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An automated one-step one-pot [^{18}F]FCWAY Synthesis: development and minimization of chemical impurities

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Abstract

[^{18}F]FCWAY (*N*-{2-[4-(2-methoxyphenyl)piperazino]}-*N*-(2-pyridinyl)*trans*-4-fluorocyclohexanecarboxamide) has been prepared routinely as a serotonin 5-HT_{1A} receptor ligand for clinical human studies. We have developed an automated one-step radiosynthesis using a modified Nuclear Interface C-11 Methylation System. The chemical synthesis of an appropriate methanesulfonate precursor for the single-step nucleophilic substitution with [^{18}F]fluoride ion and adaptation of the radiochemical synthesis to an automated production module were accomplished. Following purification of the substrate using counter current chromatography, the radiochemical yield increased from $18.9 \pm 0.3\%$ to $21.9 \pm 2.2\%$. In addition, reduction of chemical impurities from about 40% to about 20% of total mass was observed. Further improvements in chemical purity were achieved by minimization of side reactions by modification of reaction conditions and optimization of the HPLC method for purification of the final radiopharmaceutical. The optimized automated synthesis produced [^{18}F]FCWAY in a radiochemical yield of $28\% \pm 6\%$, of chemical purity 99.3% based of absorbance of FCWAY at 254nm and with a specific activity of 3433 ± 1015 mCi/ μmol at the end of bombardment, EOB, all calculated from the same 50 runs.

Keywords

[^{18}F]FCWAY; 5-HT_{1A} receptors; WAY 100635 analogue; Automated synthesis; PET Radiopharmaceutical; chemical impurities

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1. Introduction

FCWAY (*N*-{2-[4-(2-methoxyphenyl)piperazino]}-*N*-(2-pyridinyl)*trans*-4-fluorocyclohexanecarboxamide) is a *trans*-4-fluorocyclohexane analogue [1,2] of WAY 100635, *N*-(2-(1-(4-(2-methoxyphenyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)cyclohexane carboxamide, a proven high-affinity 5-HT_{1A} silent antagonist [3,4]. [¹⁸F]FCWAY was initially developed in our laboratories using a multiple step synthetic sequence [1]. This radiopharmaceutical has been applied to human clinical trials in several protocols exploring the involvement of 5-HT_{1A} receptors in neurophysiologic and neuropsychiatric disorders. Using [¹⁸F]FCWAY in Positron Emission Tomography (PET) studies, Theodore et al. reported the reduction of 5-HT_{1A} in temporal lobe epilepsy [5] and elucidated the effect of antiepileptic drugs [6]. Bain et al. studied 5-HT_{1A} bindings in post traumatic stress disorder [7] and in bipolar depression [8]. Drevets et al. revealed for the first time in vivo evidence for the implication of 5-HT_{1A} in the pathophysiology of panic disorder [9]. To facilitate data analysis, a reliable extraction method was developed to simplify metabolite analysis [10]. A human brain functional imaging model was established [11] allowing the pixel by pixel display of distribution volume with high statistical quality. The model was designed to correct spillover of the acid metabolite uptake in the brain and fluoride uptake in the skull that came from radiodefluorination of the acid metabolite [12].

Because of the significant demand for routine production of [¹⁸F]FCWAY, a onestep radiochemical synthesis was developed [13] to enable simple automation. In working out the one-pot process, we uncovered the challenges in isolating the product from the side-products of the thermal reaction. Even after re-purification of the mesylate precursor using counter current chromatography [14], much additional effort was deemed necessary in resolving the issue of chemical purity. This manuscript presents the development and optimization of an automated one-pot radiosynthesis for a simple and dependable production of consistently pure [¹⁸F]FCWAY.

2. Experimental

General Chemistry

WAY 100634 (*N*-{2-[4-(2-methoxyphenyl) piperazin-1-yl]ethyl}-*N*-(pyridine-2-yl)amine) was obtained from Med-Life Systems, Inc. Upper Darby, PA. The FCWAY reference standard sample for QC analysis was prepared as a custom synthesis reported previously [1]. Most other chemicals were purchased from commercial suppliers and used as they were received. The ¹H and ¹³C-NMR spectra were obtained on a Varian Gemini-2000 spectrometer at 200 and 50 MHz respectively. Chemical shifts were reported in parts per million downfield from tetramethylsilane (δ) and coupling constants were given in Hertz. All LC/MS analyses were performed with a Finnigan LCQ MS, coupled with an HP series 1100 HPLC system. HPLC was equipped with a Waters Symmetry C18, 5 μm, 3.9 × 150 mm column, using a linear gradient elution of 0–70% acetonitrile in 50 mM ammonium formate over 10 minutes at a fixed flow rate of 0.5 mL/min, UV absorbance set at 254 nm. QC analysis was performed routinely on a Waters Symmetry C18 column, 3.9 × 150 mm, with a mobile phase containing 60% MeOH/40% Buffer (100 mM ammonium formate) running at 1 mL/min, UV absorption at 254 nm.

