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Automated Radiosynthesis of 5-[¹¹C]L-Glutamine, an Important Tracer for Glutamine Utilization

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Abstract

Introduction: The natural amino acid *L*-Glutamine (Gln) is essential for both cell growth and proliferation. In addition to glucose, cancer cells utilize Gln as a carbon source for ATP production, biosynthesis, and as a defense against reactive oxygen species. The utilization of [¹¹C]Gln has been previously reported as a biomarker for tissues with an elevated demand for Gln, however, the previous reports for the preparation of [¹¹C]Gln were found to be lacking several crucial aspects necessary for transition to human production. Namely, the presence of unreacted precursor and the use of noncommercialized, custom built, reaction platforms. Herein, we report the development and utilization of methodology for the automated production of [¹¹C]Gln that meets institutional criteria for human use.

Methods: The preparation of [¹¹C]Gln was carried out on the GE FX₂N platform. Briefly, after trapping of [¹¹C]HCN with a solution of CsHCO₃ in DMF, the [¹¹C]CsCN was reacted with a commercially available precursor. This intermediate was then purified by HPLC and deprotected/hydrolyzed under acidic conditions. Following pH adjustment, the product was filtered to give the desired [¹¹C]Gln as a sterile injectable. The resulting product was then analyzed for quality assurance.

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Results: Automated production by this method reliably provides over 3.7 GBq (100 mCi) of [^{11}C]Gln. The resulting final drug product was found to have a >99% radiochemical purity, <5% of *D*-Gln present, no detectable impurities, and the total preparation time was roughly 45 min from the end-of-bombardment.

Conclusions: A fast, reliable and efficient automated radiosynthesis was developed using a commercially available module. Purifications used throughout allow for both a radiochemically and chemically pure final product solution of [^{11}C]Gln.

Keywords

Radiopharmaceuticals; [^{11}C]Glutamine; Automated Radiosynthesis; Metabolism; PET; Carbon-11

Introduction

Positron Emission Tomography (PET) imaging using 2-deoxy-2- ^{18}F -fluoro-D-glucose (^{18}F FDG) has become a central component for cancer diagnosis and staging since the first human scan in 1976. Despite the enhanced glycolysis observed in many tumors, elevated glucose utilization also takes place in many normal tissues such as the brain, normal muscle, cardiac tissue, and inflammation.[1, 2] In addition to dependence on glucose, cancer cells can also exhibit a metabolic shift to an increased dependency on the amino acid glutamine (Gln).[3] Gln is also known to participate in protein synthesis and serves as a precursor for glutathione biosynthesis, a cellular defense mechanism against reactive oxygen species.[4, 5] Given this, quantitative measures of glutamine uptake may reflect critical processes in oncology that are difficult to measure using existing imaging metrics. PET imaging agents targeting glutamine uptake, such as ^{18}F -4-fluoroglutamine (^{18}F 4F-Gln), have been reported and used in preclinical and clinical studies in oncology.[6–10] However, [^{11}C]Gln, which is chemically identical to naturally occurring Gln and has the same biological fate.[8]

As a prime example of a non-invasive molecular imaging technique, PET has become increasingly important both for direct clinical utilization and for helping to focus drug development efforts.[11–14] In terms of the clinical utilization, the radiopharmaceuticals must be produced under current Good Manufacturing Practices (cGMP) which can be aided by the use of automated reaction modules. These modules, many of which are commercially available, allow for a high degree of reproducibility and standardization for the radiosynthesis. Furthermore, they allow the use of high levels of radioactivity to be utilized since they can be operated remotely while contained in a radiation shielded enclosure (i.e. “hotcell”). Through the use of automated synthesis modules the preparation of a radiopharmaceuticals can be transferred to another production facility with identical equipment with relative ease, avoiding the time-consuming and expensive redevelopment that must occur. Once the radiopharmaceutical has been produced, a series of specific quality control tests must be completed to enable the drug to be released for injection. These tests, known as release criteria, commonly include: pH, residual solvents, bacterial endotoxin, final filter integrity, chemical purity, radiochemical purity, radiochemical identity/purity, and sterility (a post-release test).[15]

