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A facile automated synthesis of *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) for ^{18}F-labeled cell-penetrating peptide as PET tracer

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A fully automated synthesis of *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) was carried out by a convenient three-step, one-pot procedure on the modified TRACERlab FX$_{FN}$ synthesizer, including [^{18}F]fluorination of ethyl 4-(trimethylammonium triflate)benzoate as the precursor, saponification of the ethyl 4-[^{18}F]fluorobenzoate with aqueous tetrapropylammonium hydroxide instead of sodium hydroxide, and conversion of 4-[^{18}F]fluorobenzoate salt ([^{18}F]FBA) to [^{18}F]SFB treated with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU). The purified [^{18}F]SFB was used for the labeling of Tat membrane-penetrating peptide (containing the Arg-Lys-Lys-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Pro-Leu-Gly-Leu-Ala-Gly-Glu-Glu-Glu-Glu-Glu-Glu-Glu sequence, [^{18}F]CPP) through radiofluorination of lysine amino groups. The uncorrected radiochemical yields of [^{18}F]SFB were as high as 25–35% (based on [^{18}F]fluoride) ($n = 10$) with a synthesis time of ~40 min. [^{18}F]CPP was produced in an uncorrected radiochemical yields of 10–20% ($n = 5$) within 30 min (based on [^{18}F]SFB). The radiochemical purities of [^{18}F]SFB and [^{18}F]CPP were greater than 95%.

Keywords: [^{18}F]SFB; [^{18}F]CPP; automated synthesis; one-pot; isotopic labeling

Introduction

Cell-penetrating peptides (CPPs), also known as protein transduction domains or membrane transduction peptides, or membrane-penetrating peptides (MPP), are of interest due to their ability to translocate across cellular membranes. CPPs, generally categorized as amphipathic or cationic depending on their sequence containing clusters of primarily arginine and also lysine residues, are increasingly drawing attention as a non-invasive delivery technology for macromolecules. Delivery of a diverse set of cargo in terms of size and nature ranging from small molecules, peptides, proteins, and oligonucleotides to nanoparticles and liposomes particulate cargo has been attempted using different types of CPPs both *in vitro* and *in vivo*. However, the internalization mechanism of CPPs is an unresolved issue to date. A key reason for the lack of consensus on the mechanism can be attributed to the methodology in deciphering the internalization mechanism.[1] Therefore, it is very important for us to investigate the internalization mechanism of CPPs with positron emission tomography (PET) imaging.

The use of biomolecules, such as peptides, proteins, antibodies, and oligonucleotides labeled with positron-emitting radionuclides, as probes to image physiologic and pathologic processes will potentially be a significant means to rapidly translate genomic and proteomic information into man.[2–4] The incorporation of ^{18}F into peptides, proteins, antibodies, and oligonucleotides is a challenge to us and usually requires the use of prosthetic groups, also referred to as bifunctional labeling agents. Currently, many ^{18}F-labeled prosthetic groups[5–7] have been developed, which can be attached to biomolecules via acylation, amidation, imidation, alkylation, photochemical conjugation, and solid-phase synthesis. However, the acylation approach with *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) is undoubtedly the most versatile ^{18}F-labeling method with respect to the *in vivo* stability and good radiochemical yield of [^{18}F]SFB.[4,8]

[^{18}F]SFB synthesis generally requires a laborious three-step procedure. In the past few years, a number of modifications, including reduced synthesis time, enhanced activation step, minimal purification steps, and improved radiochemical yields, have been made to accelerate the automated production of [^{18}F]SFB for routine clinical PET imaging.[4] Recently, in an effort to develop a simpler and more efficient approach for the routine use of [^{18}F]SFB, a fully automated preparation of [^{18}F]SFB on modified commercial modules has been described.[2,3,9] Nonetheless, every method has its own advantages and limitations, and further improvements of the [^{18}F]SFB synthesis are desirable.

