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Research Article

Development of a reliable remote-controlled synthesis of *β*-[^{11}C]-5-hydroxy-*L*-tryptophan on a Zymark robotic system

O. C. Neels[1,]*, P. L. Jager[1], K. P. Koopmans[1], E. Eriks[1], E. G. E. de Vries[2], I. P. Kema[3] and P. H. Elsinga[1]

[1] *Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, Groningen, The Netherlands*
[2] *Department of Medical Oncology, University Medical Center Groningen, Groningen, The Netherlands*
[3] *Department of Pathology and Laboratory Medicine, University Medical Center Groningen, Groningen, The Netherlands*

Summary

Precise staging of neuroendocrine tumors (NET) using positron emission tomography (PET) tracers visualizing their specific metabolic activity is of interest. Besides [^{18}F]FDOPA, staging NET with carbon-11 labeled 5-hydroxytryptophan (5-HTP) is reported in recent literature. We implemented the multi-enzymatic synthesis of enantiomerically pure [^{11}C]-*L*-5-HTP on a Zymark robotic system to compare both tracers in patient studies. [^{11}C]-5-HTP can be synthesized in up to 24% radiochemical yields (EOB). Average specific activity is 44 000 GBq/mmol in ca. 50 min from [^{11}C]methyl iodide in radiochemical purities >99 %. The synthesis of 5-HTP is difficult due to its multi-enzymatic reaction steps but typical yields can be achieved of ca. 400 MBq. [^{11}C]-5-HTP is now reliably used in ongoing studies for staging NET. Copyright © 2006 John Wiley & Sons, Ltd.

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*Correspondence to: O. C. Neels, Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands. E-mail: o.neels@pet.umcg.nl

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Introduction

Neuroendocrine tumors (NETs) are slowly growing malignant tumors with the capacity of *u*ptake and *d*ecarboxylation of *a*mine *p*recursors (APUD) such as 5-hydroxytryptophan (5-HTP) and *L*-dihydroxyphenylalanine (*L*-DOPA).[1] DOPA and 5-HTP are transported into the cell via LAT1 transporters.[2] Fluorine 18 labeled *L*-DOPA[3] is in use for staging NETs in several PET centers to measure the DOPA-pathway. ^{11}C labeled 5-HTP is also of special interest because it is a direct precursor for serotonin. As 5-HTP is decarboxylated by aromatic amino acid decarboxylase (AADC) the carbon-11 atom in carboxyl-position will be lost immediately and tumor detection will be impossible.[4] Therefore, 5-HTP has to be labeled in a position other than that of carboxyl, e.g. in the β-position which has been described by Bjurling *et al*. and applied in staging of NET.[1,4–6]

Radiosynthesis of β-[^{11}C]-5-hydroxy-*L*-tryptophan has been described earlier. First, a glycine derivate is labeled with [^{11}C]methyl iodide, hydrolyzed to racemic [^{11}C]alanine and converted by a multi-enzymatic reaction into enantiomerically pure [^{11}C]-*L*-5-HTP. Fully automated synthesis[7] is reported using immobilized[8,9] as well as free enzymes.[10,11] We now report the implementation of this production on a Zymark robotic system.

Results and discussion

[^{11}C]-5-HTP was obtained in decay corrected yields of 15 $\pm$ 12 % (Figure 1) calculated from the time of release of [^{11}C]methyl iodide. This is in the range of earlier published results. Over time these yields increased up to 24%. In a typical run approximately 400 MBq of [^{11}C]-5-HTP was synthesized in

