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Original Article

Fully automated radiosynthesis of [^{18}F]FCPPC for imaging microglia with PET

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Abstract: Colony-stimulating factor 1 receptor (CSF1R) is almost exclusively expressed on microglia in the human brain and thus, has promise as a biomarker for imaging microglia density as a proxy for neuroinflammation. [^{11}C]CPPC is a radiotracer with selective affinity to CSF1R, and has been evaluated for in-human microglia PET imaging. The flourine-18 labeled CPPC derivative, 5-cyano-N-(4-(4-(2-[^{18}F]fluoroethyl)piperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide ([^{18}F]FCPPC), was previously synthesized, however, with a low radiochemical yield using manual radiosynthesis. In this work, we report a fully automated radiosynthesis of [^{18}F]FCPPC on a Synthra RNplus research module. In a total synthesis time of 50 min, [^{18}F]FCPPC was obtained in decay corrected radiochemical yields of 26.8 ± 0.1% (n = 3) with >99% radiochemical purities. Quality control testing showed that [^{18}F]FCPPC met all release criteria. In sum, we report the first fully automated radiosynthesis of [^{18}F]FCPPC, a promising radiopharmaceutical for imaging microglia in humans.

Keywords: Colony-stimulating factor 1 receptor, [^{18}F]FCPPC, radiosynthesis, automation, PET imaging, radiopharmaceutical

Introduction

Neuroinflammation is an immune response within the Central Nervous System (CNS) associated with activation and proliferation of glial cells, especially microglia. Microglia are the resident macrophages in the human brain and play a crucial role in the development and homeostasis of the CNS [1, 2]. Upon detection of inflammatory stimuli, microglia activation is critical for the initiation of a neuroinflammatory response and restoration of homeostasis [3, 4]. Neurodegenerative diseases, such as Alzheimer's disease (AD), are associated with chronic neuroinflammation and microglial activation [5, 6]. Hence, improved understanding of the role of neuroinflammatory signaling in psychiatric and neurological disorders, including neurodegenerative diseases, may lead to novel therapeutics and improved clinical outcomes.

Colony-stimulating factor 1 receptor (CSF1R) is a subfamily of tyrosine kinase receptor activated by binding to colony-stimulating factor 1 or interleukin 34, and plays a significant role in survival, homeostatic functions, and proliferation of microglia. CSF1R is almost exclusively expressed on microglia in the human brain, and thus, holds considerable potential as a cell-specific imaging biomarker of neuroimmune state in humans [7-9]. Many lead compounds targeting CSF1R have been developed and evaluated as CSF1R inhibitors, and showed promising profiles as potential therapeutic agents to prevent neurodegeneration. As such, Positron Emission Tomography (PET) imaging of the CSF1R has been proposed as an *in vivo* technique for quantifying human microglial activation and proliferation [10-12]. Several CSF1R-targeting radiotracers have been previously developed (**Figure 1**) [12-19]. One of these radiotracers is [^{11}C]5-cyano-N-(4-(4-methylpiperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide ([^{11}C]CPPC), which has been shown to exhibit selective CSF1R-affinity as well as suitable kinetic properties for PET imaging in humans [20]. Despite its promising biological properties for CSF1R imaging, the use of [^{11}C]CPPC is limited by the short half-life of carbon-11 ($t_{1/2}$ = 20.4 min). In 2022, Lee and co-workers developed a fluoride-18 labeled CPPC derivative, 5-cyano-N-(4-(4-(2-[^{18}F]fluoroethyl)piperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide ([^{18}F]FCPPC) for CSF1R imaging [21]. In the preclinical evaluations, [^{19}F]FCPPC showed high CSF1R binding affinity with IC_{50} of 3.42 ± 0.33 nM, and [^{18}F]FCPPC exhibited significantly increased brain uptake over the control mice. The radiosynthesis of [^{18}F]FCPPC was performed manually with non-decay corrected radiochemical yields of 8-8.5%, providing an opportunity for further optimization and automated radiosynthesis.