We have developed an efficient preparation of [^{18}F]SFB based on a convenient three-step, one-pot procedure, consisting of [^{18}F]fluorination of the precursor ethyl 4-(trimethylammonium triflate)benzoate hydrolysis to give 4-[^{18}F]fluorobenzoate salt

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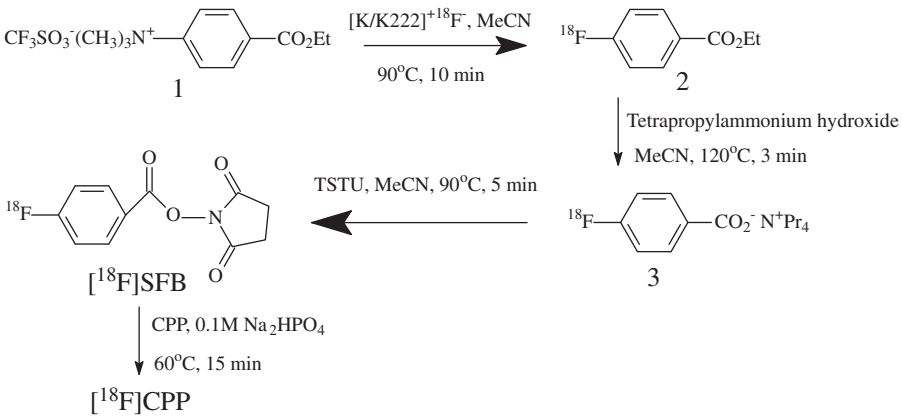
([^{18}F]FBA) with tetrapropylammonium hydroxide and activation of [^{18}F]FBA to [^{18}F]SFB in a single reaction vessel to further reduce the total synthesis time.[4] In this work, the three-step, one-pot procedure adapted to a modified automated controlled synthesis module (TRACERlab FX_{FN} synthesizer) from GE Medical Systems and ^{18}F-labeled Tat Cell-penetrating peptide ([^{18}F]CPP) are described.

Results and discussion

Radiosynthesis of [^{18}F]SFB and [^{18}F]CPP

In this work, [^{18}F]SFB was synthesized from ethyl 4-(trimethylammonium triflate)benzoate (**1**) as the precursor via three-step reactions as shown in Scheme 1. Nucleophilic substitution of compound (**1**) in MeCN with [^{18}F]$^-$ gave ethyl 4-[^{18}F]fluorobenzoate (**2**), which was used for the next hydrolysis reaction without further purification. The ester moiety (**2**) was hydrolyzed using 25% tetrapropylammonium hydroxide and the mixture was azeotropically dried to form the [^{18}F]FBA salt (**3**) with the addition of anhydrous MeCN under a stream of nitrogen, which was used for the next step without further purification. Reaction of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU) with [^{18}F]FBA salt using SEP-PAK purification gave [^{18}F]SFB. The fully automated synthesis of [^{18}F]SFB was performed using the Tracerlab $FX_{F\text{-}N}$ synthesizer via three-step, one-pot procedure (Figure 1). The [^{18}F]SFB was synthesized in an uncorrected yield 25–35%, with a synthesis time of ~40 min.

Several procedures are reported for the synthesis of [^{18}F]SFB.[2–4,8,9] However, the three-step, two-pot procedure,[2,3,8,9] including [^{18}F]fluorination of the precursor ethyl 4-(trimethylammonium triflate)benzoate, hydrolysis with sodium hydroxide to give [^{18}F]FBA and activation of [^{18}F]FBA to [^{18}F]SFB in the two reaction vessels, is the most widely used for the production of [^{18}F]SFB. We produced [^{18}F]SFB via the three-step, two-pot procedure with the SEP-PAK purification using the Tracerlab $FX_{F\text{-}N}$ synthesizer, in an uncorrected yield of 25–28% within the whole synthesis time of 85 min. Currently, Glaser *et al.*[10,11] reported a new two-step radiosynthesis of [^{18}F]SFB. Using this approach, HPLC purification furnished [^{18}F]SFB in a decay-corrected yield of 32.3% and a radiochemical purity of >99% within the total synthesis time of 2.75 h (165 min); whereas solid-phase purification gave [^{18}F]SFB in a decay-corrected yield of 50.8% with a radiochemical purity of >89% within the total



Scheme 1. Synthetic route to [^{18}F]SFB and [^{18}F]CPP.