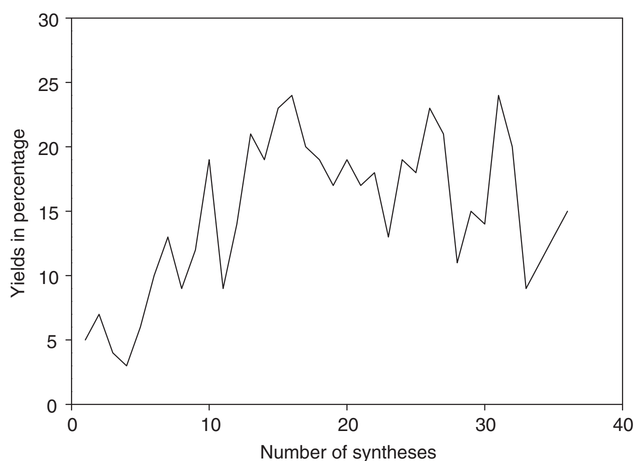

Figure 1. Yields [^{11}C]-5-HTP after trapping [^{11}C]methyl iodide corrected for decay

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approximately 50 min calculated from trapping of [^{11}C]methyl iodide (mean 9 GBq). Radiochemical purity was >99% and average specific activity was 44 000 GBq/mmol. The minimum amount of radioactive 5-HTP for a patient study was determined as 60 MBq. Average reliability after 36 syntheses was >95%. Enantiopurity of the obtained radiolabeled amino acid was assessed on a chiral HPLC column in >99%.

The most critical part during the synthesis of [^{11}C]-5-HTP is the enzymatic step where in total 4 enzymes plus co-enzymes are used in an equilibrium reaction described by Bjurling *et al.*[10] and Watanabe and Snell.[12] The concentration of the used solutions is crucial for reliable yields. Racemic [^{11}C]alanine has to be converted into [^{11}C]pyruvic acid by the use of *D*-amino acid oxidase (DAO), catalase (CAT) and glutamic pyruvic transaminase (GPT) (Figure 2) and then into [^{11}C]-5-HTP by the reversed tryptophanase reaction (Figure 3). The enzymatic reaction resulted only in good yields if the pH is adjusted in a range of 8.5–9.0. Measuring and correction of pH is therefore important and can lead to dramatic decrease in yield if omitted.

Another issue for safe human use is the origin of enzymes. For example, catalase as reported by Bjurling *et al.* was from the bovine liver. We used catalase from *Aspergillus niger* to avoid Bovine spongiform encephalopathy risk.

During the synthesis of [^{11}C]-5-HTP different parameters are playing important roles. The volume of the minivials used in the Zymark robot system is limited to 4 ml and the volume of the needle-hand has a maximum of 2.5 ml. Therefore the volume of liquids used in the solid phase extraction (SPE) cleaning step had to be scaled down as compared to reported methods. All reaction vials are newly prepared for each synthesis.

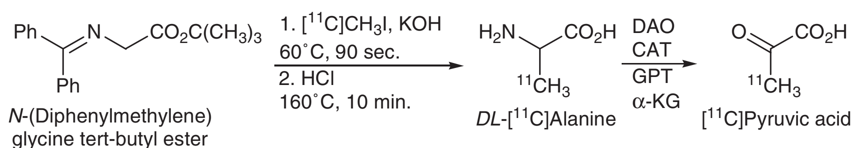

Figure 2. Synthesis of *DL*-[^{11}C]Alanine/[^{11}C]pyruvic acid

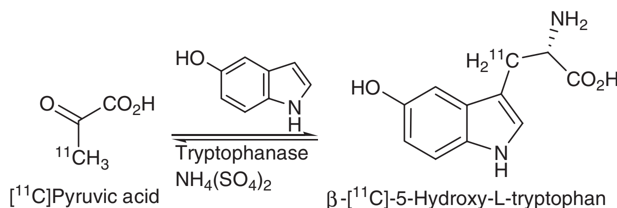

Figure 3. Synthesis of β-[^{11}C]-5-Hydroxy-*L*-tryptophan from [^{11}C]pyruvic acid

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Reduction of radiation exposure for nuclear workers is a critical point in the synthesis of [^{11}C]-5-HTP. While replacing the SPE column, measuring and adjusting pH before and adding enzymes in the last step of the synthesis, the radiochemist is exposed to high levels of radiation for a period of about 1.5 min. Radiation dose per synthesis could be restricted to an average minimum of 260 μSv for the skin and 40 μSv for the whole body by use of a fast to open and close manual sliding door from lead.

