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Purification of the precursor for the automated radiosynthesis of [^{18}F]FCWAY by counter-current chromatography

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Abstract

Radiolabeled FCWAY (*N*-{2-[4-(2-methoxyphenyl)piperazino]}-*N*-(2-pyridinyl) *trans*-4-fluorocyclohexanecarboxamide) was prepared for human positron emission tomography (PET) studies by a simple one-step radiosynthesis. The LC–MS analysis of the products indicated that it contained impurities which may interfere with FCWAY uptake of 5-HT$_{1A}$ receptors and that these impurities were derived from an impurity originally present in the precursor preparation. Since preparative HPLC failed to resolve one of the impurities from the precursor, preparative-scale high-speed counter-current chromatography (HSCCC) was used for purification of this FCWAY precursor. A suitable two-phase solvent system composed of cyclohexane–ethyl acetate–methanol–water at a volume ratio of 1:1:1:1 or 4:5:4:5 was selected based on the partition coefficients of the precursor and impurity as determined by a LC–MS method. Using the second solvent ratio of 4:5:4:5 with the organic phase as a mobile phase, a 2.57 g amount of precursor preparation was successfully purified yielding 2.2 g of the pure precursor by a single run.

Keywords: Counter-current chromatography; Preparative chromatography; FCWAY; Methoxyphenyl piperazino-pyridinyl fluorocyclohexanecarboxamide

1. Introduction

FCWAY (*N*-{2-[4-(2-methoxyphenyl)piperazino]}-*N*-(2-pyridinyl) *trans*-4-fluorocyclohexanecarboxamide), a *trans*-4-fluorocyclohexane derivative of WAY 100635, is a high-affinity 5-HT$_{1A}$ antagonist ligand [1–3]. This compound has been labeled with ^{18}F and used for human studies [1]. Recently, an automated one-step radiosynthesis procedure was developed with high yield and high specific activity [4].

This one-step labeling was carried out using a mesylate precursor of FCWAY (**1**, Fig. 1). The reaction mixture was purified by preparative HPLC with an online radioactivity detector, and the radiolabeled FCWAY peak was collected for LC–MS analysis. It was found that the target compound, [^{18}F]FCWAY, was contaminated with unlabeled impurities. Because these impurities may cross the blood-brain barrier and adversely affect the binding of the radiolabeled FCWAY, we needed to determine their structures and origin. Based on LC–MS data, it was suggested these impurities were derived during the multi-step synthetic preparation of the precursor. Because preparative HPLC failed to resolve one of these impurities from the radiolabeled FCWAY, large-scale purification of the precursor preparation were performed using high-speed counter-current chromatography (HSCCC) [5,6] to remove the minute amounts of impurities that could lower the effective specific activity.

This paper describes the LC–MS analysis of impurities and successful purification of a multigram quantity of the precursor preparation by HSCCC using a two-phase solvent system composed of cyclohexane, ethyl acetate, methanol, and water.

2. Experimental

2.1. Materials

HPLC-grade water, methanol, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA) and cyclohexane from EM Sciences (Gibbstown, NJ, USA). Unless