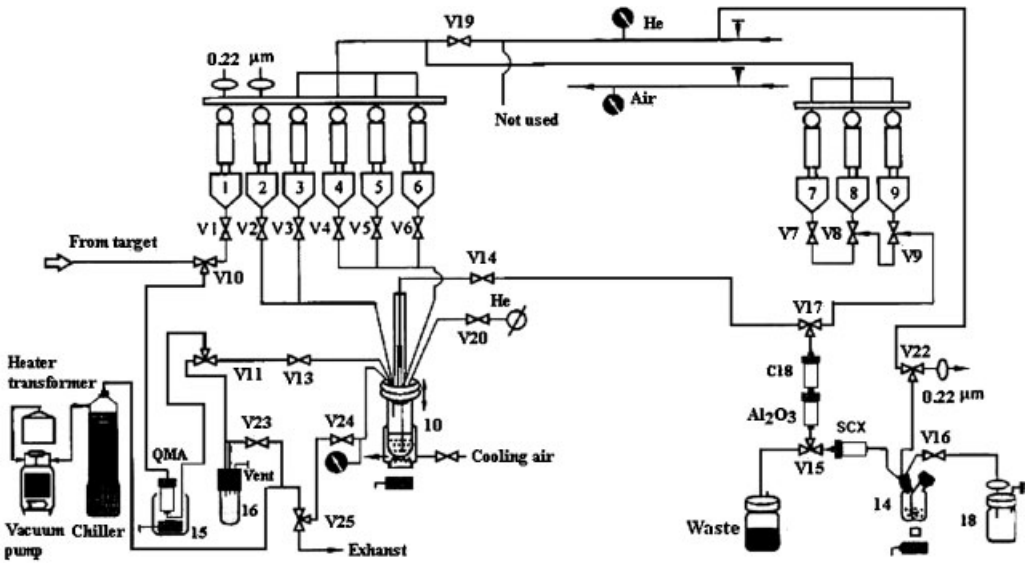


Figure 1. Schematic diagram of the automated synthesis of [^{18}F]SFB using SEP-PAK purification.

synthesis time of 1.67 h (100 min). Although this new method gave a good radiochemical yield, it is difficult to perform the automated synthesis of [^{18}F]SFB due to microwave heating and the long total radiosynthesis time. In addition, a number of papers dealing with [^{18}F]SFB synthesis were presented at the 18th International Symposium on Radiopharmaceutical Sciences(ISRS18), Edmonton, Canada, 12–17 July 2009. However, the reported [^{18}F]SFB syntheses have limitations. For example, automated microfluidic production of [^{18}F]SFB in a three-step reaction[12] only provided low radioactivity; one-pot radiosynthesis of [^{18}F]SFB via potassium tert.-butoxide hydrolysis and SPE cartridge purification[13] took a long total synthesis time of about 2 h for biomolecule labeling; one-pot radiosynthesis of [^{18}F]SFB via acidic hydrolysis and HPLC purification[14] made the automated synthesis difficult due to the necessary HPLC purification; three-step, two-pot synthesis via acidic hydrolysis[15] gave low radiochemical purity(85%); and one-step synthesis gave a low radiochemical yield 13–23% (The 17th annual symposium of the International Isotope Society's United Kingdom Group, Cambridge, UK, 9 October 2008).[16]

Compared with the three-step, two-pot procedure, the one-pot method in our work provided a simple procedure for the automated production of [^{18}F]SFB within a shorter synthesis time.[17] On the one hand, after [^{18}F]fluorination of the precursor ammonium triflate (**1**) and hydrolysis of compound (**2**) with tetrapropylammonium hydroxide instead of NaOH solution, the corresponding reaction mixture was directly used for the next step reaction without further purification. On the other hand, after the hydrolysis mixture was azeotropically dried to form [^{18}F]FBA salt with the addition of anhydrous MeCN under a stream of nitrogen. Activation of [^{18}F]FBA salt using TSTU and purification using SEP-PEK cartridges yielded the purified [^{18}F]SFB within the short synthesis time.[4] The automated synthesis of [^{18}F]SFB via the one-pot approach using tetrapropylammonium hydroxide in place of NaOH hydrolysis had advantages of one-step purification, short synthesis time, and simple operation process. Therefore, the one-pot procedure was fully adapted to automated radiosynthesis using the current commercially automated synthesis modules.