Previously, we reported an high-yield radiosynthesis of [^{11}C]CPPC for in-human microglia PET imaging [22]. In the present study, we report an automated radiosynthesis of [^{18}F]FCPPC with improved yield over the reported manual synthesis method (**Figure 2**), and three validation runs to support its clinical translation for CSF1R PET imaging. Synthra RNPlus research module is designed to allow multi-step radiosyntheses of research radiotracers and our approach utilized its versatile functions, which

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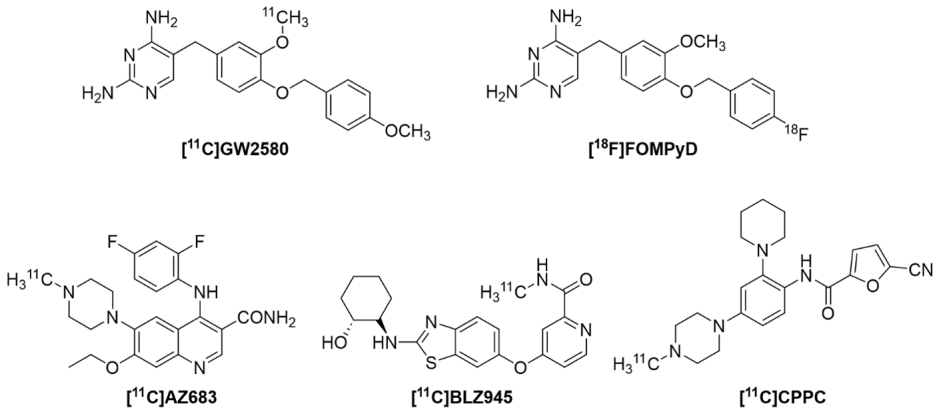

Figure 1. Representative chemical structures of CSF1R-targeting radiotracers.

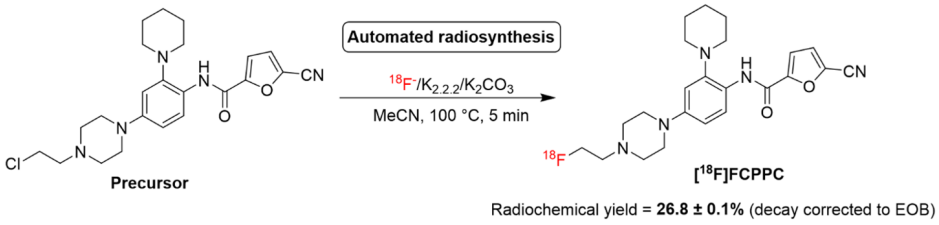

Figure 2. Scheme of automated radiosynthesis of [^{18}F]FCPPC from its Cl-form precursor. Radiosynthesis conditions: MeCN, 100 °C, 5 min. Decay-corrected yield at EOS: 26.8%.

enabled a streamlined process encompassing one-pot radiofluorination, semi-preparative HPLC purification, and solid phase extraction (SPE) assisted formulation.

Materials and methods

Chemicals and supplies

Unless otherwise stated, reagents, solvents, and chemicals were purchased from commercially available vendors and used without further purification. The 5-cyano-N-(4-(4-(2-fluoroethyl)piperazin-1-yl)-2-(piperidin-1-yl)phenyl) furan-2-carboxamide (FCPPC) reference standard and 5-cyano-N-(4-(4-(2-chloroethyl)piperazin-1-yl)-2-(piperidin-1-yl)phenyl)furan-2-carboxamide (pre-FCPPC) precursor were synthesized in-house following the reported method [21]. Acetonitrile (MeCN; anhydrous 99.8%), potassium carbonate (K_2CO_3; 99.995% trace metals basis), and 0.22 μm Millex-GV syringe-driven filter unit were purchased from Millipore Sigma (St. Louis, MO, USA). Acetonitrile (MeCN; HPLC grade) was purchased from Fisher Scientific (Hampton, NH, USA). Kryptofix 2.2.2 ($K_{2.2.2}$; chemical grade) and [^{18}O]H_2O (≥98%) was purchased from ABX (Radeberg, Germany). QMA carbonate plus light cartridge (46 mg sorbent per cartridge; 40 μm), alumina N Plus Light cartridge, and tC18 plus short cartridge (400 mg sorbent per cartridge; 37-55 μm) were purchased from Waters (Milford, MA, USA). Absolute ethanol (EtOH; USP grade) was purchased from Greenfield Global USA Inc. (Shelbyville, KY, USA). Sterile water for injection, USP and 0.9% sodium chloride (NaCl) for injection were purchased from Hospira (Lake Forest, IL, USA). Fresh deionized water (18.2 MΩ·cm at 25°C) was generated from Milli-Q Direct water purification system (Millipore Sigma, Billerica, MA, USA), and used for the preparation of all the standard and eluent solutions. The $K_{2.2.2}/K_2CO_3$ stock solution for [^{18}F]fluoride elution was prepared with $K_{2.2.2}$ (240 mg) and K_2CO_3 (40 mg) in acetonitrile (19.4 mL) and deionized water (0.6 mL), and passed through a Millex-LG PTFE filter (Millipore Sigma, St. Louis, MO, USA). Prior to use, QMA and alumina N cartridges were conditioned with 5 mL deionized water, and tC18 cartridge was conditioned with 5 mL ethanol and 5 mL deionized water. Radioactivity was determined with a Capintec® CRC-712M dose calibrator (Capintec, Inc., Florham Park, NJ, USA).