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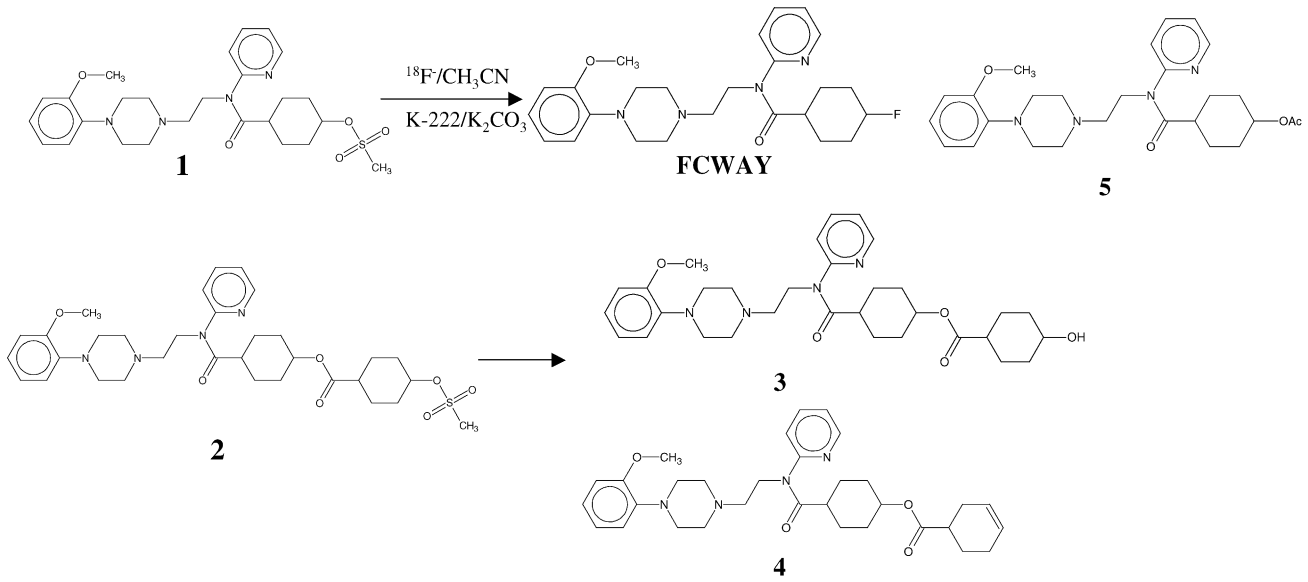

Fig. 1. Radiosynthesis of FCWAY using a mesylate precursor (**1**) and proposed structures of impurities (**2**–**5**).

otherwise specified, all other chemicals were from Aldrich (Milwaukee, WI, USA). HPLC purity was determined by UV absorbance at 254 nm and expressed as area percent of all peaks.

N-{2-[4-(2-Methoxyphenyl)piperazino]}-*N*-(2-pyridinyl) *trans*-4-fluorocyclohexanecarboxamide (*t*-FCWAY) was prepared according to the method previously reported [1,2]. The FCWAY precursor was custom-synthesized according to the method of Lang et al. [4]

2.2. HSCCC separation

Our present studies employed a commercial coil planet centrifuge (model HSCCC-1000, Pharma-Tech-Research Corp., Baltimore, MD, USA) equipped with a multilayer coil separation column made of 2.6 mm i.d. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) having a total capacity of 850 ml. This planetary centrifuge uses a rotary-seal-free flow-through system which permits continuous elution of the mobile phase through a rotating column without twisting the flow tubes [5,6].

A two-phase solvent system composed of cyclohexane–ethyl acetate–methanol–water (1:1:1:1 or 4:5:4:5) was equilibrated in a separatory funnel and the two phases were separated. The separation column was first filled with the aqueous stationary phase followed by injection of the sample solution containing 2.57 g of FCWAY precursor in 40 ml of solvent consisting of equal volumes of each phase through the sample port. The organic mobile phase was then pumped into the column at a flow rate of 4 ml/min while the apparatus was rotated at an optimum speed of 800 rpm. The effluent from the outlet of the column was continuously monitored by UV monitor (LKB Uvicord S, LKB Instruments, Stockholm, Sweden), and collected into test tubes at 2 min intervals using a fraction collector (Ultrorac, LKB Instruments). Collected fractions were analyzed by LC–MS. After the separation, the column contents were pushed out by pressured nitrogen gas (approximately 100 psi; 1 psi = 6894.76 Pa) into a graduated cylinder to determine the volume of the stationary phase retained in the column.

