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TITLE:

Solid Phase ^{11}C-Methylation, Purification and Formulation for the Production of PET Tracers

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carbon-11, radiolabeling, positron emission tomography, imaging, [^{11}C]PiB, [^{11}C]ABP688, ^{11}C-methylation, solid phase supported synthesis, solid phase extraction, automation

SUMMARY:

We report an efficient carbon-11 radiolabeling technique to produce clinically relevant tracers for Positron Emission Tomography (PET) using solid phase extraction cartridges. ^{11}C-methylating agent is passed through a cartridge preloaded with precursor and successive elution with aqueous ethanol provides chemically and radiochemically pure PET tracers in high radiochemical yields.

ABSTRACT:

Routine production of radiotracers used in positron emission tomography (PET) mostly relies on wet chemistry where the radioactive synthon reacts with a non-radioactive precursor in solution. This approach necessitates purification of the tracer by high performance liquid chromatography (HPLC) followed by reformulation in a biocompatible solvent for human administration. We recently developed a novel ^{11}C-methylation approach for the highly efficient synthesis of carbon-11 labeled PET radiopharmaceuticals, taking advantage of solid phase cartridges as disposable "3-in-1" units for the synthesis, purification and reformulation of the tracers. This approach obviates the use of preparative HPLC and reduces the losses of the tracer in transfer lines and due to radioactive decay. Furthermore, the cartridge-based technique improves synthesis reliability, simplifies the automation process and facilitates compliance with the Good Manufacturing Practice (GMP) requirements. Here, we demonstrate this technique on the

example of production of a PET tracer Pittsburgh compound B ([^{11}C]PiB), a gold standard in vivo imaging agent for amyloid plaques in the human brains.

INTRODUCTION:

Positron emission tomography (PET) is a molecular imaging modality which relies on detecting the radioactive decay of an isotope attached to a biologically active molecule to enable the in vivo visualization of biochemical processes, signals and transformations. Carbon-11 ($t_{1/2}$ = 20.3 min) is one of the most commonly used radioisotopes in PET because of its abundance in organic molecules and short half-life which allows for multiple tracer administrations on the same day to the same human or animal subject and reduces the radiation burden on the patients. Many tracers labeled with this isotope are used in clinical studies and in basic health research for in vivo PET imaging of classical and emerging biologically relevant targets – [^{11}C]raclopride for D_2/D_3 receptors, [^{11}C]PiB for amyloid plaques, [^{11}C]PBR28 for translocator protein – to name just a few.

Carbon-11 labeled PET tracers are predominantly produced *via* ^{11}C-methylation of non-radioactive precursors containing –OH (alcohol, phenol and carboxylic acid), –NH (amine and amide) or –SH (thiol) groups. Briefly, the isotope is generated in the gas target of a cyclotron *via* a ^{14}N(p,α)^{11}C nuclear reaction in the chemical form of [^{11}C]CO_2. The latter is then converted into [^{11}C]methyl iodide ([^{11}C]CH_3I) *via* either wet chemistry (reduction to [^{11}C]CH_3OH with $LiAlH_4$ followed by quenching with HI)[1] or dry chemistry (catalytic reduction to [^{11}C]CH_4 followed by radical iodination with molecular I_2)[2]. [^{11}C]CH_3I can then be further converted to the more reactive ^{11}C-methyl triflate ([^{11}C]CH_3OTf) by passing it over a silver triflate column[3]. The ^{11}C-methylation is then performed by either bubbling the radioactive gas into a solution of non-radioactive precursor in organic solvent or *via* the more elegant captive solvent "loop" method[4,5]. The ^{11}C-tracer is then purified by means of HPLC, reformulated in a biocompatible solvent, and passed through a sterile filter before being administered to human subjects. All of these manipulations must be fast and reliable given the short half-life of carbon-11. However, the use of an HPLC system significantly increases the losses of the tracer and production time, often necessitates the use of toxic solvents, complicates automation and occasionally leads to failed syntheses. Furthermore, the required cleaning of the reactors and HPLC column prolongs delays between the syntheses of subsequent tracer batches and increases the exposure of personnel to radiation.

The radiochemistry of fluorine-18 ($t_{1/2}$ = 109.7 min), the other widely used PET isotope, has been recently advanced *via* the development of cassette-based kits that obviate the need for HPLC purification. By employing solid phase extraction (SPE) cartridges, these fully disposable kits allow for the reliable routine production of ^{18}F-tracers, including [^{18}F]FDG, [^{18}F]FMISO, [^{18}F]FMC and others, with shorter synthesis times, reduced personnel involvement and minimal maintenance of the equipment. One of the reasons carbon-11 remains a less popular isotope in PET imaging is a lack of similar kits for the routine production of ^{11}C-tracers. Their development would significantly improve synthetic reliability, increase radiochemical yields and simplify automation and preventive maintenance of the production modules.

Currently available production kits take advantage of inexpensive, disposable, SPE cartridges instead of HPLC columns for the separation of the radiotracer from unreacted radioactive isotope, precursor and other radioactive and non-radioactive by-products. Ideally, the radiolabeling reaction also proceeds on the same cartridge; for example, the [^{18}F]fluoromethylation of dimethylaminoethanol with gaseous [^{18}F]CH_2BrF in the production of prostate cancer imaging PET tracer [^{18}F]fluoromethylcholine occurs on a cation-exchange resin cartridge[6]. Although similar procedures for the radiolabeling of several ^{11}C-tracers on cartridges have been reported[7,8] and became especially powerful for the radiosynthesis of [^{11}C]choline[9] and [^{11}C]methionine[10], these examples remain limited to oncological PET tracers where the separation from the precursor is often not required. We recently reported the development of "[^{11}C]kits" for the production of [^{11}C]CH_3I[11] and subsequent ^{11}C-methylation, as well as solid phase-supported synthesis[12] in our endeavours to simplify the routine production of ^{11}C-tracers. Here, we wish to demonstrate our progress using the example of the solid phase supported radiosynthesis of [^{11}C]PiB, a radiotracer for Aβ imaging which revolutionized the field of Alzheimer's disease (AD) imaging when it was first developed in 2003 (**Figure 1**)[13,14]. In this method, volatile [^{11}C]CH_3OTf (bp 100 °C) is passed over 6-OH-BTA-0 precursor deposited on the resin of a disposable cartridge. PET tracer [^{11}C]PiB is then separated from the precursor and radioactive impurities by elution from the cartridge with biocompatible aqueous ethanol. Further, we automated this method of [^{11}C]PiB radiosynthesis using a remotely operated radiochemistry synthesis module and disposable cassette kits. Specifically, we implemented this radiosynthesis on a 20-valve radiochemistry module, equipped with syringe drive (dispenser) which fits standard 20 mL disposable plastic syringe, gas flow controller, vacuum pump and gauge. Due to the simplicity of this method, we are confident that it can be modified to most commercially available automated synthesizers, either cassette-based or those equipped with stationary valves. This solid phase supported technique facilitates [^{11}C]PiB production compliant with Good Manufacturing Practice (GMP) regulations and improves synthesis reliability. The technique described here also reduces the amount of precursor required for radiosynthesis, uses only "green" biocompatible solvents and decreases the time between consecutive production batches.

PROTOCOL:

1. Preparation of buffers and eluents

1.1. Dissolve 2.72 g of sodium acetate trihydrate in 100 mL of water to prepare 0.2 M sodium acetate solution (solution A).