Chromatographic method

Semi-preparative high-performance liquid chromatography (HPLC) was carried out using an RNplus Research module (Synthra, Hamburg, Germany), which included a built-in semi-preparative HPLC system with ultraviolet (UV) and radioactivity detectors. [^{18}F]FCPPC purification was conducted on a Reeperbahn C18 column (5 μm, 250 × 10 mm; Synthra, Hamburg, Germany) at the UV wavelength of 254 nm. The HPLC loop volume was 3 mL and the mobile phase was a mixture of acetonitrile and 10 mM ammonium formate in water (v/v = 55/45) at a flow rate of 6 mL/min. The retention time of [^{18}F]FCPPC was about 15 min.

Analytical Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) equipped with ultraviolet (UV) and Ortec-556 radioactivity detectors (Oak Ridge, TN, USA) was used to determine radiochemical purities and identities, and chemical impurities on an analytical ACQUITY BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Milford, MA, USA) at the UV wavelength of 254 nm. The sample injection volume was 10 μL and the mobile phase was a mixture of acetonitrile and 50 mM ammonium formate in water (v/v = 65/35) with a flow rate of 0.3 mL/min. The retention time of [^{18}F]FCPPC on analytical HPLC was about 2.2 min.

Module configuration for [^{18}F]FCPPC radiosynthesis

The module configurations for [^{18}F]FCPPC radiosynthesis are detailed in **Figure 3**. In the optimized module configurations, vials A1-A3, C1-C3 and reaction vessel were used

