Improved, One-pot Synthesis of 6-[^{18}F]Fluorodopamine and Quality Control Testing for Use in Patients with Neuroblastoma

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ABSTRACT

6-[^{18}F]fluorodopamine ([^{18}F]F-DA) is taken into cells via the norepinephrine transporter (NET). Recent [^{18}F]F-DA PET-CT imaging of adult neuroendocrine tumors shows a dramatic improvement in sensitivity over the standard-of-care, *meta*-iodobenzylguanidine (MIBG) SPECT-CT. A new precursor (ALPdopamine™) allows no-carrier-added synthesis resulting in high molar activity [^{18}F]F-DA. Automated synthesis of [^{18}F]F-DA was performed in a single reactor using a two-step procedure; fluorination and thermolysis of a diaryliodonium salt precursor followed by acid hydrolysis. Phase transfer agents, Kryptofix® 222 and two tetraalkylammonium salts, were investigated. Optimized synthesis of [^{18}F]F-DA was achieved in 56-60 minutes (26% EOS, non-decay corrected). The product passed all FDA-required quality control testing for human use. Accumulation of [^{18}F]F-DA in the SK-N-BE(2)-C (high NET expression) cells was significantly higher than in SH-EP (minimal NET expression) cells ($p < 0.0001$). ALPdopamine™ provides an effective scaffold for the routine production of [^{18}F]F-DA for human use. Validation of uptake by neuroblastoma (NB) cell lines supports the use of [^{18}F]F-DA for imaging NB patients. A pediatric NB imaging trial using [^{18}F]F-DA PET has been approved (IND #138638) based on the methods reported here. We expect [^{18}F]F-DA will be localized in NB tumors and that high quality functional images will be obtained within minutes after injection.

Key Words: Fluorine-18, 6-[^{18}F]Fluorodopamine, F-DA, Neuroblastoma, PET

1. INTRODUCTION

Neuroendocrine tumors arise from the sympathetic nervous system and share many of the same biochemical pathways and proteins as these neurons.[1] As such, the nerve-like phenotype of these tumor cells can be exploited for functional imaging using radiotracers originally designed for neuronal applications. The norepinephrine transporter (NET) is highly expressed in most neuroblastomas (NB) and pheochromocytomas (PHEO), where NET actively transports norepinephrine and its analogs across the cell membrane.[2, 3] This uptake is also influenced by the vesicular monoamine transporter (VMAT), which is known to transport monoamines for storage in synaptic vesicles.[4]

Currently, evaluation of NB depends on a combination of anatomical imaging using computed tomography (CT) and functional imaging by single photon emission computed tomography (SPECT) using *meta*-[^{123}I]iodobenzylguanidine (MIBG). MIBG uptake into NB cells is also mediated by NET, however, several experimental parameters reduce the quality of current NB imaging, including: 1) poor image resolution of SPECT compared to PET, 2) complex quantification, and 3) the relatively high lipophilicity of MIBG prolongs non-specific retention resulting in higher background and poor contrast. The ~24 hour delay required for tumor visualization presents a practical burden to patients and limits the clinical utility of MIBG. 6-[^{18}F]Fluorodopamine, ([^{18}F]F-DA), is a catecholamine that is actively taken into cells via NET. Recent clinical PET-CT imaging of [^{18}F]F-DA in adults with neuroendocrine tumors shows that this tracer possesses high specificity and improved sensitivity vs. MIBG SPECT-CT for visualization of disseminated disease.[5-7] Because [^{18}F]F-DA is less lipophilic than MIBG, background activity clears quickly. Thus, we expect that [^{18}F]F-DA will be highly concentrated in NB tumors, and that utilizing modern PET-CT

methods, high quality functional images will be obtained within minutes after injection, eliminating the need for patients to return for next-day imaging as with MIBG.

Investigation of [^{18}F]F-DA in pediatric NB patients has been limited by the inability to produce usable quantities of tracer that pass the rigorous quality testing required for injection into children. A key stumbling block has been access to [^{18}F]F-DA that possesses high molar activity. The pharmacokinetics and pharmacodynamics of [^{18}F]F-DA have been extensively studied and validated in adults with neuroendocrine tumors[6, 8, 9]. The focus of this investigation was to develop a facile method for nucleophilic production of this tracer in high molar activity and sufficient radiopharmaceutical quality to allow pediatric use. Currently, the only synthesis of [^{18}F]F-DA for human use in the U.S. is achieved by the fluorodestannylation of a 6-aryl trimethylstannyl precursor using [^{18}F]F_2 gas.[10] Sufficient yields for patient studies are attainable, but due to the target gas containing 3% F_2, molar activities are low (50.32 GBq/mmol, 1.36 Ci/mmol). As a result, dose activities must be limited to avoid injection of a physiologically active, high mass dose of non-radioactive fluorodopamine present in the final product. This electrophilic synthesis, while direct, necessitates the purchase of a dedicated F_2 target and the appropriate equipment for handling corrosive fluorine gas for targetry and chemistry. A more recent report presented an updated method with improved molar activity of the final product while still using an electrophilic method.[9]Despite this improvement, the synthesis still requires the use of specialized equipment for corrosive fluorine gas. Furthermore, use of tin-based precursors requires additional testing during quality control to ensure that all residual tin has been effectively removed.