2.3. LC–MS analysis

All experiments were performed with a Finnigan LCQ MS System (Thermo Eltron, San Jose, CA, USA) coupled with an HP series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC utilized a Waters Symmetry C_{18}, 5 μm, 150 mm × 3.9 mm HPLC column (Milford, MA, USA), using a linear gradient elution of 0–70% acetonitrile versus 50 mM ammonium formate over 10 min followed by isocratic elution (70% acetonitrile) for an additional 10 min at a fixed flow rate of 0.5 ml/min. The entire column eluent was introduced into the electrospray ionization (ESI) MS source with a standard high flow method. Ion detection was achieved with the Finnigan classic LCQ equipped with ESI in positive ion mode.

The partition coefficient of the precursor and impurities were determined as follows: the sample (about 20 μg) was equilibrated between the two phases (1 ml each phase) in a test tube. Then, 20 μl of each phase was added to 1 ml acetontrile–water (1:1) solution. After spiking, the internal standard (FCWAY 20 μl, 1 μg/ml) into this diluted sample solution, 20 μl of solution was injected into the LC–MS system. MS–MS of molecular ions of impurity 2 (*m*/*z* 643), precursor **1** (*m*/*z* 517), and internal standard FCWAY (*m*/*z* 441) were selectively monitored. The partition coefficient of each compound was determined by comparing the area of corresponding peaks between upper and lower phases.

3. Results and discussion

3.1. Identification of impurities during the one-step radiosynthesis of [^{18}F]FCWAY

An automated one-step radiosynthesis procedure was developed to prepare radiolabeled FCWAY using a converted Nuclear Interface Methylation System [4]. As shown in Fig. 1, the mesylate (**1**) was used as the starting material for this one step labeling procedure. After the fluoride incorporation reaction, the product was purified by preparative HPLC (Beckman Ultrasphere C_{18}, 250 mm × 10 mm eluted with methanol–acetonitrile–30 mM phosphate buffer at pH 8.5, 25:20:55) with an online radioactivity detector. The fraction containing radiolabeled FCWAY and nearby fractions were collected and analyzed by LC–MS. [^{18}F]FCWAY and other proposed structures of unlabeled impurities are shown in Fig. 1. The major impurity which co-eluted with the radiolabeled FCWAY was identified as 4-({2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}(2-pyridinyl)aminocarbony)cyclohexyl 4-hydroxycyclohexanecarboxylate (**3**) by LC–MS. Another impurity which eluted before the labeled FCWAY was identified as 4-({2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}(2-pyridinyl)aminocarbonyl)cyclohexyl 3-cyclohexene-1-carboxylate (**4**). Both of these impurities (**3** and **4**) were formed from 4-({2-[4-(2-