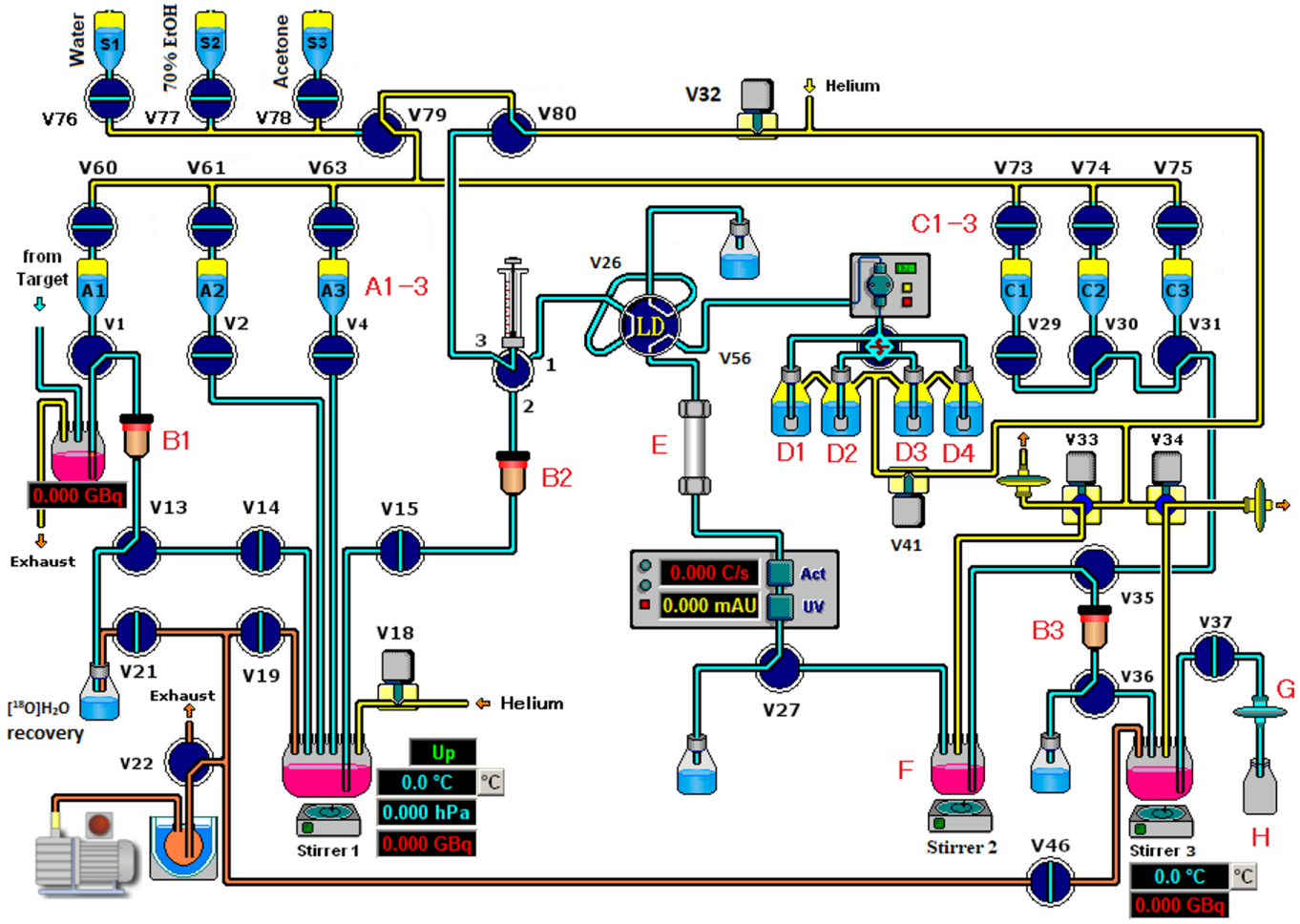

Figure 3. Diagram of synthra RNplus research module for [18F]FCPPC production. A1: 1 mL of $K_{2.2.2}/K_2CO_3$ solution; A2: 2 mg of pre-FCPPC precursor in 1 mL of MeCN; A3: 1 mL of deionized water; B1: QMA cartridge (46 mg); B2: alumina N Plus Light cartridge; B3: tC18 cartridge; C1: 10 mL sterile water; C2: 1 mL ethanol; C3: 10 mL of 0.9% NaCl; D1: HPLC eluent; E: semi-preparative HPLC column; F: 30 mL water and 1 mL 100 mg/mL ascorbic acid in water; G: Millex-GV 0.22 μm filter; and H: 30 mL product vial.

for [18F]FCPPC radiosynthesis followed by HPLC purification (**Figure 4**), and subsequently formulated via solid phase extraction (SPE). Vials S1-S3 were used for system cleaning.

Radiosynthesis, purification, and formulation of [18F] FCPPC

By irradiation of [18O]water with a GE PETtrace 800 cyclotron (15 min irradiation at 60 μA), [18F]fluoride was generated and delivered to Synthra RNPlus Research module for [18F]FCPPC radiosynthesis. The received [18F]fluoride was extracted with a QMA cartridge (46 mg) and eluted with 1.0 mL of Kryptofix 2.2.2 (12 mg, 32 μmol) and potassium carbonate (2 mg, 14 μmol) solution, and then dried by azeotropic evaporation at 70-100°C under a flow of helium and/or vacuum. The reaction vessel containing dried [18F]fluoride was cooled to 50°C. A solution of the pre-FCPPC precursor (2 mg) in anhydrous acetonitrile (1 mL) was added to the reaction vessel for radiofluorination at 100°C for 5 minutes. After cooling to 50°C, the crude [18F]FCPPC solution was diluted with 1 mL of deionized water, passed through an alumina N cartridge to remove unreacted [18F]fluoride, and loaded onto a semi-preparative HPLC column for purification. The [18F]FCPPC HPLC fraction (Rt = 15-16 min) was collected and diluted with 30 mL of water and 1 mL of 100 mg/mL ascorbic acid solution, and extracted with a tC18 cartridge. The retained [18F]FCPPC was washed with 10 mL of sterile water, and then eluted off the tC18 cartridge with 1.0 mL of ethanol and formulated with 10 mL of 0.9% NaCl. The final formulated drug product [18F]FCPPC was delivered to the 30 mL product vial through a sterilizing 0.22 μm Millex-GV filter.