1.2. Dissolve 11.4 mL of glacial acetic acid in 1 L of water to prepare 0.2 M acetic acid solution (solution B).

1.3. Combine 50 mL of solution A with 450 mL of solution B to prepare the acetate buffer at pH 3.7 (buffer 1) according to the buffer reference center[15]. Verify the pH of the buffer with pH strips or a pH meter.

1.4. Combine 12.5 mL of absolute ethanol with 87.5 mL of buffer 1 to make 12.5% aqueous EtOH solution (wash 1) in a 100 mL bottle.

1.5. Combine 15 mL of absolute ethanol with 85 mL of buffer 1 to make 15% aqueous EtOH solution (wash 2) in a 100 mL bottle.

1.6. Combine 5 mL of absolute ethanol with 5 mL of buffer 1 to make 50% aqueous EtOH solution (final eluent) and draw 2.5 mL of this solution into a 10 mL syringe.

2. Application of the precursor to the cartridge

2.1. Pass 10 mL of water followed by 5 mL of acetone through the tC18 cartridge to precondition it.

2.2. Dry the cartridge with a stream of nitrogen at 50 mL/min for 1 min.

2.3. Dissolve 2 mg of the precursor 6-OH-BTA-0 in 1 mL of anhydrous acetone.

2.4. Holding a Luer-tip 250 µL precision glass syringe downwards, withdraw 100 µL of the precursor solution and 50 µL of air cushion on top of the liquid. Remove the needle and apply the precursor solution on the tC18 cartridge from the female end by slowly pushing the plunger all the way down. Do not push the solution any further!

3. Setting up the manifold for automated synthesis

3.1. Secure the standard 5-port disposable manifold on the synthesis module and assemble it according to the **Figure 2** and steps 3.2 – 3.5 below.

NOTE: We recommend using acetone-resistant manifolds (see **Table of Materials**).

3.2. Port 1 has two positions. Connect the horizontal inlet to the automated dispenser fitted with a 20 mL syringe. Connect the vertical inlet to the bottle with wash 1.

3.3. Connect the output of the module which produces $[^{11}C]CH_3OTf$ to port 2 of the manifold.

3.4. Install the tC18 cartridge loaded with precursor 6-OH-BTA-0 between ports 3 and 4.

3.5. Port 5 has two positions. Connect the horizontal outlet to the waste bottle which must hold at least 200 mL. Connect the vertical outlet to the sterile vial for tracer collection *via* the sterile filter.

4. Radiosynthesis of $[^{11}C]$PiB

CAUTION: All manipulations involving radioactive isotopes must be performed in a lead-shielded

hot cell by personnel with adequate training to work with radioactive materials.

NOTE: This protocol does not cover the details of production of $[^{11}C]CO_2$ in the cyclotron and its conversion into $[^{11}C]CH_3OTf$ using the radiochemistry module. These procedures will depend on the individual equipment of the radiochemistry lab and are outside the scope of this protocol. Our PET centre is equipped with an IBA cyclotron, which produces carbon-11 in the chemical form of $[^{11}C]CO_2$ *via* the $^{14}N(p,\alpha)^{11}C$ nuclear reaction with a N_2/O_2 gas mixture (99.5:0.5) in the gas target, and a commercially available module for production of $[^{11}C]CH_3I$ *via* the "dry method" (catalytic reduction to $[^{11}C]CH_4$ followed by radical iodination). $[^{11}C]CH_3OTf$ is produced by passing $[^{11}C]CH_3I$ over a silver triflate column heated to 175 °C at 20 mL/min.

4.1. Deliver $[^{11}C]CH_3OTf$ into the manifold through port 2 and pass it through the loaded tC18 cartridge at 20 mL/min output flow regulated by the $[^{11}C]CH_3OTf$ module, *via* ports 3 and 4 and into the waste bottle as shown on **Figure 2A**.

4.2. Once all the radioactivity has been transferred and trapped on the tC18 cartridge as monitored by the radioactivity detector behind the cartridge holder, stop the flow of gas by closing port 2. Let the cartridge sit for 2 min to complete the reaction.

4.3. Withdraw 19 mL of wash 1 solution (see step 1.4) from the 100 mL bottle into the dispenser syringe through port 1 at 100 mL/min as shown on **Figure 2B**.

4.4. Dispense 18.5 mL of wash 1 solution from the dispenser through the tC18 cartridge *via* ports 3 and 4 and into the waste bottle at 50 mL/min as shown on **Figure 2C**. Ensure the absence of air bubbles in the manifold as they might diminish the separation efficiency.

4.5. Repeat steps 4.3 and 4.4 four times, withdrawing and dispensing 18.5 mL of wash 1 solution each time. The total volume of wash 1 solution passed through tC18 is 92.5 mL; however, it can vary within the 90 – 100 mL range depending on the particular synthesis module used.

4.6. Switch the input line on port 1 from wash 1 to wash 2 solution (see step 1.5).

4.7. Repeat steps 4.3 and 4.4 three times, withdrawing and dispensing 18.5 mL of wash 2 solution each time. The total volume of wash 2 solution passed through tC18 is 55.5 mL. However, it can vary within the 50 – 60 mL range depending on the particular synthesis module used.

4.8. Toggle valve 5 towards the final vial as shown on **Figure 2D**. Disconnect the line from the dispenser and connect it to the 10 mL syringe containing 2.5 mL of the final eluent solution (50% aqueous EtOH, see step 1.6) and 7.5 mL of air.

4.9. Holding the syringe downwards, manually push the final eluent solution (2.5 mL) followed by air (7.5 mL) through the tC18 cartridge *via* ports 3 and 4 and into the sterile vial for tracer collection *via* the sterile filter as shown on **Figure 2D**.

4.10. Disconnect the empty syringe, connect the 10 mL syringe containing 10 mL of the sterile phosphate buffer (recipe not included as it may vary) and push the entire volume through the tC18 cartridge into the sterile vial as described above (**Figure 2D**). Disconnect the syringe and flush the line with 10 mL of air using the same syringe.

4.11. Withdraw 0.7 mL of the final tracer formulation and collect samples for quality control procedures (0.1 mL), bacterial endotoxin test (0.1 mL) and sterility (0.5 mL).

5. Quality control procedures

CAUTION: Each batch of the radiotracer must be subjected to the appropriate quality control procedures (QC) prior to release to the PET imaging site for administration into human or animal subjects. The authors of this manuscript are not responsible for the compliance of the radiotracer produced at other centers with local health authority regulations.

5.1. Perform pre-release QC procedures, which must include tests for radiochemical identity (RCI), radiochemical purity (RCP), chemical purity and molar activity of the tracer as well as residual solvent content and pH of the formulation.

5.2. Determine the RCI, RCP, chemical purity and molar activity by means of analytical HPLC system equipped with UV (monitoring at 350 nm) and radioactivity detectors, and a reversed-phase column. Determine the retention times of 6-OH-BTA-0 and 6-OH-BTA-1 and calibrate the instrument to quantify the content of each compound.

5.3. Determine the residual solvent content by means of analytical gas chromatography system equipped with a capillary column. Determine the retention times of acetone and ethanol and calibrate the instrument to quantify the content of each solvent.

