 20-min period and plots of the counts obtained from HPLC analysis showed that the majority of the H^{14}CN had been consumed after 3 min. The reduction in H^{14}CN corresponded to the formation of the hydantoin and a small amount of DL-leucine. The production of DL-leucine at this point in the synthesis could be attributed to the basicity of the solution (pH = 9.0).

In view of the above information, in the second set of experiments, the addition of alkali for 3 min into the reaction, and analysis of the aliquots after a 20-min period, indicated that the optimum time needed for the complete conversion of the hydantoin to the DL-amino acid was fifteen minutes. Shorter times resulted in small amounts of unhydrolyzed hydantoin. These times were then applied to the DL-[1-^{11}C]leucine experiments which, as expected, duplicated the C-14 results. It was thus obvious that the hydrolysis portion of the reaction was the most difficult and required longer reaction times. However, the overall time required to synthesize DL-leucine in the open vessel was less than that previously used in the stainless steel vessel (*8*), and therefore can serve as an alternative method for producing many radiolabeled DL-amino acids.

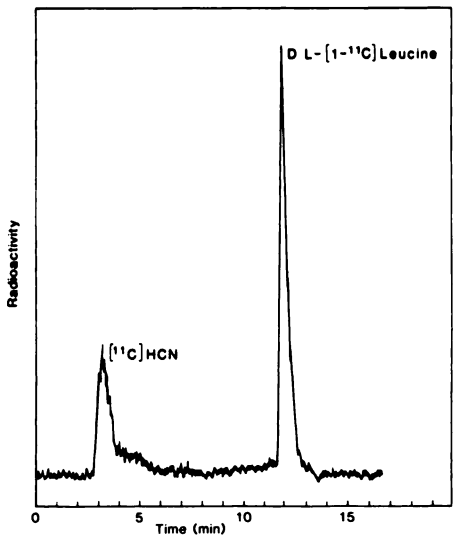

FIG. 3. DL-[1-^{11}C]leucine: Chromatographic analysis of crude reaction mixture after alkaline hydrolysis, using *o*-phthaldialdehyde (OPT) precolumn fluorescence derivatization.

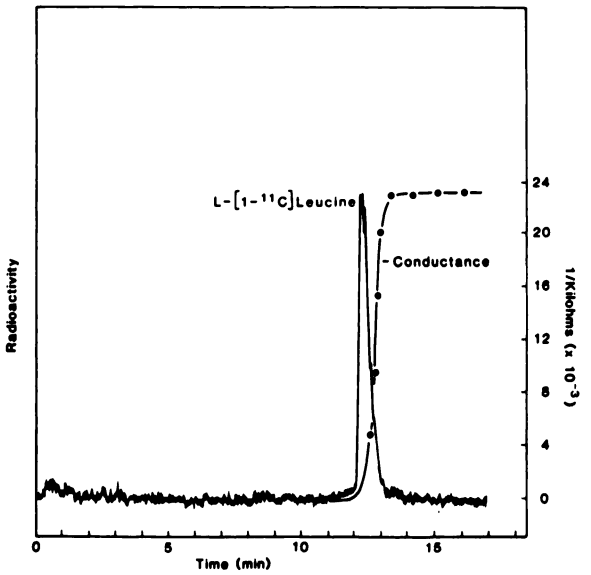

FIG. 4. DL-[1-^{11}C]leucine: Chromatographic profile of product after ion-retardation column using OPT precolumn fluorescence derivatization.

Based on the above open-vessel results, we were certain that we could reduce the times previously used for the high-pressure reaction (*8*). Through a series of reactions using H^{11}CN, we found that the optimum times required for the formation of the hydantoin, and its subsequent hydrolysis to form the DL-leucine, were 3 and 4 min, respectively. Reducing the hydrolysis time to three minutes resulted in unreacted hydantoin. Using these findings we were able to reduce the total time necessary for the synthesis of radiolabeled DL-leucine to 7 min. Reversed-phase HPLC (OPT precolumn fluorescence derivatization) of the completed reaction indicated that 75 to 95% of the total radioactivity resided in DL-[1-^{11}C]leucine (Fig. 3).