Quality control and stability test of [18F]FCPPC

A sample of ~0.5 mL was taken from the finished final product for QC tests following United States Pharmacopeia and GMP guidelines.

Results

Radiosynthesis

Three consecutive batches of [18F]FCPPC product were obtained in 7.2 ± 0.1 GBq (196 ± 3 mCi) at the end of

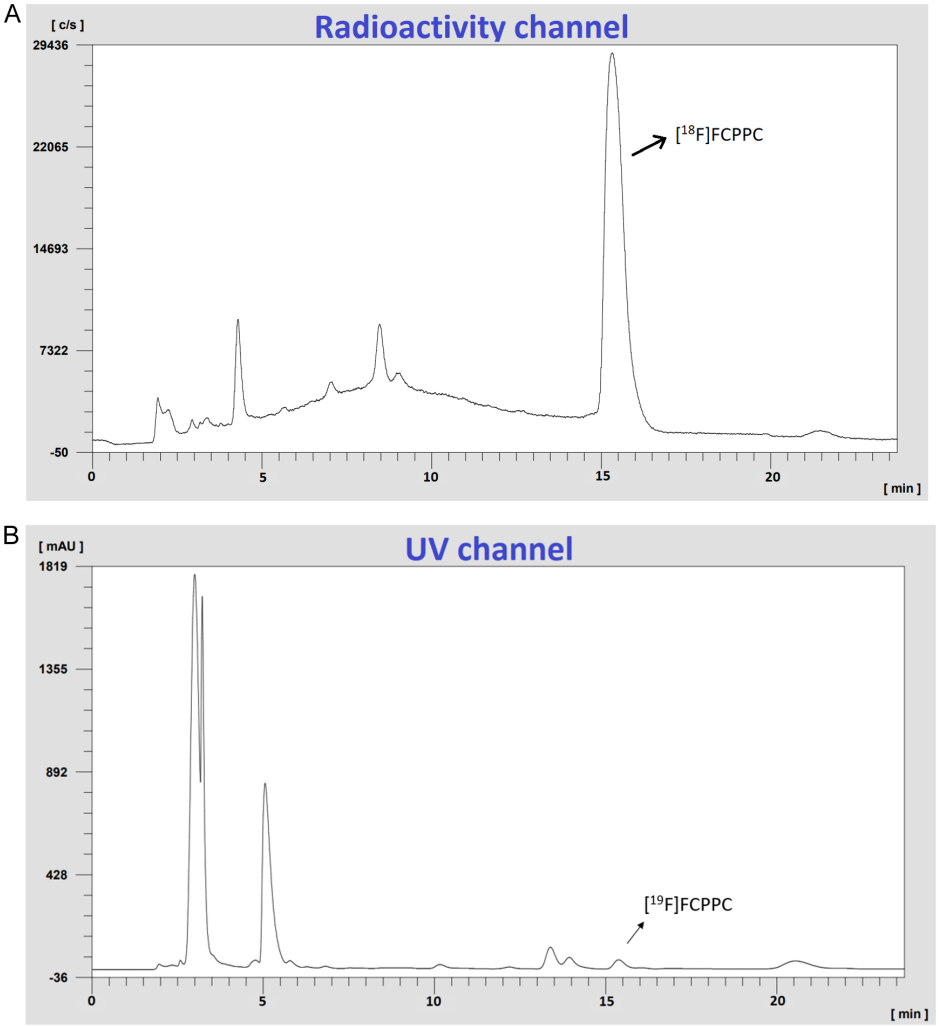

Figure 4. Representative semi-preparative HPLC chromatograms for [18F]FCPPC purification. (A) Radioactivity channel and (B) UV channel.

synthesis (EOS), from estimated starting activity of 37 GBq (1000 mCi) in three consecutive validation runs. [18F]FCPPC radiosynthesis were completed in a total synthesis time of 50 min from the end of bombardment (EOB), with 15 min irradiations at 60 μA. The final product was formulated in volumes of 10.5 ± 0.2 mL containing 10 mL 0.9% NaCl and 6% ethanol. The radiochemical yields were 26.8 ± 0.1% decay corrected to the end of bombardment (EOB).