While the preparation of [^{11}C]Gln has been previously reported, the methodology utilized required further development to facilitate automation and translation of the drug product from preclinical to clinical use.[7, 16–18] The previously published pre-clinical preparations of [^{11}C]Gln utilized a simple solid phase extraction protocol to purify the radiolabeled synthetic intermediate, which was never designed nor shown to be effective at removing the unreacted starting material prior to moving to the final steps of the procedure.[7, 19, 20] The presence of this impurity has the undesired consequence of lowering of the overall chemical purity of the final drug product solution. In addition to the purity concerns, all methodologies reported for the preparation of [^{11}C]Gln have been accomplished using noncommercialized, custom built, reaction platforms. In an effort to allow for the seamless transfer of technology from production site to production site, development of this product on a commercially available platform is a high priority. Furthermore, the previously reported preparations did not address the feasibility of transitioning the [^{11}C]Gln into a clinically ready product, nor were any of the additional preparation metrics addressed (*i.e.*, sterility, chemical purity, pyrogenicity). Herein, we report the development and utilization of methodology for the automated production of [^{11}C]Gln that meets cGMP criteria for human use.

Materials and Methods

All chemicals were obtained from commercial sources and were of analytical, ACS, or USP quality (Sigma-Aldrich, USA; APP Pharmaceutical, USA) and were used without further purification. (*S*)*Tert*-Butyl 2-((*tert*-butoxycarbonyl) amino)-4-Iodobutanoate was purchased from Advanced Biochemical Compounds (ABX, Germany) in pre-aliquoted 2 mg fractions. (*S*)-*Tert*-butyl-2-((*tert*-butoxycarbonyl)amino)-4-cyanobutanoate was synthesized according to literature methods.[21] The $\text{CsHCO}_3/18\text{-crown-6}$ solution was prepared by mixing 18-crown-6 in acetonitrile (MeCN) (18-crown-6, 160 mg, 0.61 mmol dissolved 17 mL of MeCN) with CsHCO_3 in water (H_2O)(CsHCO_3 , 60 mg, 0.31 mmol dissolved in 3 mL of sterile H_2O). The mixture was gently mixed to provide a clear and colorless solution, and stored at 10 °C. Solid phase extraction (SPE) cartridges (Sep-Pak® C18 Plus, HLB) were obtained from Waters (Waters® Association, MA, USA), and conditioned with 6 mL 200 proof ethanol (EtOH) followed by 20 mL of sterile H_2O prior to use. AG® 11-A8 ion retardation resin was purchased from BIO-RAD, USA. All radioactivity was measured using a CAPINTEC CRC-15 Dual PET, CRC-15 PET, or CRC-25 Dual PET. The radiochemical purity and enantiomeric purity (percentages of *L* and *D* isomers) of the final product [^{11}C]L-Gln were determined by analytical radio-HPLC with a Phenomenex® Chirex 3126 (*D*)-penicillamine column (150 × 4.6 mm.), mobile phase: 1 mM CuSO_4 aqueous solution, flow rate 1.0 mL/min, $\lambda = 254$ nm, RT of [^{11}C]L-Gln = 9.1 min, RT of [^{11}C]D-Gln = 13.6 min.