5.4. Perform the bacterial endotoxins test using a cartridge reader equipped with suitable cartridges.

5.5. Perform the sterility analysis of the sample at least 14 day after the synthesis to ensure the absence of bacterial growth or send the sterility sample to a laboratory accredited by the local health authority.

REPRESENTATIVE RESULTS:

To summarize a typical radiosynthesis of [^{11}C]PiB, gaseous [^{11}C]CH_3OTf is first passed through a tC18 cartridge preloaded with a solution of precursor (**Figure 1**). Separation of the reaction mixture is then achieved by successive elution with aqueous ethanol solutions as follows. First, 12.5% EtOH elutes the majority of unreacted [^{11}C]CH_3OTf and 6-OH-BTA-0, then 15% EtOH washes out the residual impurities, and finally a 50% ethanol solution elutes the desired [^{11}C]PiB into a sterile vial. The tracer is then diluted with sterile phosphate buffer and undergoes strict QC procedures before release to the PET imaging site. Typical analytical HPLC UV and radioactivity chromatograms of the [^{11}C]PiB batch suitable for administration are represented in **Figure 3**.

The total radiosynthesis time is 10 min starting from the delivery of [^{11}C]CH_3OTf, the RCY of [^{11}C]PiB using 0.2 mg of precursor is 22% (starting from [^{11}C]CH_3OTf, not corrected for decay) and the molar activity is 190 GBq/µmol. The tracer must comply with all QC specifications of the multicenter Dominantly Inherited Alzheimer Network Trials Unit (DIAN-TU) for clinical trials: the radiochemical purity must be above 95%; the non-radioactive impurities content must be below 1.3 µg per 10 mL dose; the pH must be within the 4 – 8 range; and the ethanol and acetone contents must be below 10% and 3000 ppm, respectively. The samples must also be sterile and endotoxin free. The results of four typical radiosynthesis runs are summarized in **Table 1**.

For the reported technique to work properly, care must be taken during several critical steps described above. To apply the precursor on the tC18 cartridge (step 2.4) the solution must not be pushed towards the output, so as to not shorten the effective path for separation of the [^{11}C]PiB from the unreacted starting materials and possible side products. The flow of [^{11}C]CH_3OTf through a cartridge during the transfer must not exceed 20 mL/min (step 4.1). Once the elution begins (step 4.4), it is very important to keep the cartridge wet and not let air through to avoid channeling effects which might result in lower purity of the tracer or lower RCY due to the losses of [^{11}C]PiB in the waste. If the 5-port manifold used in the radiosynthesis (step 3.1) is not resistant to acetone, such as a standard polycarbonate manifold like ACC-101, the amount of acetone must not exceed 100 µL as larger volumes might damage the manifold during the activity transfer and result in failed synthesis. In case the pH does not meet the specifications, the tC18 cartridge may optionally be rinsed with 10 mL of sterile water between steps 4.7 and 4.8 into the waste bottle.

FIGURE AND TABLE LEGENDS:

Figure 1. Radiosynthesis of [^{11}C]PiB by ^{11}C-methylation of 6-OH-BTA-0 precursor with [^{11}C]CH_3OTf. [^{11}C]PiB is one of the most widely used radiotracers for imaging of amyloid plaques associated with AD and other neurodegenerative conditions by PET. This tracer is commonly synthesized *via* ^{11}C-methylation of the aniline precursor called 6-OH-BTA-0 using [^{11}C]methyl triflate ([^{11}C]CH_3OTf) either in solution or in the dry HPLC injection loop (solvent captive technique).

Figure 2. Step-by-step synthesis and purification of [^{11}C]PiB on a tC18 cartridge. (**A**) Gaseous [^{11}C]CH_3OTf is passed through the cartridge loaded with 6-OH-BTA-0. As described in steps 4.1 and 4.2, [^{11}C]CH_3OTf is trapped on the cartridge containing the precursor and reacts with the precursor at room temperature for 2 min. (**B**) Wash 1 or wash 2 solution is withdrawn into the dispenser syringe. As described in step 4.3, the syringe pump of the module pulls the plunger of the clipped syringe upwards, withdrawing a solution of either eluent through a line connected to port 1 of the manifold. (**C**) The impurities are washed out into a waste bottle. As described in step 4.4, the syringe pump of the module moves the plunger of the clipped syringe downwards, pushing the withdrawn wash solution through the tC18 cartridge *via* ports 1, 3 and 4 of the manifold into a waste bottle. Steps represented on diagrams B and C are repeated in a cycle several times to wash out all unreacted materials from the cartridge, as described in steps 4.5 – 4.7. (**D**) [^{11}C]PiB is eluted with the final eluent into a sterile vial through a sterile filter. As described in steps 4.8 and 4.9, the syringe clipped into a syringe pump is disconnected from the

line and replaced first with a 10 mL syringe containing 2.5 mL of 50% aqueous ethanol. Port 5 of the manifold is then toggled towards the sterile vial and [^{11}C]PiB is eluted from the tC18 manually. The empty syringe is then replaced with another syringe containing 10 mL of sterile phosphate buffer and the entire contents are pushed through the tC18 to rinse the lines as described in step 4.10. The sterile vial now contains [^{11}C]PiB in a 12.5 mL 10% buffered aqueous ethanol solution. This figure has been modified from Boudjemeline et al.[12].

Figure 3. Quality control analytical HPLC of [^{11}C]PiB. (**A**) The retention times of [^{11}C]CH_3OH (from hydrolysis of [^{11}C]CH_3OTf), unreacted [^{11}C]CH_3OTf, and tracer [^{11}C]PiB on the radioactivity chromatogram are 2.1, 4.0 and 6.6 min, respectively. The analysis of the radioactivity trace shows that the RCP of [^{11}C]PiB is 98.0%. (**B**) The retention times of 6-OH-BTA-0 (precursor) and 6-OH-BTA-1 (tracer peak) on the UV chromatogram are 3.6 and 5.9 min, respectively. The analysis of the UV trace shows residual precursor concentration below the acceptable limit (1.3 µg) and the absence of other non-radioactive impurities. Thus, the radiochemical and chemical purity of the tracer is acceptable for clinical PET studies. HPLC conditions – column (**Table of Materials**): 5 µm, 100 x 4.0 mm; mobile phase: 40:60 acetonitrile/water flow rate: 0.7 mL/min.

Figure 4. Optimization of 6-OH-BTA-0 precursor amount. The lowest amount (0.1 mg) provides [^{11}C]PiB in a moderate radiochemical yield (RCY) of 18.1±3.8%. Radiosynthesis starting from 0.2 mg provides [^{11}C]PiB an RCY of 22.0±3.1%, while increasing the amount to 0.3 mg further improves the RCY to 32.1±3.7%, at the expense of a slightly higher amount of the precursor in the final product. All RCY's are not corrected for decay (radiosynthesis time of 10 min) starting from the radioactivity of the [^{11}C]CH_3OTf trapped on tC18 cartridge.

Figure 5. Quality control analytical HPLC of [^{11}C]ABP688. (**A**) Radioactivity chromatogram shows RCP of combined (*E*)- and (*Z*)-[^{11}C]ABP688 of 98.1%. (**B**) UV chromatogram shows residual precursor concentration above 10 µg. While the chemical purity might be acceptable for clinical PET studies, relatively low effective molar activity (A_m < 37 GBq/µmol) requires further purification optimization.

