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Automation of the radiosynthesis and purification procedures for [¹⁸F]Fluspidine preparation, a new radiotracer for clinical investigations in PET imaging of σ_1 receptors in brain.

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Abstract (80 words)

The radiosynthesis of [¹⁸F]Fluspidine, a potent σ_1 receptor imaging probe for pre-clinical/clinical studies, was implemented on a TRACERlabTM FX F-N synthesizer. [¹⁸F]**2** was synthesized in 15 min at 85 °C starting from its tosylate precursor. Purification *via* semi-preparative RP-HPLC was investigated using different columns and eluent compositions and was most successful on a polar RP phase with acetonitrile/water buffered with NH₄OAc. After solid phase extraction, [¹⁸F]Fluspidine was formulated and produced within 59 ± 4 min with an overall radiochemical yield of 37 ± 8%, a radiochemical purity of 99.3 ± 0.5% and high specific activity (176.6 ± 52.0 GBq/μmol).

Keywords: [¹⁸F]Fluspidine; one-step automated radiosynthesis; positron emission tomography; σ_1 receptors.

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1. Introduction

σ_1 receptors, initially postulated as opioid receptors, are remarkably distinct from all previously characterized mammalian proteins (Kekuda et al., 1996; Martin et al., 1976). These interesting ligand-operated molecular chaperones are widely distributed in peripheral organs such as liver, lung or heart and they are particularly expressed in the central nervous system (Alonso et al., 2000; Kekuda et al., 1996; Kitaichi et al., 2000). As the σ_1 receptors are involved in several neurological diseases such as depression, dementia, schizophrenia, Alzheimer's or Parkinson's diseases (Hayashi et al., 2011; Su et al., 2010; van Waarde et al., 2010), they represent an attractive target for the development of novel diagnostic imaging agents in the brain (Brust et al., 2013). To date, no optimal PET radiotracer for clinical imaging of the σ_1 receptors is available. Although [^{11}C]SA4503 has recently been studied as a σ_1 receptor tracer for use in humans (Ishikawa et al., 2007; Kimura et al., 2007; Sakata et al., 2007; Toyohara et al., 2009) there is still a need to develop more specific ^{18}F -labelled derivatives which would allow broader clinical application by use of the satellite concept. Therefore, we recently developed a series of fluorinated radiotracers with a spiro[benzofuran-piperidine] scaffold (Große Mastrup et al., 2009; Große Mastrup et al., 2011; Große Mastrup et al., 2009; Maisonial et al., 2011). Among them, [^{18}F]Fluspidine demonstrated a high potential for neuroimaging of σ_1 receptors with PET (Scheme 1). Preliminary preclinical studies (Fischer et al., 2011; Maisonial et al., 2012) revealed a high specific brain uptake and metabolic stability in mice. Further detailed preclinical and first clinical studies with this radiotracer are scheduled by our group and require the transfer and adaptation of the production of [^{18}F]Fluspidine to an automated radiosynthesis module (Figure 1). Indeed, automation is an essential feature for routine preparation of ^{18}F -labelled radiopharmaceuticals for human applications, as it ensures reliable radiolabelling yields, optimal radiochemical purities, and the decrease of radiation exposure to medical personnel. As prerequisite for the application of [^{18}F]Fluspidine in humans, we herein report on the development of a cGMP (Current Good Manufacturing Practice)-oriented automated radiosynthesis of this radiotracer. The optimisation of the one-step nucleophilic substitution reaction from the corresponding tosylate precursor **1** as well as investigations of the semi-preparative RP-HPLC purification and subsequent formulation are described.

2. Materials and methods

2.1. General

Solvents and reagents were purchased of highest commercially available quality and applied without further purification from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Co. LLC. (Taufkirchen, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), VWR International GmbH (Darmstadt, Germany) and Fisher Scientific GmbH (Schwerte, Germany).