Carbon-11 was produced on a commercial cyclotron (GE Healthcare, PETtrace 880) as [^{11}C]CO₂ by proton bombardment of a gas target containing a mixture of N₂ and O₂ gas (99% N₂ with 1% O₂) to induce the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction. Typical irradiation current used for the bombardment was 60 μA for 20–30 minutes followed by conversion of the [^{11}C]CO₂ to [^{11}C]HCN using the GE Chemical Process Cabinet (Procab). Briefly, the [^{11}C]CO₂ is transferred out of the target *via* a stainless steel delivery line, trapped at room temperature on molecular sieves (4Å), which is then heated under a stream of N₂/H₂ to elute

the pure $[^{11}\text{C}]\text{CO}_2$. This gas mixture is then passed through a nickel catalyst pre-heated to 450 °C, thereby converting the $[^{11}\text{C}]\text{CO}_2$ to $[^{11}\text{C}]\text{CH}_4$ which is then mixed with ammonia gas (NH_3) and immediately passed through platinum mesh pre-heated to 950 °C providing the desired $[^{11}\text{C}]\text{HCN}$ gas. The $[^{11}\text{C}]\text{HCN}$ was then delivered through a teflon delivery line to the desired reaction module for further use.

The synthesis was carried out on a GE FX2N automated reaction module loaded as follows: vial 1: DMF (0.5 mL); vial 2: MeCN (2 mL); vial 3: 70% MeCN in 20 mM phosphate buffer (2.3 mL); vial 4: 70% MeCN in 20 mM Phosphate buffer (1.5 mL); vial 5: precursor **1** ((S)-*tert*-Butyl 2-((*tert*-butoxycarbonyl) amino)-4-Iodobutanoate) (2 mg) in DMF (0.2 mL); vial 7: MeCN (2 mL); vial 8: sterile water (4 mL); vial 10: 4:1 TFA/ H_2SO_4 (0.2 mL); vial 11: sterile water (2 mL); vial 13: MeCN (1.5 mL); vial 14: sterile water (10 mL); Reactor 1: CsHCO_3 / 18-C-6 solution (1 mL); round-bottom flask (RBF) loaded with sterile water (20 mL); product flask: 1X Phosphate Buffered Saline (PBS) (4 mL); C18 2: C18 Plus Sep-Pak; Al_2O_3 2: HLB SepPak Plus above a column of AG[®] 11-A8 Resin. The C18 Plus and HLB Plus Sep-Paks were both preconditioned with 6 mL ethanol, followed by 20 mL sterile water. The AG[®] 11-A8 resin column was preconditioned with 20 mL 0.5 M NaCl followed by 100 mL of sterile water.

Prior to the end of bombardment (EOB) and delivery of the $[^{11}\text{C}]\text{HCN}$, a 1 mL of the CsHCO_3 /18crown-6 solution described above was added to reactor 1 and azeotropically dried by iterative additions (2X) and evaporations of acetonitrile (1 mL, 90 °C) under vacuum with a helium gas flow. *N,N*-Dimethylformamide (DMF) is then added to complete the trapping solution. The $[^{11}\text{C}]\text{HCN}$ was then delivered to the reaction module through V10 (normally closed position of the valve) with a N_2 flow rate of 150 mL/min (Figure 1). Upon completion of delivery, precursor **1** was added as a solution in DMF and the reaction vessel heated at 90 °C for 8 min. The reaction mixture was then cooled by active air cooling, diluted with HPLC mobile phase (70% Acetonitrile in 20 mM aqueous phosphate buffer), and loaded onto an HPLC purification column (Phenomenex, Luna C18(2) 250 × 10 mm). The desired radioactive peak was collected into the round bottom flask containing sterile water, which was then transferred through a C18 Sep-Pak Plus cartridge to isolate the radioactivity. This reaction intermediate is then eluted from the Sep-Pak with acetonitrile (1.5 mL) into the second reaction vessel. The acetonitrile is then azeotropically dried by iterative additions (2X) and evaporations of acetonitrile (1 mL, 90 °C) under vacuum with a helium gas flow. Once completely dry, a solution of trifluoroacetic acid / sulfuric acid (4:1) was added to the reaction vessel and the mixture is heated at 90 °C for 5 min. The resulting solution was diluted with sterile water for injection (2 mL) and passed through an HLB Sep-Pak attached to a AG[®] 11-A8 resin packed column into the product vial, containing 1X PBS, followed by washing of the column with additional sterile water for injection. The final drug solution is then passed through a 0.22 μm sterilizing filter into the final vial, to provide the $[^{11}\text{C}]\text{Glutamine}$ as a ready to inject solution. The radiosynthesis requires approximately 45 minutes from the EOB to the drug product solution being deposited in the final vial.