Semi-automated synthesis of [^{18}F]CPP was performed from [^{18}F]SFB as shown in Scheme 1. Typical radio-HPLC chromatograms of the reaction mixture of [^{18}F]SFB with CPP and typical UV HPLC chromatograms of the reaction mixture of [^{19}F]SFB with CPP are shown in Figure 2. In UV chromatograms, retention time (Rt) for CPP, [^{19}F]CPP-2, [^{19}F]CPP-1, [^{19}F]FBA, and [^{19}F]SFB was 1.8, 2.4–2.6, 2.6–2.8, 4.0–4.4, and 6.3–6.5 min, respectively. In radioactivity chromatograms, Rt for $^{18}F^{-}$, [^{18}F]CPP-2, [^{18}F]CPP-1, [^{18}F]FBA, and [^{18}F]SFB was 1.8–2.0, 2.6–2.8, 2.8–3.2, 4.0–4.3, and 6.5–6.7 min, respectively. As there were two lysine residues at adjacent position in CPP, we couldn't differentiate between [^{18}F]CPP-1 and [^{18}F]CPP-2 in [^{18}F]CPP. Uncorrected labeling yields of 10–20% for [^{18}F]CPP-1 ($n = 5$) and 1–3% for [^{18}F]CPP-2 ($n = 5$) were obtained from [^{18}F]SFB using HPLC purification after 30 min of the reaction time. [^{18}F]CPP-1 was used for the next experiments. There was no significant difference in the yields between the one-pot procedure and the two-pot procedure in our work. Adding a small amount of acetonitrile to dissolve the [^{18}F]SFB prior to the addition of CPP improved the peptide-labeling yield (15–20% vs 10–15%). Also, CPP labeling reaction with [^{18}F]SFB in 0.1M borate buffer (pH 8.5) reduced the yield of [^{18}F]FBA and improved the peptide-labeling yield (30–40% vs 15–20%).

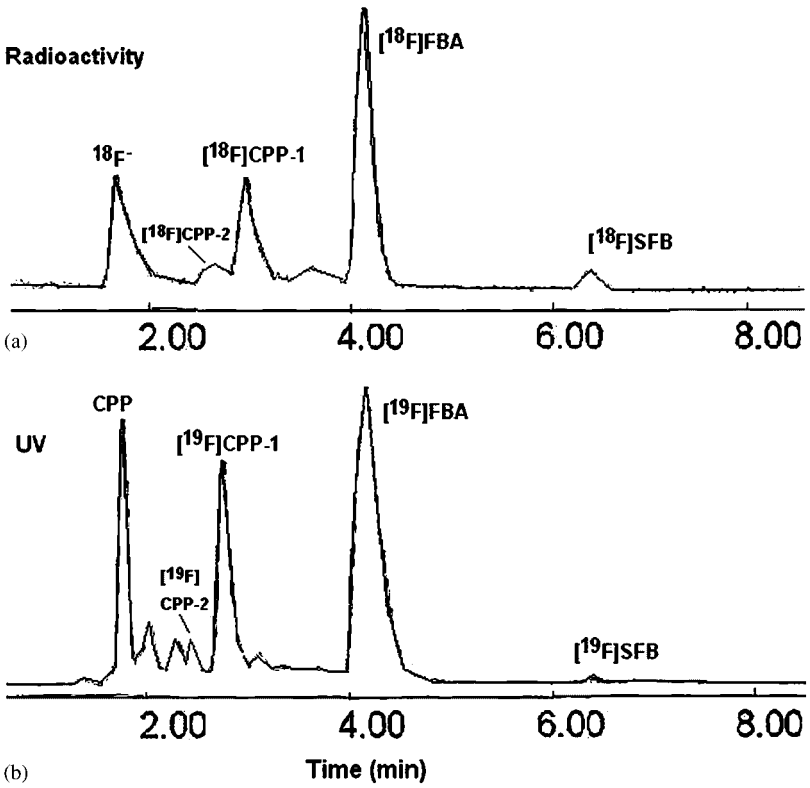


Figure 2. Typical HPLC chromatograms of the reaction mixture of [^{18}F]SFB with CPP (a) and [^{19}F]SFB with CPP (b).

Quality control of [^{18}F]SFB and [^{18}F]CPP

The identity of [^{18}F]SFB and [^{18}F]CPP was confirmed by comparison of the chromatograms with unlabeled reference compounds. The radiochemical purity of [^{18}F]SFB produced by three-step, one-pot approach was above 99%, confirmed by using radio-TLC and radio-HPLC. We did not find any radiochemical and chemical impurities in the [^{18}F]SFB solution by using the analytical radio-HPLC. The typical HPLC chromatograms of the final purified [^{18}F]SFB solution are shown in Figure 3 (Rt was 6.5–6.7 min for [^{18}F]SFB and Rt was 4.2–4.5 min for [^{18}F]FBA). Color spot test for K_{222} by using TLC also showed no detection of K_{222} in final [^{18}F]SFB solution. [^{18}F]SFB in MeCN and [^{18}F]CPP ([^{18}F]CPP-1) in Na_2HPO_4 solution showed good stability with over 95% radiochemical purities at 6 h after synthesis. The radiochemical purity of [^{18}F]CPP-1 (Rt = 2.4 min) was above 99% for all preparations, confirmed by using TLC and HPLC.