Table 1. Representative results of [^{11}C]PiB production runs under optimized conditions. All batches are compliant with requirements for tracers intended for clinical PET studies.

DISCUSSION:

Despite the recent emergence and FDA approval of several ^{18}F-labeled PET tracers, such as florbetapir, florbetaben and flutemetamol, [^{11}C]PiB remains a gold standard tracer for amyloid imaging due to the fast brain uptake and low non-specific binding. Currently this tracer is synthesized *via* either wet chemistry[16] or using a "dry loop" approach[4,17]. Both methods require HPLC purification followed by reformulation in aqueous ethanol, which takes approximately 20 – 30 min starting from [^{11}C]CH_3OTf. Inspired by some of the previous reports on solid phase supported ^{11}C-methylation techniques and the clinical importance of [^{11}C]PiB, we aimed to develop a radiosynthesis of this tracer using inexpensive disposable solid phase extraction (SPE) cartridges as a "3-in-1" entity for reaction, purification and formulation.

The most critical steps for successful production of PET tracers for in vivo imaging in human subjects are: 1) incorporation of the radioactive isotope into a tracer molecule; 2) separation of the tracer from unreacted radioactive and non-radioactive species; 3) reformulation of the tracer in a biologically compatible solvent; 4) compliance with quality control procedures. Based on the previously reported solvent captive method, we expected that the SPE-supported technique would require a lower amount of precursor compared to ^{11}C-methylation in solution. In particular, previously reported solvent captive procedures for the radiosynthesis of [^{11}C]PiB require 0.5 – 1.0 mg of the precursor[4,17]. Thus, we investigated not corrected for decay radiochemical yields of [^{11}C]PiB starting from [^{11}C]CH_3OTf at three different amounts of 6-OH-BTA-0: 0.1, 0.2, and 0.3 mg. Even the lowest amount (0.1 mg) provides a moderate amount of [^{11}C]PiB, albeit at relatively low and less reliable RCY (18.1±3.8%). Radiosynthesis starting from 0.2 mg provides an RCY of [^{11}C]PiB (22.0±3.1%), while increasing the amount to 0.3 mg further improves RCY (32.1±3.7%), at the expense of a slightly higher amount of the precursor in the final product. In all cases, the radiosynthesis was completed in 10 min. Thus, the optimal precursor amount depends on the desired RCY and purity of [^{11}C]PiB at particular PET centers. The results of the radiochemical yield optimization experiments based on precursor amount are summarized in **Figure 4**. Notably, radiosynthesis attempts using [^{11}C]CH_3I as a methylating agent or ethanol as a reaction solvent did not yield the desired [^{11}C]PiB (data not shown).

The quantitative separation of the radiosynthesis reaction mixture on a short SPE cartridge was the most challenging part of the described technique. We hypothesized that aromatic amines 6-OH-BTA-0 and 6-OH-BTA-1 predominantly exist in their protonated forms in acidic media and therefore would have sharper elution profiles from the reversed-phase solid phase. Hence, all aqueous ethanol solutions were prepared using 0.2 M acetate buffer at pH 3.7. Next, we determined that aqueous ethanol solutions with EtOH concentration up to 15% gradually elute unreacted precursor 6-OH-BTA-0 and [^{11}C]CH_3OTf, while radiolabeled [^{11}C]PiB remains trapped on the tC18 cartridge. In order to prevent tailing of those impurities into a final tracer formulation the ethanol concentration was increased from 12.5% to 15% in a gradient elution. After all the impurities had been washed out of the cartridge, tracer elution was achieved using a minimal amount (2.5 mL) of the concentrated ethanol solution (50%). In order to keep the ethanol content under the 10% limit and to bring the pH of the formulated tracer within the acceptable range for human injection (4 – 8), the tracer was diluted with sterile phosphate buffer.

Following conditions optimization, the radiosynthesis of [^{11}C]PiB was automated using a commercially available automated synthesis unit (ASU), equipped with dispenser syringe and disposable manifold. The manifold setup for this particular ASU is straightforward as described in steps 3.1 – 3.5. Notably, this methodology can be easily implemented on most of the other available ASU's following the recipes described above. Under optimized conditions, batches of [^{11}C]PiB suitable for clinical application are synthesized with final activities ranging from 1.4 to 2.4 GBq (38 – 61 mCi).

More recently, we applied the "3-in-1" technique for the radiolabeling of [^{11}C]ABP688, a PET tracer for the imaging of metabotropic glutamate receptors type 5 (mGlu5)[18,19]. Radiosynthesis of this tracer relies on the ^{11}C-methylation of the –OH group in the oxime; therefore, addition of

base is required to deprotonate the desmethyl precursor. Tetrabutylammonium hydroxide (as a 1 M solution in MeOH) was selected as a base because it is soluble in most polar organic solvents. In a preliminary radiolabeling experiment, a solution of precursor (0.5 mg) in DMSO (100 µL) was mixed with 1 M TBAOH in MeOH (20 µL) and the mixture was carefully applied on the tC18 cartridge as described above (see step 2.4). Gaseous [^{11}C]CH_3I was passed through the cartridge as described in steps 4.1 – 4.2 and the reaction was allowed to proceed at room temperature for 5 min. Sequential elution with dilute ethanol solutions in 0.2 M sodium bicarbonate buffer (pH 8.5 – 9.0) – 92 mL of 15% EtOH followed by 92 mL of 20% EtOH – washed out the unreacted [^{11}C]CH_3I and residual precursor. Radiochemically pure [^{11}C]ABP688 (RCY = 18.2%, RCP >98.0%) was then eluted with 50% EtOH solution in the same buffer through a sterile filter as described in steps 4.9 – 4.11. Despite the fact that over 98% of the precursor is removed with dilute ethanol washes, the presence of some unreacted precursor in the final tracer (up to 20 µg) requires further optimization of the radiosynthesis procedure. This optimization is ongoing, and the results of this project will be published in due course. Representative analytical HPLC UV and radioactivity chromatograms of the [^{11}C]ABP688 batch is shown on **Figure 5**.

In conclusion, we have developed an efficient solid phase supported carbon-11 radiolabeling procedure using readily available inexpensive SPE cartridges as "3-in-1" entities for radiosynthesis, purification, and formulation of PET tracers used for clinical imaging. Tracers suitable for human injection are produced within 10 min starting from the addition of ^{11}C-methylating agent ([^{11}C]CH_3OTf or [^{11}C]CH_3I) in high RCY and molar activity. We fully automated this technique to make it compliant with Good Manufacturing Practice (GMP) regulations imposed by health and radiation safety authorities. Solid phase supported radiosynthesis requires a low amount of precursor, avoids the use of toxic solvents, decreases the synthesis time and radiation dose sustained by the personnel. Furthermore, avoiding HPLC-related failures improves radiosynthesis reliability and allows for development of disposable kits for routine tracer production.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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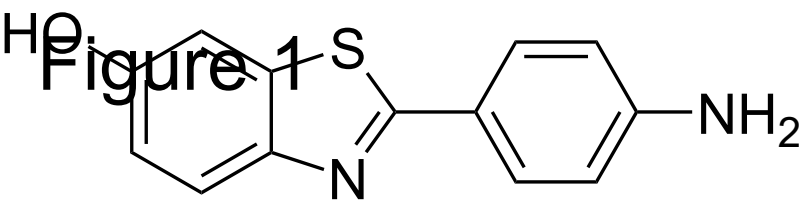
Figure 1