Experimental

General

For the synthesis of [^{11}C]-5-HTP we used a Zymark robotic system controlled by Easylab software (Hopkinton, MA, USA). Easylab software could easily be configured for [^{11}C]-5-HTP syntheses by adapting existing steps and changing parameters as reaction times and temperature. The robotic arm is centered in the hot cell and surrounded by workstations, e.g. two ovens for heating (ambient temperature to 160°C) and cooling (−20 to +60°C), racks to hold septa-sealed (mini)-vials, air-pressured SPE purification and an injection unit for HPLC. Both ovens are combined with compatible needle devices that can shift up and down pneumatically and is controlled by Easylab software. Via the needle devices [^{11}C]methyl iodide as used in this synthesis can be trapped. Also helium can be streamed through the reaction vial by gas flow for evaporation. For standard procedures a finger-hand and a 0.2–2.5 ml needle-hand are used. The sterile vial containing synthesized [^{11}C]-5-HTP is placed in a lead container situated near the sliding door of the hot cell.

Chemicals and solvents were obtained from Sigma (Zwijndrecht, The Netherlands), Merck (Amsterdam, The Netherlands), Janssen (Geel, Belgium) and Rathburn (Walkerburn, Scotland). Enzymes were purchased from Sigma except tryptophanase (TRP). DAO from *porcine kidney* was dissolved in 3.6 M $NH_4(SO_4)_2$ and pH adjusted to 6.5. CAT from *Aspergillus niger* in 3.2 M $(NH_4)_2SO_4$ solution pH 6.0 and GPT from *porcine heart* in 1.8 M $(NH_4)_2SO_4$ solution pH 6.0 were used without further treatment. TRP dissolved in 20 mM potassium phosphate buffer pH 7.5, 0.1 mM pyridoxal 5′-phosphate, 0.1 mM dithiotheitol and 20% glycerol was purchased from Ikeda (Hiroshima, Japan) and used without further treatment. *Tris*(hydroxymethyl)-aminomethane (TRIS), ammonium sulfate and α-ketoglutaric acid were dissolved in distilled water. Pyridoxal 5′-phosphate and flavin adenine dinucleotide were dissolved in 0.1 M sterile phosphate buffer pH 7.2 and 5-hydroxyindole (HIn) was dissolved in ethanol.

Preparation of the synthesis is very time-consuming compared to other PET tracers. Most of the necessary solutions have to be prepared manually the day before synthesis and are stable between 1 day and 4 weeks when cooled or

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frozen. Depending on the frequency of syntheses in a period of time, preparation can be more or less time-consuming by repeated use of the reagents.

Preparative HPLC was performed on a Waters (Etten-Leur, The Netherlands) 515 system using an Alltech (Breda, The Netherlands) 250×10 mm Econosphere C18 10 μm column (5 ml/min) with 0.1 M NaH_2PO_4 containing 2% ethanol as mobile phase in series with a Waters 481 UV detector (280 nm) and an Eberline (Erlangen, Germany) RM25 radiation detector. Analytical HPLC was performed on a Waters 515 system equipped with a Waters 150×3.9 mm NovaPak C18 4 μm column (flow 1 ml/min) in series with a Waters 486 UV detector (280 nm) and an Eberline RM25 radiation detector and 0.1 M NaH_2PO_4 as mobile phase. Enantiomeric purity was assessed by HPLC on a Waters 600 system using a Serva (Heidelberg, Germany) pre-conditioned (0.1 M $CuNO_3$) 250×4.6 mm ChiralProCu 6 μm column (1 ml/min) in series with a Waters 2487 UV detector (280 nm) and a Bicron (Paris, France) frisk-tech radiation detector with 0.1 M NaH_2PO_4 as mobile phase. C18 Solid phase extraction columns were pre-conditioned with 10 ml ethanol and 10 ml distilled water.