Quality Control

Radiochemical and chemical purities were analyzed by analytical HPLC ($[^{11}\text{C}]\text{Gln}$ RT = 9.1 min)(1 mM CuSO_4 (aq), 1 mL/min, 254 nm). The chemical purity of the product was >95%. Analysis for residual organic solvents was carried out using an Agilent 7890B GC (Agilent Technologies Inc., USA) with a capillary column (length 30 m, ID 0.520 mm, DB-WAX 1.0 μm , Agilent Technologies Inc., USA). Apyrogenicity (Charles River Laboratories Inc., USA) tests were performed in-house to ensure that doses of $[^{11}\text{C}]\text{Gln}$ contained <8.75 endotoxin units (EU) per ml. ColorpHast® pH indicator strips (EMD Chemicals Inc., USA) were used to determine pH of the final product. Final filter integrity testing was performed on the sterilizing filter by standard bubble-point along with sterility testing via direct inoculation into growth media. Radionuclidic purity was confirmed by half-life determination of the final product. Stability testing of the $[^{11}\text{C}]\text{Gln}$ product was performed at periodic times over two hours, testing for radiochemical purity by HPLC and pH

Results & Discussions

Automated Radiosynthesis Optimization

The radiosynthesis of $[^{11}\text{C}]\text{Gln}$ is shown in Scheme 1. Our approach was based upon two previous reports of the radiosynthesis of $[^{11}\text{C}]\text{Gln}$, as well as a recently reported preparation of $[^{18}\text{F}]\text{4-F-Gln}$. [7, 9, 20] Previously $[^{11}\text{C}]\text{Gln}$ was prepared by alkylation with $[^{11}\text{C}]\text{HCN}$ of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl) amino)-4-Iodobutanoate, followed by a C18 Sep-Pak trap and release, deprotection/partial hydrolysis with TFA/ H_2SO_4 and finally desalting to provide the final product.[7] This method was then improved upon by Qu and co-workers, with a systematic examination of the trapping and deprotection conditions.[20] Unfortunately, the process that was reported did not allow or account for the purification of the desired product away from the unreacted precursors or any other non-radioactive impurities that were generated throughout the reaction process.

Our initial attempts at automation of the literature process started from the optimized procedure, directly applying the procedure on a GE FX₂N. Unfortunately in our hands the yields were quite low, typically providing as little as 0.93 GBq (25 mCi) of final product from 59.2 GBq (1600 mCi) of trapped $[^{11}\text{C}]\text{HCN}$. Furthermore, the use of TFA/ H_2SO_4 for the second reaction step caused rapid degradation of several permanent components of the reaction module, namely the PEEK connections which were exposed to the solution.

Zhang and co-workers, examining the related automation radiosynthesis of $[^{18}\text{F}]\text{4-FGln}$ developed a process using the GE FX₂N.[9] Small modifications to the modules standard flow-paths allowed for the HPLC purification of the intermediate, and subsequent acidic deprotection to produce the desired final product. We applied this method to the synthesis of $[^{11}\text{C}]\text{Gln}$, with a similar reconfiguration of a GE FX₂N (Figure 1), that allows for HPLC purification of the intermediate, subsequent trap & release, acidic deprotection, and reaction to give the final product. To overcome the hardware destruction caused by the TFA/ H_2SO_4 solution we replaced the PEEK connection between Vial 10 and Valve 34 with a custommade teflon replacement of exactly the same dimensions as the PEEK connector and removed the PEEK filter located inside of the connector. This teflon replacement has proven