Quality control

The QC results showed that the produced [18F]FCPPC met all the release criteria. As indicated in **Table 1**, all three batch products were clear, colorless solutions, and free from particulate matter. The pH and half-life values were within the ranges of 4.0-7.0 and 105-115 min. From analytical HPLC results (**Figure 5**), excellent radiochemical and chemical purities were achieved. The radiochemical purities were over 99% and the molecular activity was 1330 ± 200 GBq/μmol (36 ± 5 Ci/μmol) at EOS. The non-radioactive mass of [19F]FCPPC were 0.22 ± 0.02 μg/mL and the total unknown impurities were 0.40 ± 0.03 μg/mL. The radionuclidic purities of over 99% were determined by non-presence of long-lived isotopes in the product after 72 hours decay. The residual solvents in the product were determined to be 6.0-6.2% ethanol by gas chromatography. The integrity of the final filter was demonstrated by a bubble-point filter test with holding ≥45 psi pressure. The formulated products were sterile and nonpyrogenic from the sterility and endotoxin results. Stability at 4 hours after EOS was evaluated by performing the repeated assessment of appearance, radiochemical identity/purity, chemical purity, pH, and bacterial endotoxin. A summary of the stability tests was given in Table S1, showing no significant changes at 4 hours post EOS.

Discussion

During manual optimization, the mass of pre-FCPPC precurosr, solvent, reaction temperature and time were evaulated to improve the conversion yield. The highest conversion yield on radio-TLC (~88%) was achieved using 2 mg of pre-FCPPC precursor in 1.0 mL acetonitrile and heating at 100°C for 5 min (Table S2, Entry 7 and Figure S1). The conversion yield slightly decreased with more pre-FCPPC precursor or higher temperature. These optimized conditions were used for automated radiosynthesis of [18F]FCPPC in the present study.

With a starting activity of ~37 GBq (1000 mCi) obtained from a 15 min irradiation at 60 μA, the automated radiosynthesis of [18F]FCPPC produced 7.2 ± 0.1 GBq (196 ± 3 mCi) of labeled compound, with a yield of 26.8 ± 0.1% (decay corrected to EOB), which is higher than the reported manual radiosynthesis (8-8.5%, non-decay corrected) [21]. The radiosynthesis yield may be higher than the calculated yield because the starting activity for yield calculation was not calibrated at arrival of Synthra RNPlus module, but obtained from the cyclotron bombardment report. Limited by the facility layout, the hot cell and module for [18F]FCPPC synthesis were located 60 meters from the cyclotron, resulting in activity loss during [18F]fluoride delivery.

In the automation test run, we observed a radiochemical impurity on analytical HPLC (~10%; rt = 0.9 min; Figure

Table 1. Summary of QC results from three [^{18}F]FCPPC validation runs

QC Test	Acceptance Criteria	Result		
		Run 1	Run 2	Run 3
Appearance	Clean, colorless and no particles	Pass	Pass	Pass
Concentration	≥74 MBq/mL (2 mCi/mL) at EOS	703 MBq/mL (19 mCi/mL)	666 MBq/mL (18 mCi/mL)	703 MBq/mL (19 mCi/mL)
Filter integrity	Bubble point ≥310 kPa (45 psi)	Pass	Pass	Pass
Radionuclidic identity	Half-life: 105-115 min	110.2 min	109.8 min	109.6 min
Radionuclidic purity	≥99.5% observed gamma emission should correspond to 0.511 and 1.022 MeV	Pass	Pass	Pass
pH	pH value: 4.0-7.0	5.0	5.0	5.0
Radiochemical purity	[^{18}F]FCPPC peak: ≥90%	>99%	>99%	>99%
Radiochemical identity	RSD of [^{18}F]FCPPC Rt values: ≤10%	4.1%	4.6%	4.4%
Chemical purity	FCPPC mass: ≤10 μg/mL	0.25 μg/mL	0.22 μg/mL	0.19 μg/mL
	Impurities: ≤10 μg/mL	0.36 μg/mL	0.41 μg/mL	0.43 μg/mL
Chemical purity: residual solvent	Ethanol ≤10% (w/v)	6.1%	6.2%	6.0%
	MeCN ≤0.041% (w/v)	0%	0%	0%
Chemical purity: $K_{2.2.2}$	Intensity is less than $K_{2.2.2}$ STD	Pass	Pass	Pass
Pyrogen test	LAL Endotoxins test: ≤175 EU/vial	Pass	Pass	Pass
Sterility test	No growth after 2 weeks incubation	Pass	Pass	Pass