No-carrier-added [^{18}F]fluoride was produced *via* the [$^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$] nuclear reaction by irradiation of a [^{18}O]water target (> 97%-enriched, 2 mL) on a PETtrace cyclotron (16.5 MeV proton beam, GE Healthcare). Radiosyntheses were performed on a TRACERlabTM FX F-N synthesizer (GE Healthcare, Waukesha, WI, USA) equipped with a S1021 pump (SYKAM Chromatographie Vertriebs GmbH, Fürstenfeldbruck, Germany), WellChrom K-2001 UV detector (KNAUER GmbH, Berlin, Germany), NaI(Tl)-counter and automated data acquisition (NINA software version 4.8 rev. 4, Nuclear Interface GmbH, Dortmund, Germany). Chromafix[®] 30 PS-HCO₃⁻ (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and Sep-Pak[®]Plus C₁₈ cartridges (Waters Corporation, Milford, MA, USA) were used for solid phase extraction. TLC analyses were performed on POLYGRAM[®] SIL G/UV254 plates, 40 × 80 mm (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Processed TLC plates were exposed to ^{18}F -sensitive storage phosphor screens (BAS-TR2025, FUJIFILM Co., Tokyo, Japan), and image plates were analyzed using a bioimaging analyzer system (BAS-1800 II, BASReader 2.26 Beta and AIDA 2.31 Image Analyzer software; Raytest GmbH, Straubenhardt, Germany, and FUJIFILM Co., Tokyo, Japan). Analytical HPLC was carried out on a computer assisted LC-2000*Plus* system from JASCO International Co., Ltd. (including LC-NetII/ADC Netbox, DG-2080-54 4-Line Degasser, LG-2080-04S quaternary gradient unit, PU-2080*Plus* HPLC Pump, AS-2055*Plus* Sampler, UV-2070*Plus* UV/Vis Detector), coupled with a radioactivity-HPLC-flow-monitor (Gabi Star, Raytest GmbH, Straubenhardt, Germany).

2.2. Radiosynthesis of [^{18}F]2

2.6-7.4 GBq (70-200 mCi) of n.c.a. [^{18}F]fluoride in an aqueous solution were trapped on an anion exchange cartridge (Figure 1, Entry 1) on the TRACERlabTM FX F-N synthesis module.

The activity was released from the cartridge with a solution of 1.8 mg potassium carbonate (13 μmol) in a mixture of acetonitrile:water (1:1, v/v, 0.4 mL) and eluted to the reactor, followed by the addition of 11.2 mg of Kryptofix[®] (K₂₂₂, 28 μmol) in 1 mL of acetonitrile (Figure 1, Entries 2-3). The K[¹⁸F]F-K₂₂₂-carbonate complex was formed under a stream of helium at 55 °C for 7 min, followed by the evaporation of the solvents at 85 °C under vacuum for 2 min. The tosylate precursor **1** (1.7-2.5 mg, 3.5-5.2 μmol), available from the group of B. Wünsch on request, in 1 mL of anhydrous acetonitrile (Figure 1, Entry 4) was added to the dried complex at 60 °C, and the mixture was heated under helium atmosphere at 85 °C for 15 min. After cooling the reactor to 35 °C, 3 mL of water were added (Figure 1, Entry 5) and the crude reaction mixture was injected to the semi-preparative HPLC system. During isocratic elution on an RP-column (Figure 1, Entry 6, details see section 2.3.), the [¹⁸F]**2** fraction was collected and diluted with 10 mL of water, placed in the collection vial before the synthesis procedure (Figure 1, Entry 7). The resulting solution was passed through an activated Sep-Pak[®] Plus C₁₈ cartridge (Figure 1, Entry 8). The cartridge was washed with 2 mL of water (Figure 1, Entry 9) and eluted with 2 mL of ethanol (Figure 1, Entry 10) to provide 1.0-2.6 GBq (26-69 mCi) of [¹⁸F]**2**. All radiotracer batches were shown to be identical to the authentic non-radioactive material and to be free of significant chemical and radiochemical impurities by radio-TLC (EtOAc/petroleum ether/NH₃ aq. 25%, 3/7/0.1, v/v/v, R_f = 0.54) and analytical radio-HPLC with the following conditions (Scheme 2): gradient of 55 min with 0-10 min 100% eluent A, 10-40 min up to 100% eluent B, 40-45 min up to 100% eluent A and 45-55 min 100% eluent A; gradient of 80 min with 0-10 min 100% eluent A, 10-60 min up to 100% eluent B, 60-65 min 100% eluent B, 65-70 min up to 100% eluent A and 70-80 min 100% eluent A (Eluent A: 10% MeCN/H₂O 20 mM NH₄OAc_{aq.}, eluent B: 90% MeCN/H₂O 20 mM NH₄OAc_{aq.}, flow rate 1 mL/min, λ = 264 nm; t_{R,55 min} = 36 min, t_{R,80 min} = 45 min; ReproSil-Pur[®] C18-AQ, 5 μm , 250 × 4.6 mm, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The formulation of [¹⁸F]**2** for injections *in vivo* was made by concentration of the ethanol content using evaporation under a stream of argon and subsequent dilution with an isotonic NaCl solution in order to achieve a maximum of 5% of ethanol in the final preparation.