6-OH-BTA-0

[11C]PIB ([11C]6-OH-BTA-1)

Figure 2

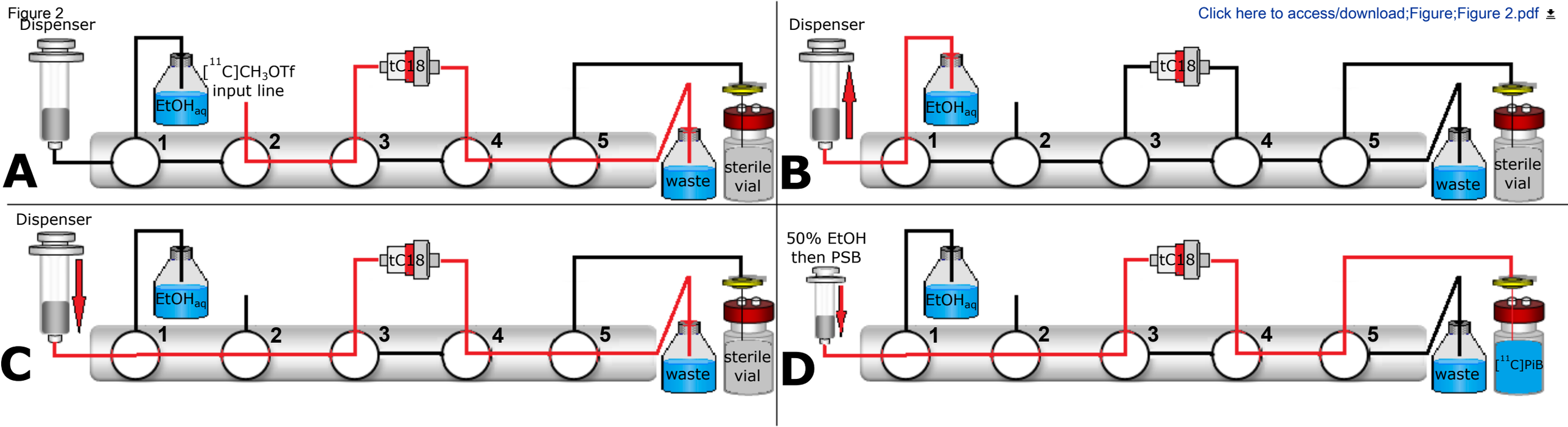


Figure 3

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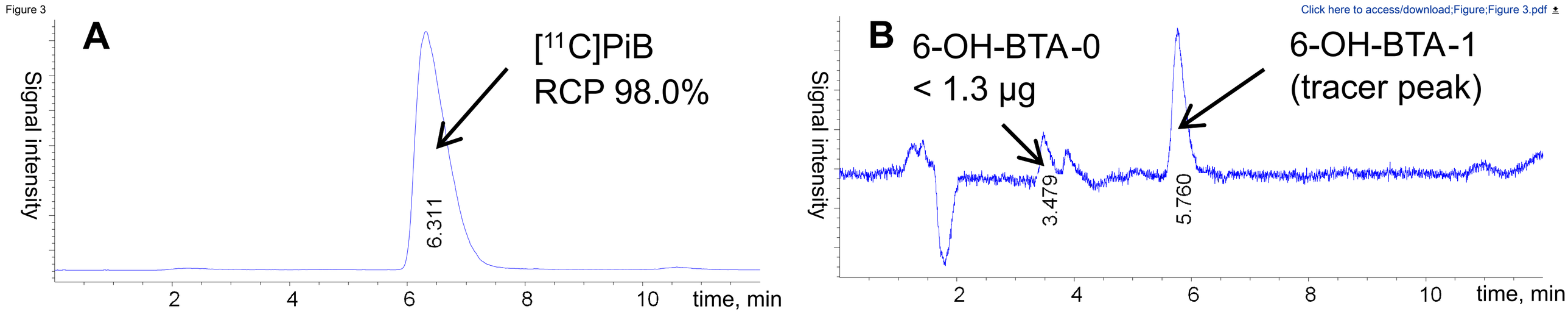

Figure 4

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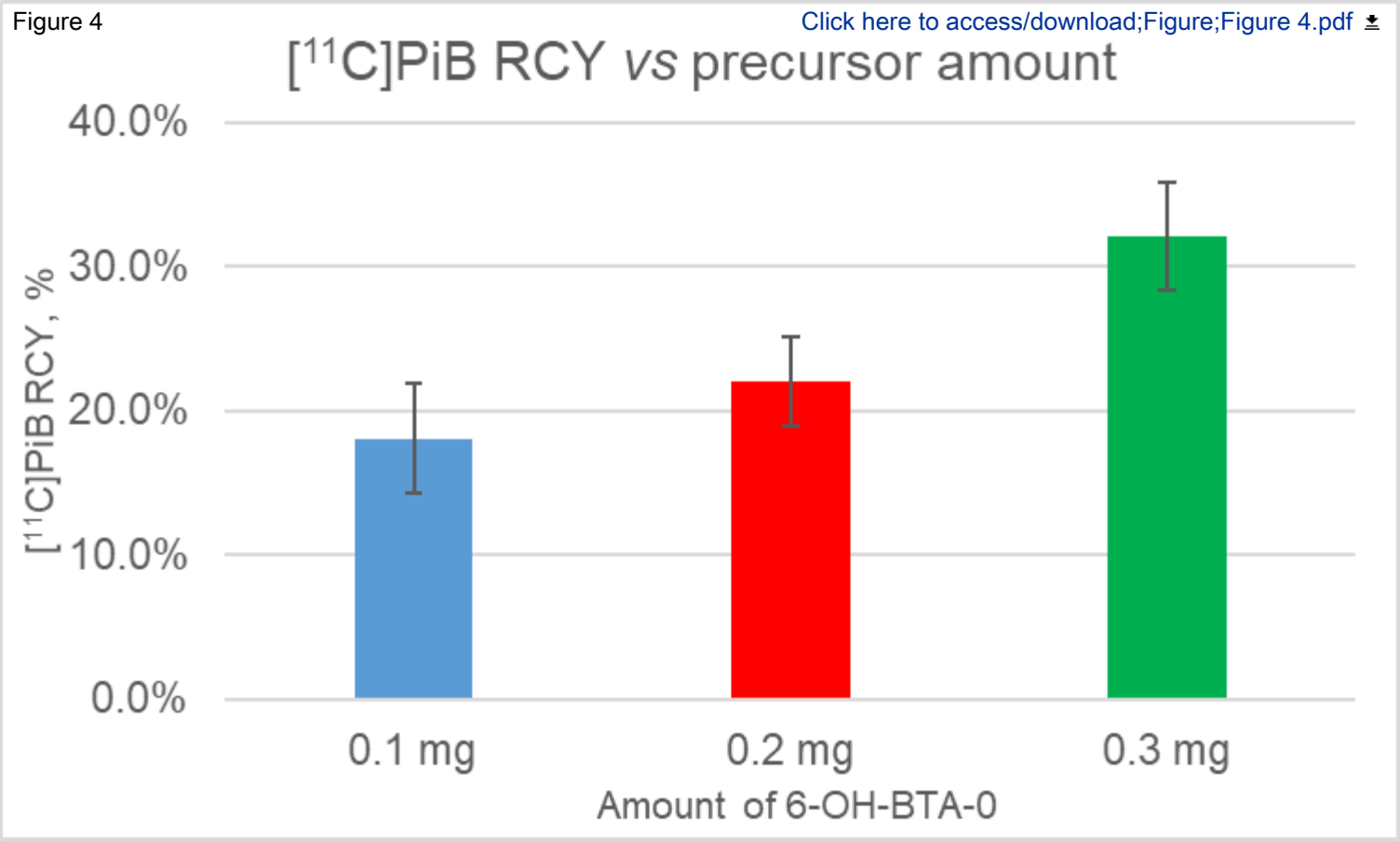