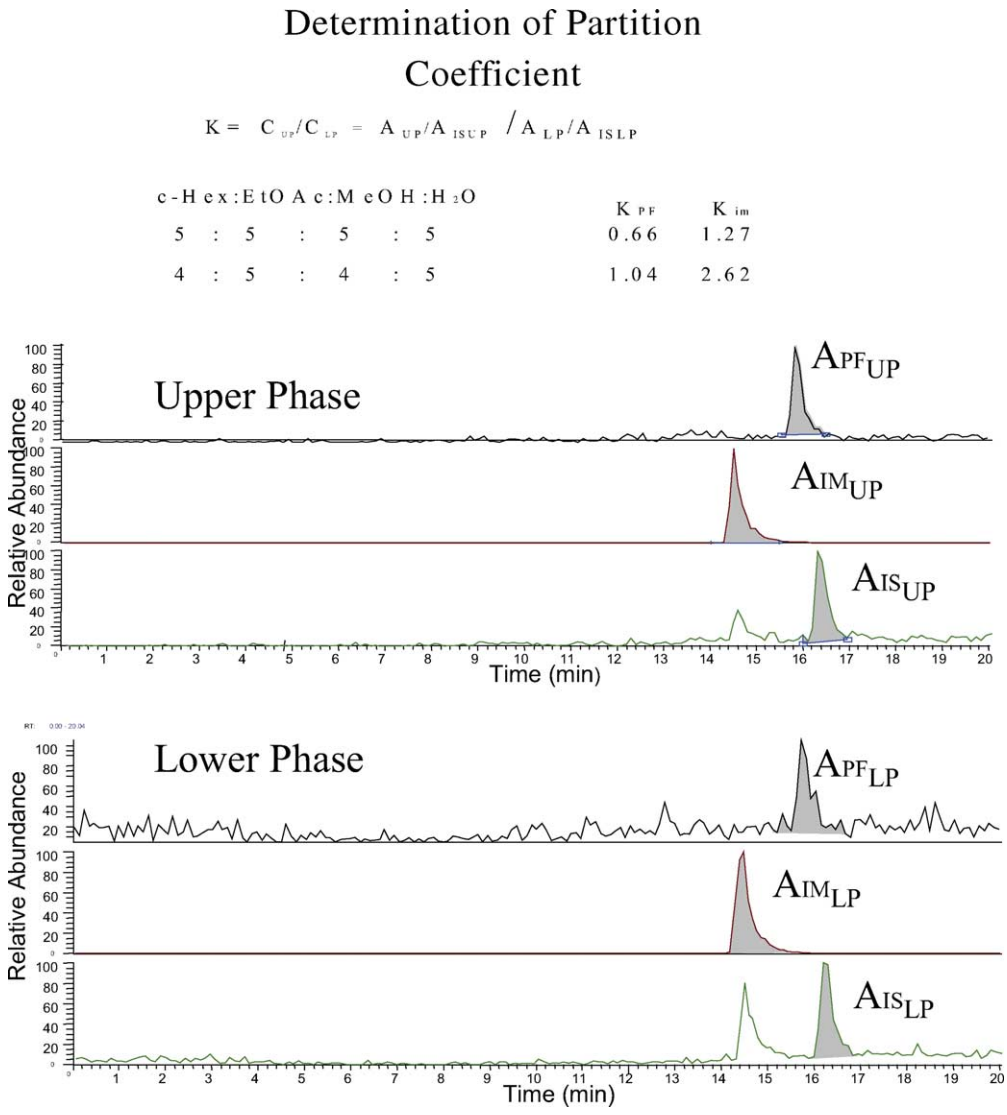

Fig. 2. Determination of partition coefficient by LC–MS. The sample (about 20 μg) was equilibrated between the two phases (1 ml each phase) in a test tube. Then, 20 μl of each phase was added to 1 ml acetontrile–water (1:1) solution. The internal standard (FCWAY 20 μl, 1 μg/ml) was spiked into the diluted sample solution, and 20 μl of solution was injected into the LC–MS system. MS–MS of molecular ions of impurity **2** (*m*/*z* 643), precursor **1** (*m*/*z* 517) and internal standard FCWAY (*m*/*z* 441) were selectively monitored. The partition coefficient of each compound was determined by comparing the area of corresponding peaks between upper and lower phases. A_{PF}: peak area of precursor (**1**); A_{IM}: peak area of impurity (**2**); A_{IS}: peak area of internal standard; UP: upper phase; LP: lower phase; K_{PF}: partition coefficient of precursor (**1**); K_{IM}: partition coefficient of impurity (**2**).

methoxyphenyl)-1-piperazinyl]ethyl}(2-pyridinyl)aminocarbonyl)cyclohexyl 4-[(methylsulfonyl)oxy]cyclohexanecarboxylate (**2**) which was present as a minute impurity in the starting material. The impurity eluted after the FCWAY was identified as 4-({2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl}(2-pyridinyl)aminocarbonyl)cyclohexyl acetate (**5**).

Although impurities **4** and **5** can be eliminated by selectively collecting the fractions by preparative HPLC, impurity **3** overlaps with the FCWAY fraction. Purification of the precursor (**1**) is most desirable since it contains the minor impurity **2** that forms impurities **3** and **4** during the radiosynthesis. In order to purify 3.57 g of this starting material, HSCCC was selected because of its high sample loading capacity [5,6].