2.2 Precursor for the one step radiosynthesis of [¹⁸F]FCWAY

cis Pentamethylbenzyl 4-acetoxycyclohexanecarboxylate (2)—To 3.0 g (10 mmol) of cis pentamethylbenzyl 4-hydroxycyclohexanecarboxylate prepared according to previously published procedure [1] was added 40 mL CH₂Cl₂, 1.4 mL of acetyl chloride and 1.4 mL of

triethylamine. The mixture was refluxed overnight. The solvent was evaporated and the product **2** was purified on silica gel column using 20% ethyl acetate/hexane. Yield: 85%. ^1H NMR (CDCl_3) δ 1.49-1.95 (m, 8H), 2.05 (s, 3H), 2.23-2.28 (m, 15H), 2.33-2.45 (m, 1H), 4.93 (m, 1H), 5.25 (s, 2H).

cis 4-Acetoxycyclohexanecarboxylic acid (3)—To 1.05 g (3 mmol) of compound **2** was added 0.66 mL of anisole and 10 mL of trifluoroacetic acid (TFA). The mixture was stirred for 20 min and TFA was evaporated under reduced pressure. The residue was loaded on a silica gel column and eluted with 75% hexane in ethyl acetate to give 0.34 g of the title compound **3**. Yield: 60%. ^1H NMR (CDCl_3) δ 1.56-1.97 (m, 8H), 2.06 (s, 3H), 2.45 (m, 1H), 4.95 (m, 1H).

cis 4-Acetoxy-N-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-N-(2-pyridyl)cyclohexanecarboxamide (5)—Compound **3** (0.32 g, 1.72 mmol) was dissolved in CH_2Cl_2 (10 mL) and treated with dichloromethyl methylether (0.6 mL, 6.63 mmol). The mixture was refluxed for 2 hours. The solvent was evaporated under reduced pressure and the product **4** was used without further purification. Compound **4** was dissolved in 5 mL of CH_2Cl_2 and added to a 15 mL CH_2Cl_2 solution containing WAY 100634 (0.54 g, 1.73 mmol) and 0.3 mL of triethylamine. The mixture was stirred at room temperature overnight. At the end of the reaction, the solvent was evaporated and the product was purified on silica gel column with ethyl acetate containing 1% triethylamine to give 0.58 g of oil (compound **5**). Yield: 70%. ^1H NMR (CDCl_3) δ 1.22-1.40 (m, 2H), 1.47-1.35 (m, 2H), 1.80-1.98 (m, 4H), 2.05 (s, 3H), 2.59-2.66 (m, 6H), 3.00 (m, 4H), 3.85 (s, 3H), 3.99 (t, 2H), 4.91 (m, 1H), 6.83-7.02 (m, 4H), 7.22-7.34 (m, 2H), 7.78 (m, 1H), 8.53 (m, 1H).

cis 4-Hydroxy-N-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-N-(2-pyridyl)cyclohexanecarboxamide (6)—To compound **5** (0.32 g, 0.67 mmol) in 10 mL of CH_3OH was added 90 mg (0.65 mmol) of potassium carbonate. The mixture was stirred at room temperature overnight. After evaporating the CH_3OH , the product was purified on silica gel column using ethyl acetate containing 1% triethylamine to give 240 mg of oil (compound **6**). Yield: 82%. ^1H NMR (CDCl_3) δ 1.22-1.40 (m, 2H), 1.47-1.35 (m, 2H), 1.80-1.98 (m, 4H), 2.05 (s, 3H), 2.59-2.66 (m, 6H), 3.00 (m, 4H), 3.85 (s, 3H), 3.99 (t, 2H), 4.91 (m, 1H), 6.83-7.02 (m, 4H), 7.22-7.34 (m, 2H), 7.78 (m, 1H), 8.53 (m, 1H).