Figure 5

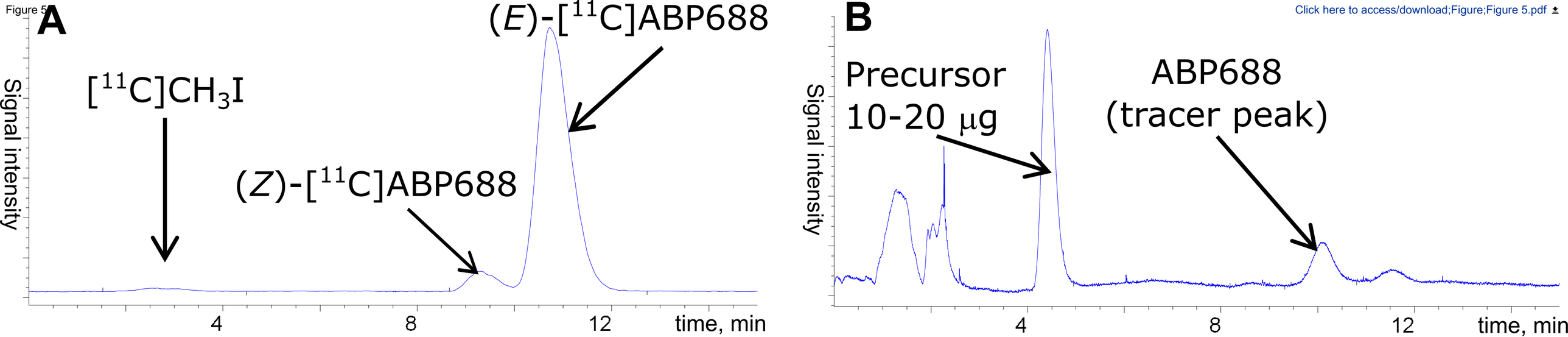

Table 1

Batch	Run 1	Run 2	Run 3	Run 4
[^{11}C]CH_3OTf, GBq	9.21	11.25	7.84	6.44
[^{11}C]PiB, GBq	2.26	2.37	2.11	1.41
RCY, %*	24.5	21.1	26.9	21.8
RCP, %	98	97.2	97.8	99.2
Molar activity, GBq/µmol	154.6	322.6	121.1	162.1
Residual precursor, µg	0.32	0.55	0.58	0.87
pH	5	5	5	5
EtOH content, %	9.4	8.8	7.7	8.1
Acetone content, ppm	33	38	46	33
BET test	N/A	<10 EU/mL	<10 EU/mL	<10 EU/mL
Sterility test	N/A	No Growth	No Growth	No Growth

* Footnote: From [^{11}C]CH_3OTf, not corrected for decay

Name of Material/Equipment	Company
6-OH-BTA-0	ABX advanced biochemical compounds
6-OH-BTA-1	ABX advanced biochemical compounds
Agilent 1200 HPLC system	Agilent
Ethanol absolute	Commercial alcohols
Hamilton syringe (luer-tip, 250 µL)	Hamilton
MZ Analytical PerfectSil 120	MZ-Analysentechik GmbH
Perkin Elmer Clarus 480 GC system	Perkin Elmer
polycarbonate manifold	Scintomics
Restek MTX-Wax column (30 m, 0.53 mm)	Restek
Scintomics GRP module	Scintomics
Sep-Pak tC18 Plus	Waters
solvent-resistant manifold	Scintomics
Spinal needle	BD
Sterile extension line	B. Braun
Sterile filter	Millipore
Sterile vial (20mL)	Huayi
Sterile water	Baxter
Synthra MeIplus Research	Synthra
Syringe (10 mL)	BD

Syringe (1mL)	BD
Syringe (20 mL)	B. Braun
Vent filter	Millipore

Catalog Number	Comments/Description	
5101	Non-radioactive precursor of [^{11}C]PiB	
5140	Non-radioactive standard of [^{11}C]PiB	
Agilent 1200	Analytical HPLC system	
432526		
HAM80701		
MZ1440-100040	Analytical HPLC column	
Clarus 480	Gas chromotograph	
ACC-101	Synthesis manifold	
70625-273	Analytical GC column	
Scintomics GRP	Automated synthesis unit	
WAT020515	Solid phase extraction cartridge	
ACC-201	Synthesis manifold	
405181		
8255059		
SLLG013SL		
SVV-20A		
JF7623		
MeIplus Research	[^{11}C]CH_3I/[^{11}C]CH_3OTf module	
309604		

309659		
4617207V	Dispenser syringe	
TEFG02525		

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Author(s):	Thomas A. Singleton, Mehdi Boudjemeline, Robert Hopewell, Dean Jolly, Hussein Bdair, Alexey Kostikov

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We added the space lines as suggested

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The filmable content was highlighted

5. Step 5.1-5.5: Please write these steps in the imperative tense.

Steps have been rewritten

6. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We added description of figures and tables where necessary.

7. Please do not abbreviate journal titles for references.

We now provide full journal names in the references.

8. Please revise the table of materials to include all essential supplies, reagents, and equipment.

9. Please sort the items in alphabetical order according to the name of material/equipment.

We have added missing essential reagents and materials to the table and organized them in alphabetical order.

10. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite lines 99-101.

We have rewritten the lines 99-101 to avoid overlap with our previously published work.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This submission from Dr. Kostikov's lab demonstrates a method for solid-phase radiopharmaceutical synthesis using carbon-11 methylation. The method developed is a significant advance that could improve accessibility of some key radiotracers like [11C]PiB. As the work deals heavily with a new technical approach to conducting these reactions and purifications, it is very suitable for a video manuscript.

We thank the reviewer for the positive evaluation of our work and suggested improvements.

Minor Concerns:

The title and abstract of the manuscript are appropriate. The title could be improved by emphasizing that the solid-phase cartridges are not merely reactors, but also media for purification and reformulation, as described in the abstract.

We thank the reviewer for this suggestion and we changed the title of this manuscript to "**Solid phase ^{11}C-methylation, purification and formulation for the production of PET tracers.**"

The authors discuss other applications of their work ([11C]ABP688). I am hesitant to suggest further iterations, as they would know the possibilities and limitations more intimately.

Indeed, the application of this methodology to labeling of [^{11}C]ABP688 tracer is not complete at the moment due to the presence of residual, albeit relatively small (10-20 µg, which is <4% of the initial load), amount of the precursor in the final tracer formulation. Furthermore, two diastereomers (*E*)- and (*Z*)-[^{11}C]ABP688 are not separable on a short cartridge and they are difficult to separate even by HPLC, please see more detailed comment below. Nevertheless, the RCY we obtained shows the general applicability of this procedure to ^{11}C-methylation of hydroxyl group in a presence of a strong organic base TBAOH as a proof of principle.