3.2. Determination of partition coefficient by LC–MS

Optimization of sample partition coefficient in the two-phase solvent system is the crucial step for successful CCC separation [5–8]. A simple manual procedure of equilibrating the sample between the two phases in a test tube followed by UV absorbance measurement of each phase is applicable only when the pure standard of the target compound is available. HPLC or TLC may be applied for a mixture of compounds by measuring the absorbance of an aliquot of each phase and comparing the peak height (or area under the peak) between the corresponding peaks, provided that peaks are well resolved. In addition to UV and visible wavelengths, more specific parameters such as fluorescence, radioactivity, and bioassay can also be used for determination of partition coefficients [8,9]. However, none of these methods could be applied to the present studies, because the minor impurity (**2**) peak co-eluted with the major precursor (**1**) peak in HPLC and TLC analysis.

This situation prompted us to take an approach to use LC–MS for determination of partition coefficients. This method allows the selective determination of partition coefficients of multiple components without the baseline separation of HPLC peaks. The method works as follows: MS–MS of molecular ions of impurity **2** (*m*/*z* 643) and precursor **1** (*m*/*z* 517) were selectively monitored by LC–MS (Fig. 2). The partition coefficient of each compound was determined by comparing the area of corresponding peaks obtained between the two mass spectra from each phase. In order to reduce the system error, the internal standard of FCWAY was spiked into the diluted sample solution of each phase and injected into the LC–MS system.

Using the above technique, we have selected a suitable two-phase solvent system composed of cyclohexane, ethyl acetate, methanol, and water at two different volume ratios of

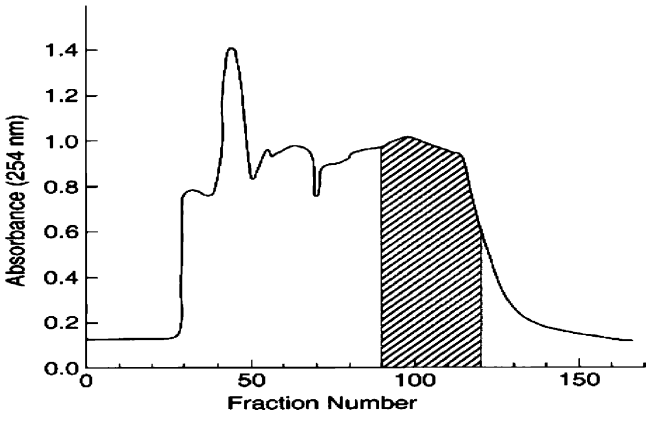

Fig. 3. The separation of 2.57 g of FCWAY precursor by HSCCC. The shade fractions (90–120) were precursor (**1**) labeled on the basis of identification of LC–MS. Experimental conditions are as follows: apparatus, Type-J high-speed CCC centrifuge (Pharma-Tech Research Corp., Baltimore, MD, USA); column, multiplayer coil, 2.6 mm i.d. and 850 ml capacity; revolution, 800 rpm; sample, 2.57 g of FCWAY precursor in 40 ml of solvent used for separation; solvent system, cyclohexane–ethyl acetate–methanol–water (4:5:4:5, v/v); mobile phase, upper organic phase; flow rate, 4 ml/min; detection, 254 nm; retention of the stationary phase, 48.7%.