Purification of DL-[1-^{11}C]leucine via chromatographic column (C), and analysis of the elution product by reversed-phase HPLC, showed only the DL-amino acid (Fig. 4). The ability of the ion-retardation column to retain ions while allowing DL-leucine to pass through depends on the pH of the mixture. The pH was adjusted (~9.0–9.5) by the addition of a fixed amount (0.55 ml) of glacial acetic acid to the stainless-steel or open-vessel reaction product just before transferring the solution to column (C). Subsequent elution of the DL-[1-^{11}C]leucine was monitored by a small shielded gamma detector at

the base of column (C). Typical radiochemical yields of pure DL-[1-^{11}C]leucine at this point were 240–350 mCi, with synthesis times of 15 min (stainless-steel vessel) and 25 min (open vessel) after cyclotron production of C-11-labeled HCN.

Production of the L-[1-^{11}C]leucine required purification and separation of the DL stereoisomers, which could, a priori, be done using either chromatographic (*24*) or enzymatic procedures (*25*). We have chosen the latter because enzymatic procedures are compatible with the simplicity of the remote systems designed in our laboratory (*14,15,18*) combined with minimum radiation exposure to the operator.

Final isolation of the radiolabeled L-isomer of leucine was achieved rapidly with immobilized D-amino acid oxidase, which is a flavoprotein oxidase that selectively deaminates D-amino acids and is most active with D-isomers of neutral amino acids such as D-leucine, D-phenylalanine, and D-methionine (*9*). The L-amino acid counterparts are the amino acids of choice to study cerebral protein synthesis, due to their high blood-brain barrier permeability (*26*) and traceable metabolic pathways (*7*). Furthermore, porcine kidney D-amino acid oxidase is commercially available and ready for immobilization without any further purification steps. Other available enzymatic procedures for the resolution of amino acids include the use of their acetyl derivatives and either acylase I or carboxypeptidase (*27*). While effectively applied to the resolution of C-14-labeled amino acids (*27*), this procedure is impractical within the time frame imposed by carbon-11.

Preparation of the DL-[1-^{11}C]leucine for entry into the enzyme column (F) involved buffering to pH 8–10 (the optimum pH for porcine-kidney D-amino acid oxidase activity (*28,29*)), in a final solution of sodium pyrophosphate (30 m*M*)/FAD (10 μM). Hydrogen peroxide, which is also produced by deamination of the D-amino acid, serves as an oxygen source in the presence of catalase ($H_2O_2 \rightleftarrows \frac{1}{2}O_2 + H_2O$) which is co-immobilized with the D-amino acid oxidase. D-amino acid oxidase has a high degree of specificity for O_2 as a hydrogen acceptor (*9*), which is required for continued enzymatic activity (*29*). Given these conditions, the DL-[1-^{11}C]leucine solution is passed through column (F) at the rate of 1–2 ml/min, yielding L-[1-^{11}C]leucine and [1-^{11}C]alpha-ketoisocaproic acid from the deaminated D-[1-^{11}C]leucine (Fig. 5). Selective removal of the [1-^{11}C]alpha-ketoisocaproic acid was accomplished by passing the mixture from column (F) (previous pH adjustment) directly through an anion-exchange column (H), which retains all the [1-^{11}C]alpha-ketoisocaproic acid (Fig. 6). Washing column (H) with 30 m*M* phosphate buffer (pH 5.6) accelerates the release of L-[1-^{11}C]leucine from the column and maintains the pH of the final solution at 7.0. The solution was then made isotonic and passed through a 0.22-μm sterile filter, making it suitable for injection.

In summary, L-[1-^{11}C]leucine has been prepared, in amounts sufficient for PET studies (40–50 mCi) and with >99% radiochemical purity, using either a closed stainless-steel-vessel (total synthesis time = 30 min), or an open-vessel reaction (total synthesis time = 40 min).