cis 4-Methanesulfonyloxy-N-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-N-(2-pyridyl)cyclohexanecarboxamide (7)—To a flask containing 0.23 g (0.53 mmol) of compound **6** in 5 mL of CH_2Cl_2 were added 45 μL of methanesulfonylchloride and 100 μL of triethylamine. The mixture was stirred at room temperature overnight. After evaporating the CH_2Cl_2 , the product was purified on a silica gel column using ethyl acetate containing 1% triethylamine to give 0.18 g of oil (compound **7**). Yield: 66%. ^1H NMR (CDCl_3) δ 1.34-1.68 (m, 5H), 1.85-2.13 (m, 5H), 2.35 (m, 1H), 2.58-2.65 (m, 6H), 3.02 (m, 7H), 3.82 (s, 3H), 3.98 (t, 2H), 4.92 (s, 1H), 6.82-7.01 (m, 4H), 7.23-7.30 (m, 2H), 7.78 (td, 1H), 8.53 (tt, 1H). ^{13}C NMR (CDCl_3) δ 23.71, 30.27, 39.13, 40.73, 45.64, 50.78, 53.58, 55.54, 56.33, 111.46, 118.29, 121.15, 122.21, 122.59, 123.06, 138.54, 141.50, 149.52, 152.45, 156.03, 175.28. MS/ESI m/z 517.

2.3 One Step Manual Synthesis of [^{18}F]FCWAY

The mesylate precursor **7** was used without further purification. 3-4 mg of **7** in 500 μL 1% aqueous acetonitrile was added to a dried mixture of [^{18}F]HF, K_{222} (8 μmoles) and K_2CO_3 (4 μmoles) in a 1 mL V-vial. The mixture was heated in a 105°C hot block for 15 minutes, cooled, diluted with 1.5 mL HPLC Eluent A, {(30/22/48 MeOH/ACN/buffer (20 mM triethylamine/20 mM NaH_2PO_4 /10 mM Na_2HPO_4)) and injected onto the semi-preparative column

(Beckman C18 10 × 250 mm) flowing at 4.5 mL/min. The HPLC instrument consisted of a Perkin-Elmer series 200 HPLC pump, a Waters 486 UV Detector at 254 nm and a Beckman 170 radioactivity detector. The major radioactivity peak eluting around 30 minutes had the same retention time as that of a standard FCWAY and its identity was confirmed by LC/MS analysis.

2.4 Automated Synthesis

Automation was performed on a Nuclear Interface Synthesizer modified from an original C-11 Methylation System as described previously [15] with one additional adaptation. [¹⁸F]fluoride was trapped by a strong anion-exchange (SAX) column and eluted into the second reaction vessel (RV2). The precursor **7** was further purified before use by Counter Current Chromatography, as previously described [14]. Initial conditions, solvents, and HPLC eluents for the automation procedure were based on the initial manual conditions. The reaction was conducted at 105 °C for 15 min, the reaction was cooled to 40 °C prior to quenching with the aqueous HPLC buffer. In the manual synthesis, the cooling temperature was not a controlled variable. Semipreparative HPLC used eluent A as described in the manual synthesis. The standard QC method was used to analyze all batches of product. To assist in the removal of chemical impurities, fractions were collected from the semipreparative HPLC before, during, and after elution of the desired FCWAY. These fractions were analyzed by HPLC-MS, using conditions described in the general experimental section, to assist in the identity of chemical impurities.

2.5 Optimized Automated procedures

An automated clean-dry procedure prepared the module and the system set up for the synthesis. The [¹⁸F]fluoride trapped on the SAX Bio-Rad AG 1-X8 (Bio-Rad Lab., Richmond, CA), supported in an Upchurch pre-column (Upchurch Scientific, Oak Harbor, WA) was eluted with 0.5 mL 0.01M K₂CO₃ (0.5 μmoles) into RV2 already containing 0.5 mL acetonitrile and 8 μmoles K222. The [¹⁸O]OH₂ was recovered for recycle. After azeotropic drying, the purified precursor **7** (3.0 mg in 0.5 mL anhydrous acetonitrile) was added to the dried and cooled residue. The reaction mixture was heated at 105°C for 15 minutes and then cooled to 26°C. The reaction was diluted with 1.35 mL of acetonitrile/water (15:120), and transferred onto a semi-preparative HPLC column (Beckman Ultrasphere C18, 5 μm, 10 × 250 mm). The column was eluted at 7.5 mL/min with Eluent B, 33:67 acetonitrile/buffer (20 mM triethylamine/20 mM NaH₂PO₄/10 mM Na₂HPO₄). The radioactive peak of FCWAY collected at 30 minutes was passed through a C-18 SEP-PAK Light cartridge (Waters, 130 mg from Waters Corps., Milford, MA) to isolate the product from the HPLC eluent. The cartridge was washed with 2 mL HPLC grade water and dried in an argon stream for 3 minutes. The retained product was quantitatively eluted with 0.5 mL ethanol into a collection vessel, followed by passage of 10 mL normal saline via the cartridge into the same vessel. The formulated product was sterilized via passage through a sterile 0.22 μm filter into the final serum vial.