to be resistant to the acidic corrosion with over fifty runs to-date. We also added an HLB Sep-Pak Plus in-line with the ionretardation column. The addition of the HLB Sep-Pak Plus added to the inlet of the desalting column was found to be necessary to aid in lowering the overall flow-rate of the product solution through the desalting column, avoiding an over-pressurization scenario and providing lower flow-rate through the resin thereby providing a consistent removal of the acid used in the previous step. This, in theory, has the added benefit of further sequestration of any organic impurities that result from the deprotection performed in the prior step. The final modification was to change the formulation of the final product from 100% water, to 0.4X phosphate-buffered saline. This modification ensures the final product consistently has a pH that is acceptable for human administration.

With the addition of the HPLC purification of the intermediate, not only were we able to produce a chemically pure product, but the radiochemical yield was significantly improved to over 70% decay corrected and to 15% non-decay-corrected (based upon the [^{11}C]HCN trapped). The identity and radiochemical purity of final product was confirmed by HPLC (RT = 9.1 min), with the only detectable radioactive impurity being [^{11}C]D-Gln (RT = 13.6 min). (Figure 2) Furthermore, the process was analyzed by ion-pair HPLC chromatography to determine the level of [^{11}C]L-Glutamate byproduct produced; < 1% [^{11}C]L-Glutamate was observed under these conditions. (Supplementary Figure 1) The results of the three consecutive qualification runs are shown in Table 1.

Conclusion

We have successfully developed an automated radiosynthesis of [^{11}C]Gln using a commercially available reaction platform and commercially available materials. Addition of the HPLC purification of the intermediate allows removal of unreacted precursor as well as any other impurities; in addition to allowing for an increased yield and production of [^{11}C]Gln in suitable quantities for clinical use. This allows production of pure [^{11}C]Gln without fear of contamination with the difficult to detect impurities. The final [^{11}C]Gln passes all clinically required release criteria and has been approved for human

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Modifications (shown as orange lines and red X's) to the GE FX₂N for the production of $[^{11}\text{C}]\text{Gln}$.

Figure 2:

Typical HPLC chromatogram of [^{11}C]Gln product. The top trace (panel A) is the UV absorbance at 254 nm of the [^{12}C]Gln standard; the bottom trace (panel B) is a representative radioactive trace of a final drug product solution.

Scheme 1:

Initial automated synthetic pathway for the radiosynthesis of [^{11}C]Gln.

Scheme 2:
Optimized Radiosynthesis of [^{11}C]Gln.

Table 1:

Results of the Qualification Runs administration as part of two first-in-man clinical trials (NCT03275974 & NCT03263429).

Quality Control Test	Requirements for Pass	Qual Run 1	Qual Run 2	Qual Run 3
Appearance	Clear, colorless, no particulates	Clear, colorless, no particulates	Clear, colorless, no particulates	Clear, colorless, no particulates
Filter Integrity (Bubble Point)	Meets pressure specified by Manufacturer	Pass	Pass	Pass
pH	4.5 – 8.5	6.5	7.0	7.0
Radiochemical Purity (%)	> 90%	97.48%	99.85%	97.00%
Radiochemical Identity (% difference from Reference Std)	< 5%	1.99%	0.13%	0.66%
Amount of Product	N/A	4.31 GBq (116.6 mCi)	2.84 GBq (76.7 mCi)	4.68 GBq (126.6 mCi)
Residual Acetonitrile Level, ppm	< 400	3.2	23.3	24.5
Residual DMF Level, ppm	< 800	0.0	0.0	0.0
Radionuclidic Identity (t _{1/2} , minutes)	19.38 – 21.42 min	20.36 min	20.33 min	20.38 min
Bacterial Endotoxin Levels (EU/mL)	< 8.75 EU/mL	< 1.00 EU/mL	< 1.22 EU/mL	< 1.00 EU/mL
Sterility (Observed growth after 14 d)	No growth observed in 14 days	No Growth	No Growth	No Growth