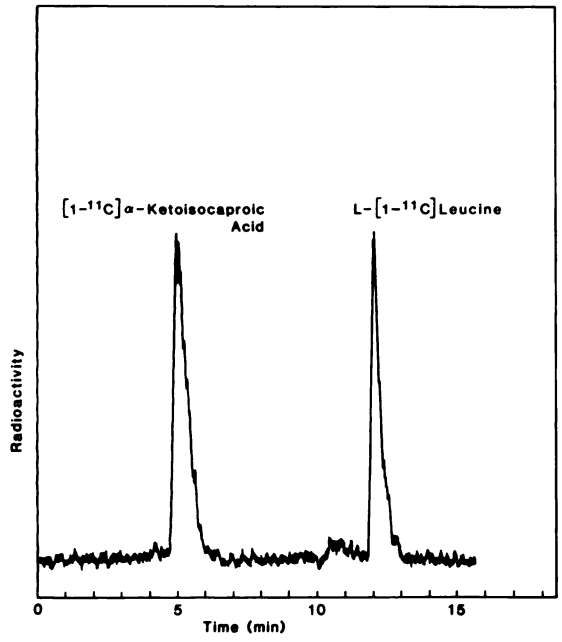

FIG. 5. Chromatographic analysis (OPT precolumn fluorescence derivatization) of DL-[1-^{11}C]leucine after treatment with immobilized D-amino acid oxidase.

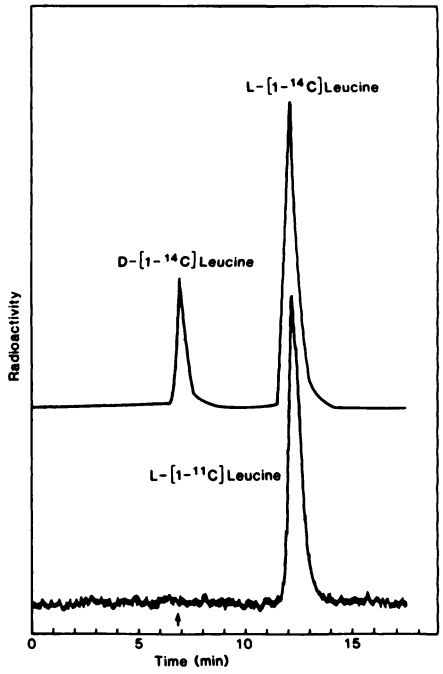

FIG. 6. L-[1-^{11}C]leucine: Purity of final product is routinely analyzed by reversed-phase HPLC using Cu^{2+}/L-proline buffer as mobile phase (experimental conditions described in text). Retention times of C-14 markers are indicated.

The use of the enzyme complex D-amino acid oxidase/catalase achieved our objective of isolating the L-isomer rapidly and effectively with minimum radiation exposure (<1 mR) to the chemist. The remote semiautomated synthesis system developed for the synthesis of L-[1-^{11}C]leucine is an extension of those previously constructed for the preparation of 2-[^{18}F]fluoro-2-deoxy-D-glucose (*30*), 2-deoxy-D-[1-^{11}C]-glucose (*18*), [1-^{11}C]palmitic acid (*31*), and other carbon-11 and nitrogen-13 L-amino acids (*14,15*). Finally, the initial kinetic data from PET studies after intravenous injections of L-[1-^{11}C]leucine (*7*) (which permits the calculation of transport and pool turnover rates, ratio metabolism/protein synthesis rate, rate constants for each process, and cerebral protein synthesis rates in monkeys and man) demonstrate the usefulness of this radiopharmaceutical and the applicability of the synthetic approach described in this work for routine production of L-[1-^{11}C]leucine (*32*).

FOOTNOTES

* Sigma Chemical Co., St. Louis, Mo.
† Pharmacia, Uppsala, Sweden.
‡ Ultrasphere ODS, 5 μm.

ACKNOWLEDGMENT

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