3 Results

The precursor (**7**) with the mesylate leaving group was prepared in 8 steps, starting from the commercially available ethyl 4-hydroxycyclohexanecarboxylate.

Manual radiochemical synthesis

The radiochemical yield of 6 exploratory manual runs averaged $6.0 \pm 2.7\%$ at end of synthesis (EOS) based on the starting [¹⁸F]HF radioactivity. The product was not analyzed for chemical purity.

Automated radiochemical synthesis

It was realized in early process development that this one step synthesis produced a FCWAY product which contained chemical impurities. HPLC-MS results allow formation of the hypothesis that the primary impurity, **8**, was derived from **12** (Figure 1). **12** was identified as a contaminant in the precursor and was removed from the substrate using Counter Current Chromatography (CCC) [14]. Once purified, the substrate was analyzed by LC-MS and found to contain no **12** but a small amount of the hydroxy derivative, **11**, which proved not to be detrimental to final product purity.

Initial test runs were made with [^{18}F]HF added directly into RV2. HPLC product fractions were counted without formulations. Yields and product purities were compared before and after CCC purification. Before CCC, yield was $18.9 \pm 0.3\%$ at EOS, and $31.8 \pm 1.3\%$ decay corrected, $n = 3$. Presence of chemical impurities was estimated to be 25-40% by LC-MS. After purification of the precursor, yield was $21.9 \pm 2.2\%$ at EOS, and $37.1 \pm 3.5\%$ decay corrected, $n = 3$, all based on the starting radioactivity of [^{18}F]HF. About 16% increase in the radiochemical yield was observed. Total impurities were estimated to be 10-20% of the UV area of the FCWAY peak and compound **8** was still detected by LC-MS, among other contaminants shown in Figure 1. In order to improve product purity, additional modifications were made both to the synthesis method and the purification method, with the results summarized in Table 1.

The entire synthesis process took 85 minutes. The specific activity obtained after method optimization was found to be 3433 ± 1015 mCi/ μmol at EOB, which meets the criterion of the current clinical receptor studies.

4 Discussion

The initial automated procedure allowed for routine clinical production of [^{18}F]FCWAY. However, product purity emerged as a critical issue, not uncommon with thermal reactions, in a one-pot, one-step, no-carrier-added radiosynthesis. In our case, major effort was required to provide a product of sufficient chemical purity for human studies. Mass spectroscopy proved to be an invaluable tool to identify the UV detectable impurities and to better understand and control the synthesis chemistry.

We observed an impurity in our final product we believed to be **8** based on MS interpretation. In preliminary work, we discovered that **12** was present in our precursor [14], and proposed that base hydrolysis of **12** produced **8**. Purification of the precursor using CCC [14] removed **12** from the precursor. However, we still observed **8** in the radiochemical product. We can rationalize its formation from **7** during the reaction. We hypothesize that hydrolysis at the carbonyl could free an alkoxyl cyclohexyl carboxylate, producing a nucleophile that could react with additional **7** to form the double cyclohexyl analogue, **8**. Based on this hypothesis, reaction conditions were changed to minimize base mediated hydrolysis, in which water could be a culprit.

Modification to the Synthesis Method

Anhydrous acetonitrile was used to introduce the precursor instead of the 1% aqueous acetonitrile. In addition, the reaction vessel was cooled from 40°C to 26°C before quenching with the aqueous HPLC eluent. Early work in the manual synthesis indicated that a small amount of water facilitated the nucleophilic substitution. The elimination of this 1% water in the substrate solution effectively precluded the formation of **8** as none was detected by HPLC analysis of the HPLC fractions adjacent to or containing product.