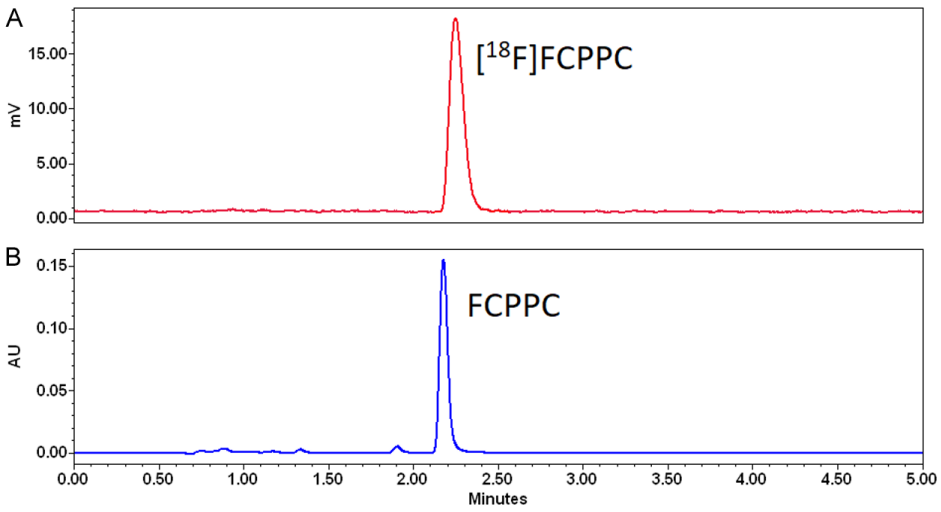

Figure 5. Representative analytical HPLC chromatograms of [^{18}F]FCPPC and reference co-injection. (A) Radioactivity channel and (B) UV channel.

S2) due to the radiolysis at increased radioactivity concentration, which was inhibited by addition of 1 mL 100 mg/mL solution of ascorbic acid along with 30 mL of water in HPLC fraction collection vial during the purification. We successfully achieved >99% radiochemical purity in the final product, and observed excellent stability at 4 hours post EOS. Radiolysis was also inhibited by the presense of ethanol used in the formulation of [^{18}F]FCPPC product.

The pre-FCPPC precursor is highly reactive and gradually decomposes in presence of water. Monitoring via HPLC analysis, gradual decomposition of the pre-FCPPC precursor (dissolved in 50% of acetonitrile in water) was observed in injections of the same sample at different intervals. Therefore, the pre-FCPPC should be stored free from mositure while not in use.

It is worth mentioning that [^{18}F]FCPPC is a lipophilic compound and may stick to plastic materials due to precipitation. In our practice, [^{18}F]FCPPC dose was prepared just prior to use, and avoided unnecessary dilution, and left-over activity in syringe and/or extension loop was measured and subtracted to get the corrected activity.

Conclusions

A fully automated production of [^{18}F]FCPPC was achieved with excellent radiochemical and chemical purity as well as radiochemical yield. Three consecutive [^{18}F]FCPPC validation runs and quality control results demonstrated the efficacy of our automated approach for future CSF1R PET imaging applications.

Acknowledgements

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Disclosure of conflict of interest

None.

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Manual optimization

We performed manual optimization to find the suitable condition for radiolabeling of FCPPC. [^{18}F]Fluoride was transferred to a reaction vial from QMA cartridge (46 mg), using 1.0 mL of Kryptofix 2.2.2 (12 mg, 32 µmol) and K_2CO_3 (2 mg, 14 µmol) solution and dried by azeotropic evaporation at 100°C under gentle flow of N_2. The reaction vial was then cooled down to 50°C and a solution of precursor was added to it. Radiolabeling was done by heating the reaction mixture to a particular temperature for several minutes. Crude conversion yield was obtained from radio-TLC. Several manual radiosynthesis were performed with varying solvent, precursor weight, reaction temperature, solvent volume, and reaction time (Table S2). The highest conversion yield (88%) was achieved using 2 mg of pre-FCPPC precursor in 1.0 mL of acetonitrile and heating at 100°C for 5 min (Table S2, Entry 7). We used this optimized condition for the automated radiosynthesis.