Not all of the required materials are listed in the table: for example ethanol, water (DI, sterile? should be specified). Should the instrument(s) used for automation be listed (scintomics and syringe pump)? The levels of activity used would seem to preclude manual work with the manifold during the synthesis. Also, equipment and supplies for quality control may be important to note. Are the manifolds altered in any way, such as adding longer tubing for manual elution in step 4.9, and if so, the materials used should be described.

We added ethanol, sterile water, sterile extension line, synthesis module and other supplies to the table of materials. We are hesitant to add quality control equipment to this table as this will depend on the individual setup of the radiochemistry lab. However, we noted our QC equipment

in section 5 of the protocol.

The steps listed should lead to the desired outcome, though radiochemistry is always challenging to reproduce exactly. For example, 2.4 appears to be a critical step and it would be important to get this right. This step should be highlighted and more detail provided.

This is an excellent point and we have rewritten this and other steps in a lot more detail.

As mentioned above, some steps could be described in more detail, such as set up of the instrument, when valves are changed and how, and how the dispenser operates. Also, in figure 2, parts B and C are mislabeled (or the text is wrong). See steps 4.3 and 4.4 to compare. After 4.9 and 4.10, are the lines flushed with air/gas to release eluent still on the cartridge or in tubing? This will have an effect based on tubing volumes and could change the final concentration of the product.

We thank the reviewer for catching that the B and C labels on figure 2 were reversed, this has been fixed. We also specified that the lines should be flushed with air after steps 4.9 and 4.10.

While the likelihood of success is high, it would be useful to suggest some controls or approaches to troubleshooting if the synthesis fails or yields and purities are lower than reported.

We have added the troubleshooting paragraph to the representative result section of the manuscript as follows: "*For the reported technique to work properly, care must be taken during several critical steps described above. To apply the precursor on the tC18 cartridge (step 2.4.) the solution must not be pushed towards the output as to not shorten the effective path for separation of the [^{11}C]PiB from the unreacted starting materials and possible side products. The flow of [^{11}C]CH_3OTf through the cartridge during the transfer must not exceed 20 mL/min (step 4.1). Once the elution begin (step 4.4.) it is very important to keep the cartridge wet and not let air through to avoid channeling effect which might result in lower purity of the tracer or lower RCY due to the losses of [^{11}C]PiB in the waste. If the 5-port manifold used in the radiosynthesis (step 3.1.) is not resistant to acetone, such as a standard polycarbonate manifold ACC-101, the amount of acetone must not exceed 100 µL as larger volumes might damage the manifold during the activity transfer and result in failed synthesis. In case the pH does not meet the specifications, the tC18 cartridge maybe optionally rinsed with 10 mL of sterile water between steps 4.7 and 4.8 into the waste bottle.*"

The anticipated results are useful to readers. Some numbers are reported in ways that do not conform to the latest "consensus nomenclature", such as ndc RCY. It should be made clear throughout and in Fig4 how RCY is calculated. Likewise "[11C]kits" is technically contrary to the "consensus".

We have rewritten one sentence in the Discussion section as follows: "*Thus, we investigated not corrected for decay radiochemical yields of [^{11}C]PiB starting from [^{11}C]CH_3OTf at three different amounts of 6-OH-BTA-0: 0.1, 0.2, and 0.3 mgs.*". We have added another sentence regarding the synthesis time: "*In all cases, the radiosynthesis was completed in 10 minutes.*". Because the short synthesis time is one of the strengths of our procedure, we wanted to emphasize the RCY not corrected for decay, which is still used in some publications. As for the "[11C]kits" terminology, while it is currently technically incorrect, it refers to the previously published paper

which has this term in its title.

A couple stray concerns: figure 5b really doesn't look like 98% RCP. What is the big peak at 9-10 minutes? Since separation of precursor is a challenge and limitation for this method, perhaps a discussion is in order regarding what situations this would be important for apparent molar activity in PET imaging.

This is a very good point. Due to the asymmetrically substituted C=N double bond in its structure, [^{11}C]ABP688 tracer has two diastereomers (*E*)- and (*Z*)-, which are not separable on a short cartridge. We have revised Figure 5 to indicate the presence of 2 diastereoisomers. At the time we were not able to separate them even by HPLC, although lately we developed a new procedure for production of diastereomerically pure (*E*)-[^{11}C]ABP688 (an active form of this tracer), which we currently employ in our PET centre. Hence, the data on [^{11}C]ABP688 tracer synthesis in this manuscript serves as a proof of principle of applicability towards labeling of other C-11 labeled PET tracers on resin, rather than a complete procedure. If in the reviewer's opinion this data cannot be included in the manuscript as is, we will remove it.

References to the most prevalent methods for synthesis of [11C]PiB would be welcome, such as the Radiochemical Syntheses Vol 1, or other sources showing clinical implementation.

The references for both "reactor" method (Philippe et al. Radiochemical Syntheses Vol 1) and "dry loop" method (Shao et al. Radiochemical Syntheses Vol 2 and Wilson et al. J Labelled Compd. Radiopharm.) have been added.

I recommend for moving forward with video production after addressing the suggestions above.

Reviewer #2:

Manuscript Summary:

The submitted manuscript by Singleton et al. describes a novel procedure where [11C]methylation reactions could be performed rapidly on SPE cartridge. The described procedure improves the reproducibility, reduces production time and ultimately increases the overall radiochemical yield of 11C-labeled radiopharmaceuticals. As a proof-of-concept, the well-known radiopharmaceutical, [11C]PIB, was produced, purified and finally formulated all using a single C18 SPE cartridge. The produced [11C]PIB was shown to fulfill all quality control criteria for in Human use.

The manuscript is well written and the results are presented in a nice way. This referee recommends acceptance of the manuscript after some corrections.

We thank the reviewer for the positive evaluation of our work and suggested improvements.

Major Concerns:

A general comment to the Protocol section 4 (Page 3-4, Line 146-182). Figure 2 does not correspond to what is described in the text. For example, point 4.3) it is written, "Withdraw 19 mL of wash 1 solution (see 1.4.) from the 100 mL bottle into the dispenser syringe through port 1 at 100 mL/min as shown on figure 2, B.". However, Figure 2, B does not show that step. Make sure that figure 2 illustrates what is being described in the text. This is very important.

We thank the reviewer for catching that the labels B and C on figure 2 were reversed, this has been fixed.

Minor Concerns:

1) Introduction (Page 1, line 66): This referee recommends the author includes the original article which describes 11C-MeI to 11C-MeOTf.
Jewett DM (1992) A simple synthesis of [11C]methyl triflate. Int Appl Radiat Isot 43:1383-1385

We thank the reviewer for this suggestion, this reference has been now replaced

2) Protocol (Page 2, Line 132): This referee recommends that the part number of the Hamilton Syringe is specified, as Hamilton syringes comes in many different forms and sizes. For example, does this version have a laur-lock fitting to connect to the SPE cartridge. Please clarify point 2.4.

Thank you for pointing this out, we specified the type of Hamilton syringe being used in this step and also added a part number to the table of materials.