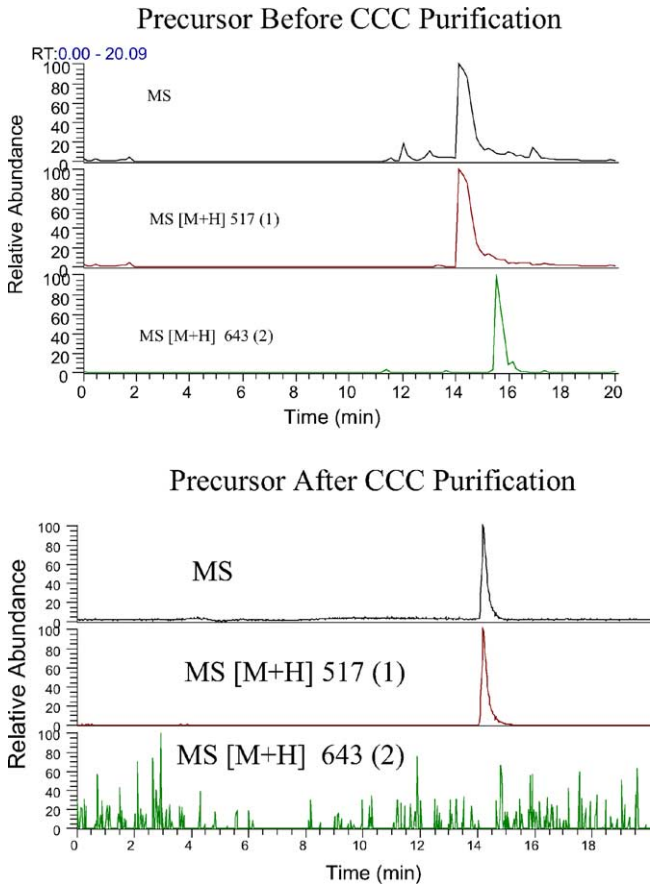

Fig. 4. LC–MS analysis of precursor before and after CCC purification. HPLC utilized a Waters Symmetry C_{18}, 5 μm, 150 mm × 3.9 mm HPLC column using a linear gradient elution of 0–70% acetonitrile vs. 50 mM ammonium formate over 10 min followed by isocratic elution (70% acetonitrile) for an additional 10 min at a fixed flow rate of 0.5 ml/min. The entire column eluent was introduced into the ESI-MS source with a standard high flow method. Ion detection was achieved with the Finnigan classic LCQ equipped with ESI in positive ion mode.

1:1:1:1 and 4:5:4:5. The volume ratio of 1:1:1:1 gave partition coefficients ($K = C_{\text{upper-phase}}/C_{\text{lower-phase}}$) of FCWAY precursor (**1**) and impurity (**2**) of 0.66 and 1.27, while those for the volume ratio of 4:5:4:5 gave partition coefficients of 1.04 and 2.62, respectively.

3.3. HSCCC purification of the precursor

After selecting a suitable two-phase solvent system based on the partition coefficient of the FCWAY precursor (**1**) and the impurity (**2**), a preparative HSCCC column (850 ml capacity) was used for the purification of the precursor.

In the reverse-phase separation using the two-phase solvent system at the 1:1:1:1 volume ratio where the organic phase was used as the stationary phase, a 1 g amount of sample was eluted with the aqueous mobile phase at a flow rate of 4 ml/min by collecting 8 ml fractions. This resulted in elution of precursor (**1**) first in fractions 90–100, where 0.8 g of pure precursor was recovered after extracting with dichloromethane from the pooled aqueous fraction followed by vacuum evaporation of the organic solvent. The retention of the stationary phase was about 31.5%.

In this reverse-phase separation, precursor (**1**) (mesylate) was eluted in the aqueous phase. Because mesylate (**1**) was found to be unstable in the aqueous phase during the vacuum evaporation, a normal-phase separation was designed for the elution of the precursor (**1**) using the upper organic phase as the mobile phase. This second separation was performed with the two-phase solvent system at the volume ratio of 4:5:4:5 which gives partition coefficient values of precursor (**1**) and impurity (**2**) at 1.04 and 2.62, respectively. The column was loaded with 2.57 g of FCWAY precursor sample dissolved in 40 ml of solvent consisting of equal volumes of each phase. The precursor (**1**) was eluted in fractions 90–120 where 2.2 g of pure product was recovered after vacuum evaporation of the organic solvent (Fig. 3). The retention of the stationary phase in this normal-phase elution was about 49%. The purity of the precursor was determined by LC–MS (Fig. 4).

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