The stability of [¹⁸F]Fluspidine ([¹⁸F]**2**) was controlled by incubation of the formulated solution at 40 °C for 15, 30, 45, 60, 90, and 120 minutes. At each time points samples were analysed by radio-TLC and analytical radio-HPLC, allowing the determination of the percentage of unchanged radiotracer in the solution.

2.3. Semi-preparative HPLC purification of [¹⁸F]2

Semi-preparative HPLC-purification experiments with [¹⁸F]2 were performed on four different RP columns: Multospher[®] 120 RP-18 AQ, 5 μm (CS-Chromatographie Service, Langerwehe, Germany), ReproSil-Pur[®] C18-AQ, 7 μm and ReproSil-Gold[®] 120 C18, 10 μm, (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany), 50 × 10 mm plus 150 × 10 mm; Nucleodur[®] C-18 pyramid, 7 μm, 250 × 10 mm (Macherey-Nagel, Düren, Germany). Three types of eluent mixtures were tested: 56% MeCN/20 mM NH₄OAc_{aq.} (pH ~ 7), 56% EtOH/20 mM NH₄OAc_{aq.} (pH ~ 7), and 35% MeCN/25 mM NaH₂PO_{4aq.} (pH ~ 5). Elution was carried out on isocratic mode with a flow rate of 4-5 mL/min, UV detection at 254 nm and radioactivity detection. The buffer concentrations stated in mM correspond to the concentration in the aqueous component of an eluent mixture.

3. Results and discussion

3.1. Radiosynthetic procedure

[¹⁸F]2 was prepared starting from its corresponding tosylate precursor **1** (Scheme 1) using a TRACERlab[™] FX F-N synthesis module. The synthesizer setup is depicted in Figure 1. Briefly the incorporation of [¹⁸F]fluoride in the precursor **1** was performed in acetonitrile at 85 °C for 15 minutes. Compared to the manual procedure we previously reported (Fischer et al., 2011), higher labelling efficiencies of 83 ± 9% (n = 5) vs. 60-70% were achieved. The reaction time could be reduced from 25 to 15 min. Moreover, these results were obtained using slightly smaller amounts of precursor (1.7-2.5 mg vs. 2.5-3 mg). As a result, the purification process of the crude reaction mixture was simplified, since an intermediate step of a pre-purification on a SPE cartridge to remove most of the non-reacted precursor was not necessary. The crude reaction mixture could be applied directly on the semi-preparative HPLC column, equipped with a small pre-column. Radioactivity balance showed that only 4.3 ± 0.8% (n = 6) of the radioactivity was lost in the reactor after transfer to the semi-preparative HPLC system. To remove the HPLC eluent and render possible the final formulation of [¹⁸F]2, the collected HPLC fraction was diluted with water. The desired radiotracer was then trapped on an equilibrated Sep-Pak[®] Plus C₁₈ cartridge, washed with water and finally eluted using absolute ethanol, instead of formerly used methanol (Fischer et al., 2011). Using these

optimized conditions, only $1.6 \pm 0.7\%$ ($n = 7$) of the total radioactivity was lost on the cartridge during the SPE procedure.