Material and methods

General

Ethyl 4-(trimethylammonium triflate)benzoate was synthesized in our laboratory. All other reagents used in the synthesis were commercial products and were used without further purification unless otherwise indicated. SEP-PAK light QMA cartridges, SEP-PAK plus C18 cartridges, and SEP-PAK light alumina cartridges were obtained from Waters (Milford, MA). Thin layer chromatography (TLC) was carried out using precoated aluminum-backed silica gel 60 F254 TLC plates (E. Merck Company, Darmatadt, Germany) to verify the product purities. For [^{18}F]SFB or [^{18}F]CPP purification, HPLC separation was carried out at the TRACERlab FX_{F-N} synthesis module built-in HPLC system with a semi-preparative reverse-phase C18 column (10 mm × 250 mm) and C18 precolumn equipped with a UV detector and a radioactivity detector. For the quality control, HPLC analysis was carried out on a modular HPLC system with a reverse-phase analytical C18 column (4.6 mm × 150 mm; Shimadzu Corporation of Japan), consisting of two LC-10ATvp pump (Shimadzu Corporation of Japan) and a variable wavelength SPD-10ATvp UV detector (Shimadzu Corporation of Japan), a LB 508 Radioflow Detector with two channels analyzer (EG &G, Germany), and a computer (Japan). The UV signal was monitored with a UV detector at 254 nm.

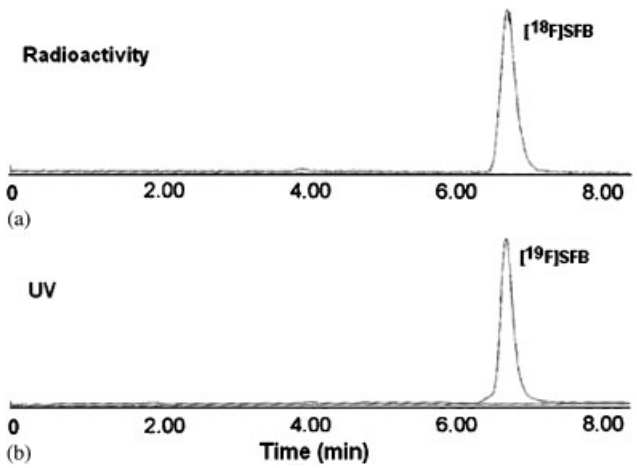


Figure 3. HPLC chromatograms of [^{18}F]SFB (a) and standard [^{19}F]SFB (b). The peaks at a retention time (Rt) of 6.5–6.7 min were standard [^{19}F]SFB and [^{18}F]SFB, respectively.

Automated synthesis of [^{18}F]SFB

The fully automated synthesis of [^{18}F]SFB was carried out on the TRACERlab FX_{F-N} synthesizer via a simplified three-step, one-pot procedure as shown in Figure 1. Before delivery of [^{18}F]fluoride to the synthesizer, Vial 1 was filled with a mixture of 15 mg of Kryptofix 2.2.2 (K_{222}), 3 mg of K_2CO_3, 1 ml of acetonitrile, and 0.5 ml of water; Vial 2 was filled with tetrapropylammonium hydroxide (25% in water, 20 μl) and acetonitrile (MeCN, 2 ml); Vial 3 was added with ethyl 4-(trimethylammonium triflate)-benzoate (**1**) (5 mg) dissolved in anhydrous MeCN (1 ml); Vial 4 was added with 9 ml of 5% acetate acid; Vial 5 was filled with a solution of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU; 12 mg) in anhydrous MeCN (1 ml); Vial 6 was added with 15 ml of water; Vial 7 was added with 15 ml of 10% MeCN in water; and Vial 9 was added with 2 ml of anhydrous MeCN.