Modification to the Purification Method

With the exclusion of **8**, other contaminants, **7**, **9** and **10**, remained detected by LC/MS in the preparative HPLC purified product. A more selective HPLC method was required. Using a Beckman analytical column (C18, 4.6 × 150 mm, 5 μm), HPLC eluent conditions were modified (Figures 2-4) in order to improve chromatographic resolution. Eluant B (Figure 3) provided satisfactory separation of FCWAY from other chemical components.

With a satisfactory analytical chromatographic system available, the scale-up to preparative HPLC still required work. Following collection of the radioactivity peak from the semi-prep, the analytical HPLC showed unacceptable levels of precursor. Tailing of the precursor, present in milligram quantities, resulted in microgram amounts co-eluting with the desired product in spite of a 10 minute difference in retention time using Eluent B. The elimination product EP, **10**, which eluted 10 minutes *after*, was also detected in the HPLC product fraction, probably coming from elimination of the tailed mesylate at the eluent's basic pH.

To minimize tailing, the amount of precursor was reduced. Reduction of the precursor quantity from 4.5 to 2 mg gave a lower radiochemical yield of 7%, based on the [¹⁸F]HF trapped on the SAX. The radiochemical yield was not significantly reduced with 3 mg of precursor but the precursor still was found to be 7% of the FCWAY UV absorbance upon HPLC analysis.

We hypothesized that the matrix of the HPLC injectate could be responsible for peak broadening and tailing. The reaction was carried out in 0.5 mL acetonitrile. To load it onto the HPLC loop (volume 2mL), 1.35mL of the eluent had been added, resulting in a sample matrix of 51% acetonitrile. To correct this discrepancy in solvent strength, the reaction was diluted with 1.35mL acetonitrile/water (15/120). The matrix of the injected sample now was brought to 35% acetonitrile, much closer to the 33% in the mobile phase. Figure 4 shows the analytical chromatogram of FCWAY product from the final optimized synthesis and purification procedure.

A comparison of yield and purity of the initial automation conditions to the optimized conditions were tabulated in Table 1. A higher yield was achieved from the accumulated changes during optimization, including revision of the automated clean/dry procedures.

5. Conclusion

We have developed an automated [¹⁸F]FCWAY production that is both simple and reliable; achieving high radiochemical yield and minimal radiation exposure to the operator. Using the chemical structure information obtained from HPLC-MS as a guide, we succeeded in minimizing the presence of unlabeled components that could have a physiological and pharmacological effect. As a result, the one-step one-pot radiosynthesis was able to routinely deliver [¹⁸F]FCWAY with high chemical purity and specific activity.

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Figure 1. Structures of FCWAY and proposed chemical impurities

The structures are assigned based on MS data and a chemist's rationalization.

Figure 2. FCWAY product UV chromatogram on an analytical Beckman C18 @254nm from a synthesis using preparative HPLC Eluent A, precursor in 1% aqueous ACN

Note: The mobile phase used in the analysis was also Eluant A at 1mL/min, similar linear velocity as in production. This showed that Eluent A was an inadequate mobile phase for FCWAY purification.

Figure 3. Analytical chromatogram of a reaction mixture using Eluent B showing resolution of FCWAY from the chemical components, **7** (precursor), **8** (DC-OH), **9** (the acetate, OAc) and the elimination product EP, **10**, fractions identified by LC/MS.

Figure 4. Analysis using Eluent B of product FCWAY from an optimized procedure ready for clinic production and human use. LC/MS detected only one component with m/z of 441.

Table 1

Comparison of yield and purity before and after method modification

Conditions	n	Yield EOS	Yield EOB	%UV purity	%Radio. purity
4.5 mg substrate in 0.6 mL 1% aq. ACN 1.35 mL injectate of eluent A w/o salt, Prep-HPLC Eluent A	12	11 ± 3	20 ± 4	82.2 ± 14.1	> 99
3 mg substrate in 0.5mL anhydrous ACN 1.35 mL injectate of ACN/H ₂ O 15:120 Prep-HPLC Eluent B	50	28 ± 6%	49 ± 50	99.3 ± 3.0	> 99

Note: All yields based on [¹⁸F] fluoride activity trapped in the SAX and were taken from complete runs including fluoride exchange and formulations described in Section 2.4. Purity was obtained by the QC method described in Section 2.1 based on the UV absorption of FCWAY @ 254nm.