Table S1. Summary stability results at 4 hours after EOS of [^{18}F]FCPPC validation runs

QC Test	Acceptance Criteria	Result		
		Run 1	Run 2	Run 3
Appearance	Clean, colorless and no particles	Pass	Pass	Pass
Radionuclidic identity	Half-life: 105-115 min	109.4 min	109.5 min	109.6 min
pH	pH value: 4.0-7.0	5.0	5.0	5.0
Radiochemical purity	[^{18}F]FCPPC peak: ≥90%	100%	100%	100%
Radiochemical identity	RSD of [^{18}F]FCPPC Rt values: ≤ 10%	4.2%	4.7%	4.8%
Chemical purity:	FCPPC mass: ≤ 10 µg/mL	0.23 µg/mL	0.25 µg/mL	0.20 µg/mL
	Impurities: ≤ 10 µg/mL	0.40 µg/mL	0.42 µg/mL	0.43 µg/mL
Chemical purity: $K_{2.2.2}$	Intensity is less than $K_{2.2.2}$ STD	Pass	Pass	Pass
Pyrogen test	LAL Endotoxins test: < 175 EU/vial	Pass	Pass	Pass

Table S2. Manual optimization for the radiosynthesis of [^{18}F]FCPPC

Entry	Solvent	Vol. of solvent (mL)	Precursor wt. (mg)	Temp. (°C)	Time (min)	%Conversion
1.	DMSO	0.5	1.0	100	10	25.8
2.	MeCN	0.5	1.0	100	10	40.9
3.	DMF	0.5	1.0	100	10	20.7
4.	MeCN:^{t}BuOH (1:1)	0.5	1.0	100	10	2.0
5.	MeCN	0.5	1.0	100	5	53.3
6.	MeCN	0.5	2.0	100	5	65.9
7.	MeCN	1.0	2.0	100	5	88.0
8.	MeCN	1.0	3.0	100	5	80.4
9.	MeCN	1.0	2.0	110	5	79.4
10.	MeCN	1.0	2.0	90	5	69.1

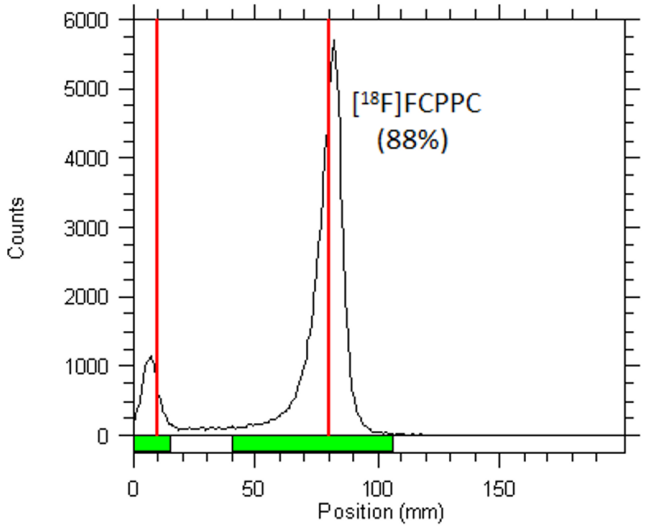

Figure S1. Radio-TLC of crude [^{18}F]FCPPC during manual optimization.

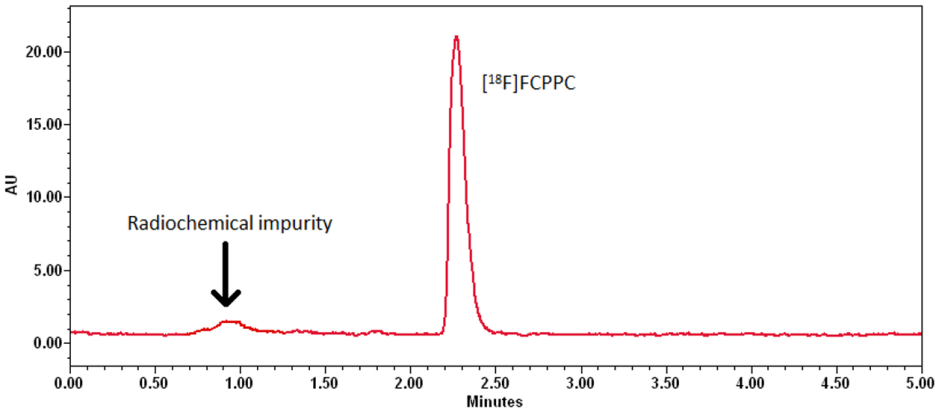

Figure S2. Analytical HPLC chromatograms of [^{18}F]FCPPC at increased radioactivity concentration in radioactivity channel.