[^{11}C]Methyl iodide

[^{11}C]Methane was produced in an aluminum target by the $^{14}N(p,\alpha)^{11}C$ nuclear reaction in N_2, containing 10% H_2, using 17 MeV protons and a Scanditronix MC-17 cyclotron (1 h, 30 μA). After trapping [^{11}C]methane in a software-controlled module, [^{11}C]methyl iodide was formed in average yields of 50% by reaction with iodide vapor at 725°C in 10 min. Obtained [^{11}C]methyl iodide was transferred to a second hot cell via PEEK tubings into a minivial containing the precursor solution.

β-[^{11}C]-5-Hydroxy-L-tryptophan

First, 3 mg of *N*-(diphenylmethylene)glycine *tert*-butyl ester (10 μmol) was dissolved in 290 μl dimethylformamide. Then, 7 μl potassium hydroxide (KOH) 5 M was added manually just before trapping [^{11}C]methyl iodide by the needle device at 0°C. The needle device contains one long needle to trap [^{11}C]methyl iodide and one short needle equipped with a carbosphere trap. After trapping of [^{11}C]methyl iodide the minivial containing the yellow mixture was moved to the pre-heated oven (60°C) with the finger-hand to react for 90 s. The minivial was then placed into the minivial holder and a mixture of water/0.1 M phosphate buffer pH 7.2/ethanol (2.27 ml/0.77 ml/0.3 ml) was added by the needle-hand. The cloudy liquid was transferred to the C18 SPE column, passed by air pressure via a three-way valve to a waste bottle and washed with 2 ml water. After switching the three-way valve the radioactive

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compounds were eluted with 2 ml dichloromethane to a minivial containing 200 μl HCl 6 M.

The minivial was set into a pre-heated oven (70°C) by the finger-hand and then heated up to 160°C in a stream of helium gas. During evaporation the SPE-column was replaced manually by a 6 ml syringe connected with a 22 μm sterile pore filter. After complete evaporation the minivial was placed in a cooled oven (0°C) for 1 min. Then, 1 ml of 0.1 M TRIS was added by the needle-hand after placing the minivial into the vial rack. The minivial was slightly shaken manually and 1 ml air was added by the needle-hand to avoid low pressure in the minivial after rapid cooling down.

The clear solution was transferred with the needle-hand to a minivial containing 100 μl ammonium sulfate 1.5 M, 80 μl TRIS 0.5 M pH 9, 15 μl KOH 5 M, 50 μl α-ketoglutaric acid 0.2 M, 10 μl pyridoxal 5′-phosphate 10.7 mM and 10 μl flavin adenine dinucleotide 1.8 mM. pH was measured with a hand-held pH-meter and adjusted to 8.5–9.0 by adding KOH 5 M manually with a pipette. A prepared mixture of 11 μl GPT (20 units), 20 μl CAT (3600 units), 250 μl DAO (12 units) and 50 μl TRP (8 units) was added manually with a pipette. Finally, 10 μl HIn 0.5 M were added with a pipette, slightly shaken manually and the solution was incubated for 8 min. Enzymes were denatured by manual addition of 3 drops of HCl 6 M. The suspension was transferred by the needle-hand to the SPE-station and filtered into an empty minivial via a 22 μm sterile pore filter by air pressure and washed with 0.6 ml HPLC eluens.

The minivial containing a clear solution was transferred to the HPLC injection station by the finger-hand and injected on the preparative HPLC column. *β*-[^{11}C]-5-Hydroxy-*L*-tryptophan was obtained after purification on the described preparative HPLC system with a retention time of 9 min. The radioactive fraction of 5-HTP was collected by passage through a 22 μm sterile pore filter in a sterile vial ready for use in human studies. The sterile vial was placed in a lead container near the door of the hot cell by the finger-hand.

Conclusion

Because of its multi-enzymatic steps [^{11}C]-5-HTP synthesis is difficult, but with slight modifications the end result is suitable for reliable patient studies. With a high specific activity and increasing yields this tracer can be successfully applied for patient studies.

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