No-carrier-added [^{18}F]fluoride was obtained through the nuclear reaction $^{18}O(p, n)^{18}F$ by irradiation of more than 95% [^{18}O]-enriched water target with a 16.5-MeV proton beam on the PETtrace cyclotron (GEMS). After the delivery of [^{18}F]fluoride from the cyclotron, the radioactivity passed through a QMA SEP-PAK cartridge, where [^{18}F]fluoride was trapped and [^{18}O]water was collected for recycling. The trapped $^{18}F^-$ was eluted off the SEP-PAK QMA cartridge into the reaction vessel with a solution of K222 (1.5 ml). The solvent was evaporated under a stream of helium and vaccum at 60–95°C for 4 min to form $[K/K_{222}]^{+18}F^-$ complex. Ethyl 4-(trimethylammonium triflate)benzoate (**1**) in anhydrous MeCN (Vial 3) was added to the above dried $[K/K222]^{+18}F^-$ complex. The reaction mixture was heated at 90°C for 10 min to produce ethyl 4-[^{18}F]fluorobenzoate (**2**). The ethyl ester was subsequently hydrolyzed to form the 4-[^{18}F]fluorobenzoic acid salt ([^{18}F]FBA salt, **3**) using 20 μl of tetrapropylammonium hydroxide in water and 2 ml of MeCN (Vial 2) at 120°C for 1 min, and then the mixture was azeotropically dried under a stream of helium and vacuum at 90°C for 3 min. Subsequently, a solution of TSTU in MeCN (Vial 5) was added and the solution was heated at 90°C for 5 min. After cooling, 5% acetic acid (Vial 4) and water (Vial 6) were added respectively, and the reaction mixture was passed through a SEP-PAK plus C18 cartridge and a SEP-PAK light alumina cartridge connected in series. Finally, after the cartridges was washed with 10% MeCN (Vial 7), the product [^{18}F]SFB was eluted from the cartridges with MeCN (Vial 9) and further passed through a Lichrolut SCX cartridge into a sterile vial (Vial 18). The solvent was then removed by a stream of nitrogen at 60°C using a remote control system.

Labeling CPP with [^{18}F]SFB

Tat MPP (containing Arg-Lys-Lys-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Pro-Leu-Gly-Leu-Ala-Gly-Glu-Glu-Glu-Glu-Glu-Glu-Glu sequence, CPP, 0.5 mg in 1 ml of 0.1M Na_2HPO_4), was added to the dried [^{18}F]SFB residue, and the mixture was allowed to react at 60°C for 15 min. At the end of reaction, the mixture was purified by using HPLC with a semi-preparative reverse-phase C18 column (10 mm × 250 mm) and C18 precolumn eluted with 0.01 M H_3PO_4/CH_3CN (60/40, v/v) at a flow rate of 5 ml/min, equipped with a UV (254 nm) detector and a radioactivity detector. The fraction containing [^{18}F]CPP was collected and evaporated to dryness. To the residue, 5 ml of 0.1 M Na_2HPO_4 solution was added and the resulting solution was filtered

through a 0.22-μm cellulose acetate membrane filter (Millipore) into a final product vial.

Purity determination of [^{18}F]SFB and [^{18}F]CPP

The aforementioned analytical HPLC on a C18 column was used for checking the radiochemical purity of [^{18}F]SFB and [^{18}F]CPP at a flow rate of 1 ml/min eluted with 0.01 M H_3PO_4/CH_3CN (60/40, v/v) as mobile phase. A radio-TLC (MeCN/H_2O, 90/10, v/v) was also used for checking the radiochemical purity. K_{222} detection test was performed on the silica gel 60-coated plate developed with methanol/ammonium hydroxide (9/1, v/v) as a solvent system and iodine vapor was used for staining the spots to render them visible.[18] Radiochemical stability of 5 ml solution of [^{18}F]SFB or [^{18}F]CPP injection was checked using a radio-TLC and an analytical HPLC up to 6 h.

Conclusions

A fully automated synthesis of [^{18}F]SFB, one of the most versatile ^{18}F-labeling agents used for labeling biomarkers, has been developed by the three-step, one-pot procedure using tetrapropylammonium hydroxide instead of sodium hydroxide hydrolysis to reduce the total synthesis time on a TRACERlab FX_{F-N} synthesizer. This approach could give a high uncorrected radiochemical yield of [^{18}F]SFB and good radiochemical purity within the short total synthesis time (about 40 min). The new one-pot procedure should be adaptable to the fully automated synthesis of [^{18}F]SFB using a current commercial modified automated synthesis module. [^{18}F]CPP was successfully produced from [^{18}F]SFB for further study.

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