3) Protocol (Page 3, Line 136): Please insert a reference to Figure 2 after "on the synthesis module.". Moreover, it would also be helpful if the automated synthesis module is described in more detail. What components are included, e.g. syringe pump drivers, gas-flow controlling etc.

We included the details of the Scintomics GRP synthesis module in the introduction by adding two sentences: "*In particular, we implemented this radiosynthesis on a 20-valve Scintomics GRP module, equipped with syringe drive (dispenser) which fits standard 20 mL B-Braun syringe, gas flow controller, vacuum pump and gauge. Due to simplicity of this method, we are confident that it can be modified to most commercially available automated synthesizers, either cassette-based or the ones equipped with stationary valves.*"

We referenced Figure 2 in step 3.1. This step will also appear in the video protocol.

4) Protocol (Page 3, Line 161-162): The authors write, "Dispense 18.5 mL of wash 1 solution from the dispenser through the tC18 cartridge via ports 3 and 4 and into the waste bottle at 50 mL/min as shown on figure 2, C.". How is this flowrate controlled? I assume that an automated syringe driver is used. Please clarify.

We hope that the sentence describing the Scintomics GRP module which we now added to the introduction clarifies that it is equipped with a syringe drive (dispenser).

5) Protocol (Page 3, Line 168): The authors write, "Switch the input line on port 1 from wash 1 to wash 2 solution (see 1.5)." How is this physically done? Since, the module at this stage will be highly radioactive. Are the washing solutions located outside the hot cell? Please clarify.

The "methyl iodide" module (Synthra) is located in a separate hot cell in our lab and the total amount of radioactivity transferred to the hot cell where ^{11}C-methylation occurs does not exceed 300 mCi. The manual manipulation to switch the input line between two solutions takes about 3 seconds with one hand and the distance between that line and the source of activity (tC18 cartridge) is about 50 cm, thus the hand dose received during such manipulations is quite small.

Nevertheless, we stacked several lead bricks between the solvent bottles and the source of activity to further reduce the dose.

6) Protocol (Page 3, Line 173): Continuing on my previous argument. The authors write, "Disconnect the line from the dispenser and connect it to the 10 mL syringe containing 2.5 mL of the final eluent solution (50% aqueous EtOH, see 1.6.).". How is this physically done? Are all these connectors located outside the hot cell? And what about the syringe driver? Please clarify.

Similarly to our previous response, the amount of time necessary to pull out the syringe from the dispenser unit and undo the connection to the line is about 5 seconds. In combination with a 50 cm distance from the activity and lead protection, the hand radiation dose received during this manipulation is quite small. The final elution with an eluent and sterile phosphate buffer can be done from the outside of the hot cell.

7) Discussion (Page 6, Line 267-269): The authors write, "Even the lowest amount (0.1 mg) provides a moderate amount of [11C]PiB, albeit at relatively low and less reliable RCY (18.1±3.8%). Radiosynthesis starting from 0.2 mg provides excellent RCY of [11C]PiB (22.0±3.1%), while further increasing the amount to 0.3 mg improves RCY (32.1±3.7%), at the expense of a slightly higher amount of the precursor in the final product.". I would argue that saying 18.1±3.8% is a moderated RCY (at 0.1 mg) and 22.0±3.1% is an excellent RCY (at 0.2 mg) is not a fair description. It is only 4% in RCY between the two. This referee suggest to remove the word "excellent".

We fully agree with this assessment and removed the word “excellent”.

Reviewer #3:

Manuscript Summary:
The manuscript described the fully automated synthesis of very important PET radioligand for AD, [11C]PiB via on-line cartridge based 11C-methylation. This approach is widely used in PET radiochemistry in the cartridge-based synthesis of L-[11C]methyl methionine (see for example "Reliability and reproducibility of N-[11C]methyl-choline and L-(S-methyl-[11C])methionine solid-phase synthesis: A useful and suitable method in clinical practice. Nucl Med Comm 29 (8):736-740"). However the application of this approach for the preparation of PET radioligands is much more difficult due to necessity to separate the 11C-labelled product from labeling precursor. To solve the problem the authors suggest the original elution protocol using different concentration of an aqueous ethanol. Also they were able to achive high r11C-methylation yields using low amounts of precursor (0.1-0.3 mg). The novelty of this radiolabeling and purification procedures is doubtless. The authors successfully implemented their method on the commonly used cassette-based automation platform SCINTOMICS GRP. All the experiments were well planned and very well performed. The desired product formulation suits well to all the required QC parameters. The feasibility of this approach was also confirmed by the preparation of [11C]ABP688, a PET radiotracer for the imaging of metabotropic glutamate receptors type 5 (mGlu5)PET radioligand.
The paper is concise and well-organized and methods are thoroughly described. I would recommend publication provided the following concerns can be addressed:

We thank the reviewer for the positive evaluation of our work and suggested improvements.

Major Concerns:
No

Minor Concerns:

- 11C-methylating agent, [11C]CH3OTf, is produced using Synthra automated module. Please specify whether the agent is prepared via "wet" (LiAlH4/HI) method or by means of a gas-phase process? Second, which type of the gas target was used for generating 11C (methane target or CO2 target?)

We modified the preface paragraph to step 4 as follows: “Our PET centre is equipped with an IBA cyclotron (Cyclone® 18/9 IBA, Louvain-La-Neuve, Belgium), which produces carbon-11 in the chemical form of $[^{11}C]CO_2$ *via* the $^{14}N(p,\alpha)^{11}C$ nuclear reaction with a N_2/O_2 gas mixture (99.5:0.5) in the gas target (Nitra), and a commercially available Synthra module for production of $[^{11}C]CH_3I$ *via* the “dry method” (catalytic reduction to $[^{11}C]CH_4$ followed by radical iodination). $[^{11}C]CH_3OTf$ is produced by passing $[^{11}C]CH_3I$ over a silver triflate column heated to 175 °C at 20 mL/min.”

- Line 229 "the non-radioactive impurities content must be below 1.3 µg per 10 mL dose" Please provide the corresponding references supporting this limit value.

These requirements come from the “multicenter Dominantly Inherited Alzheimer Network Trials Unit (DIAN-TU)” clinical trials which we participated in. We could not find a published source of QC requirements to reference, but we specified these clinical trials in the text.

- Line 253 "Currently this tracer is synthesized via either wet chemistry or using a "dry loop" approach". Please provide the corresponding references.

The references for both “reactor” method (Philippe et al. Radiochemical Syntheses Vol 1) and “dry loop” method (Shao et al. Radiochemical Syntheses Vol 2 and Wilson et al. J Labelled Compd. Radiopharm.) have been added.

- Line 262-66. "Based on the previously reported solvent captive method, we expected that the SPE-supported technique would require lower precursor amount compared to 11C-methylation in solution. Thus, we investigated radiochemical yields of [11C]PiB at three different amounts 265 of 6-OH-BTA-0: 0.1, 0.2, and 0.3 mgs". Please compare the amounts of precursors applied in the solvent captive method (please give a reference).

We added the amount of precursor (0.5 – 1.0 mg) reported for solvent captive method by two independent groups (Shao et al. Radiochemical Syntheses Vol 2 and Wilson et al. J Labelled Compd. Radiopharm.)

- In the introduction please insert the references on the application of on-line cartridge-based 11C-methylation in the synthesis of PET radiotracers.

We have added the references for the radiosynthesis of $[^{11}C]$choline and $[^{11}C]$methionine.

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