With this entire radiosynthesis process [^{18}F]Fluspidine was produced within 59 ± 4 min ($n = 8$) with a radiochemical purity of $99.4 \pm 0.5\%$ ($n = 11$, analytical HPLC chromatogram see figure 2), a decay corrected radiochemical yield of $37 \pm 8\%$ ($n = 9$) and a specific activity of 176.6 ± 52.0 GBq/ μmol ($n = 5$, determination *via* analytical RP-HPLC using UV/mass calibration). [^{18}F]2 also showed good radioactive stability in the final solution for injections *in vivo* ($> 97.1\%$) even 2 h at 40 °C after production. Altogether, a basis could be established for the design of a procedure for GMP compliant production of [^{18}F]Fluspidine.

3.2. Semi-preparative HPLC purification

As reported previously (Fischer et al., 2011), the purification of [^{18}F]2 succeeded with a Multospher[®] RP18-AQ column using a neutral buffered, aqueous acetonitrile eluent. Thereby (i) the two appearing radioactive species were identified as radioactive fluoride (Figure 3, $t_{\text{R}} \sim 3$ min) and [^{18}F]2 ($t_{\text{R}} = 22.8$ min), and (ii) [^{18}F]2 could be separated from the UV active side products (t_{R} around 11, 14.6 and 17.7 min). Based on this achievement, we investigated the [^{18}F]2 separation by use of other stationary phases. Therewith we aimed to (i) shorten the retention of this short-lived radioligand, (ii) reduce the peak tailing resulting in a reduction of the volume collected, and (iii) further optimize the separation between UV active side products (especially the one with $t_{\text{R}} \sim 21$ min) and desired radiolabeled product.

The Multospher[®] RP18-AQ column is a RP phase with polar as well as hydrophobic properties, because hydroxyethyl and C18 groups are bound to the silica. Since this mixed characteristic seems to be appropriate for the separation of our crude reaction mixture, we selected two further columns with similar properties. The ReproSil-Pur[®] C18-AQ and the Nucleodur C18 Pyramid[®] also provide hydroxyethyl and C18 spacers, but differ regarding the silica support. With the ReproSil-Gold[®] column, we tested a strong hydrophobic phase which is base-deactivated by double bound end-capping resulting in 20% carbon content compared to 14-15% for the AQ phases. In particular regarding the peak shape this phase was promising since 2, as a basic compound due to its tertiary amine, can strongly interact with free silanol groups under neutral or basic conditions (Neue et al., 2001; Wenzel et al., 2010). Furthermore, we investigated two different buffer systems: aqueous ammonium acetate (pH

6.8) and aqueous sodium dihydrogen phosphate (pH ~ 5). With the aim to lower the risk of remaining acetonitrile in product formulations (allowed percentage according to United States Pharmacopoeia: 0.04% (Hung, 2002)) we tested ethanol as organic modifier, which would be more appropriate regarding a future clinical application.

With the ReproSil-Pur[®] C18-AQ phase similar properties and elution profiles as for the Multospher[®] RP18-AQ phase were observed. However, using 56% MeCN/20 mM NH₄OAc_{aq.} the retention time of [¹⁸F]2 was somewhat reduced and accompanied by a clear and advantageous separation from the UV active by-products (Figure 4).

Additionally, peak tailing observed with Multospher[®] RP18-AQ could be decreased significantly and resulted in a reduction of the collected volume (3 mL compared to formerly 6-7 mL). According to our previous experiences using the ReproSil-Pur[®] C18-AQ column, all investigated combinations of mobile phases exhibited separations with good peak shape properties. However, regarding the separation of [¹⁸F]2 from its UV active by-products, the MeCN/20 mM NH₄OAc_{aq.} eluent system was more suitable than EtOH/20 mM NH₄OAc_{aq.} and MeCN/25 mM NaH₂PO_{4aq.}. With ethanol as organic modifier, the back pressure of this column was observed to be in a tolerable range of 70-75 bar. Comparing the two different buffer systems, we observed a decreased retention of [¹⁸F]2 when the slightly acidic NaH₂PO₄ was used. Therefore the content of MeCN in the eluent mixture was reduced to achieve a separation from the UV by-products. This trend was observed for all columns we tested and might be caused by the slightly lower pH value or by the different counter ions used (Na⁺ vs. NH₄⁺).

Using the Nucleodur C18 Pyramid[®] column and acetonitrile as organic modifier, [¹⁸F]2 could be also separated within 20 min and a good peak shape was observed. However, a single UV by-product was eluted shortly in front of the radiotracer, which limits the quality of this separation system (Figure 5). In contrast to the ReproSil-Pur[®] C18-AQ column, a high back pressure was observed with ethanolic eluents and the flow rate had to be reduced (1-2 mL/min). This resulted in a strong increase of retention times, unsuitable for the separation of a ¹⁸F-labelled radiotracer.

Further, the strong hydrophobic ReproSil-Gold[®] C18 phase was investigated, offering lowest silanol activity and high pH-stability. Using acetonitrile as organic modifier, [¹⁸F]2 could be separated from the UV by-products within 20 min. Interestingly, independent of the buffer system used for the purification a considerable peak tailing was observed when using MeCN as organic modifier, which could be suppressed with ethanol. However, as depicted in Figure

6 the separation with ethanol was not appropriate due to the almost co-elution of a single UV by-product.

Summing up, a feasible separation of [¹⁸F]2 could be achieved on combinations out of a serial of four different commercially available stationary RP and three mobile phases. Thereof, to our opinion the most suitable system consisted of the ReproSil-Pur[®] C18-AQ phase and ammonium acetate buffered acetonitrile.

4. Conclusion

The radiosynthesis and the purification of [¹⁸F]2 was successfully transferred onto an automated synthesis module and is routinely used in our laboratory for pre-clinical studies using this radiotracer. This rapid and versatile radiosynthetic approach will enhance the accessibility of [¹⁸F]Fluspidine, a potent novel ligand for PET imaging of σ_1 receptors in the brain, for widespread production in future clinical studies. The development of the corresponding fully cGMP compliant process is currently under investigation.

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Figure Captions

Figure 1. Diagram of the configuration of the TRACERlabTM FX F-N automate for the preparation of [¹⁸F]Fluspidine: ❶ Chromafix[®] 30 PS-HCO₃⁻ cartridge; ❷ 1.8 mg of K₂CO₃ in 400 μL MeCN/H₂O, 1/1, v/v; ❸ 11.2 mg of K₂₂₂ in 1 mL MeCN; ❹ 1.7 – 2.5 mg precursor in 1 mL MeCN; ❺ 3 mL H₂O; ❻ semi-preparative RP-HPLC column, mobile phase: organic solvent/ aqueous buffer, 4 mL/min, λ = 254 nm; ❼ 10 mL H₂O; ❽ Sep-Pak[®]Plus C₁₈ cartridge; ❾ 2 mL H₂O; ❿ 2 mL EtOH.

Figure 2. Analytical HPLC chromatogram of [^{18}F]2 (radioactivity detection), $t_{\text{R}} = 33.3$ min, on ReproSil-Pur[®] C18-AQ, gradient of 10 to 90% MeCN/H₂O, 20 mM NH₄OAc_{aq.} over 55 min, 1 mL/min.

Figure 3. Semi-preparative HPLC chromatogram of [^{18}F]2, $t_{\text{R}} = 22.8$ min, on Multospher[®] 120 RP-18 AQ, 56% MeCN/20 mM NH₄OAc_{aq.}, 4 mL/min.

Figure 4. Semi-preparative HPLC chromatogram of [^{18}F]2, $t_{\text{R}} = 20.7$ min, on ReproSil-Pur[®] C18-AQ, 56% MeCN/20 mM NH₄OAc_{aq.}, 4 mL/min.

Figure 5. Semi-preparative HPLC chromatogram of [^{18}F]2, $t_{\text{R}} = 16.7$ min, on Nucleodur C18 Pyramid[®], 35% MeCN/25 mM NaH₂PO_{4aq.}, 5 mL/min.

Figure 6. Semi-preparative HPLC chromatogram of [^{18}F]2, $t_{\text{R}} = 16.5$ min, on ReproSil-Gold C18, 56% EtOH/20 mM NH₄OAc_{aq.}, 4 mL/min.

Scheme Captions

Scheme 1. Radiosynthesis of racemic [^{18}F]2, starting from its tosylate precursor 1.

Scheme 2. Gradients of 55 and 80 min for elution on analytical HPLC. Eluent A: 10% MeCN/H₂O 20 mM NH₄OAc aq., eluent B: 90% MeCN/H₂O 20 mM NH₄OAc aq.