The first nucleophilic preparation of [^{18}F]F-DA, reported by Ding and Fowler in 1991, was a manual, 120 min synthesis that provided [^{18}F]F-DA in 10% yield (EOS).[11] The impetus for this research was to investigate no-carrier-added [^{18}F]F-DA as a tracer for imaging

cardiac sympathetic denervation in baboons.[12-14]. This multistep, tour-de-force synthesis was recently automated in our lab[15]; however, the complexity of this synthesis limits the robustness and feasibility for clinical studies.

The proven utility of [^{18}F]F-DA for imaging neuroendocrine tumors has stimulated efforts to find an efficient, reliable nucleophilic route for this tracer. For example, Preshlock and coworkers described a synthetic approach in which aryl boronic esters react with [^{18}F]fluoride in the presence of copper ion catalyst.[16] While isolated yields of [^{18}F]F-DA were sufficient (29%), deprotection reaction conditions were harsh (hydroiodic acid and temperatures > 150 °C), and analyses of the final product for residual copper catalyst or Kryptofix® 222 were not included. Zlatopolskiy and coworkers described radiofluorination of an aryl nickel(II) complex by [^{18}F]fluoride in the presence of a hypervalent iodine oxidant.[17] A radiochemically pure product was achieved with a radiochemical yield of 12% (6.3% activity yield). Radiochemical yield suffered in part due to the duration of the synthesis (100 min).

Development of a highly selective radiofluorination methodology and an appropriate diaryliodonium salt precursor by Neumann and coworkers provided a reliable means for nucleophilic production of [^{18}F]F-DA.[18, 19] Since the introduction of this chemistry, improvements in precursor synthesis and protecting group strategies have enabled milder reaction conditions to be used in the production of ^{18}F-labled catecholamines.[20] The work presented here describes an efficient, one-pot automated synthesis of [^{18}F]F-DA that features milder reaction conditions, fewer synthetic steps, a new, acid-labile precursor, and improved final purification (Scheme 1). These advances have made possible the routine production of [^{18}F]F-DA, which meets USP <823> specifications and is available for pediatric human use. Finally, we show preliminary in vitro data to support the accumulation of [^{18}F]F-DA in NB cell lines.

[SCHEME 1.]

2. MATERIALS AND METHODS

2.1. General

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise noted. All aqueous solutions were prepared with distilled, deionized water (Milli-Q Integral Water Purification System, Millipore Corp.; 18.2 MΩ·cm resistivity). The purified precursor and organic solvents for radiosynthesis were stored in an anaerobic chamber (Coy Laboratory Products, Inc.) under argon to reduce exposure to atmospheric water. Radioactive samples were analyzed in a CRC-15R Dose Calibrator (Capintec, Inc.) for determination of activity (expressed in megabecquerels or millicuries). Cell uptake assay samples were counted for radioactivity (decays per minute) in a WIZARD2™ 2480 Automatic Gamma Counter (PerkinElmer Life and Analytical Sciences). Purification of the final radiotracer was achieved using a custom-built injector system and an Agilent 1100 Series high performance liquid chromatography (HPLC) pump. Thin-layer chromatography (TLC) samples were analyzed for radioactivity using an AR-2000 radio-TLC Imaging Scanner (Eckert & Ziegler - Bioscan) equipped with WinScan 3 image analysis software. Analytical HPLC was performed on an Agilent 1200 Series LC System using both diode array detection and a Bioscan Flow-Count radionuclide detector (Eckert & Ziegler - Bioscan). Gas chromatography was conducted on an Agilent 7890A GC System.

2.2 Synthesis of (2-{2-[bis(tert-butoxycarbonyl)amino]-ethyl}-4,5-bis-(ethoxy-methoxy)-phenyl)(4-methoxyphenyl)-iodanium triflate (1)

Preparation of the iodonium salt precursor, known as ALPdopamine™, was previously reported in the literature.[21, 22] Due to the high purity of precursor needed for radiochemical syntheses, a survey of different solvents and conditions led to simple recrystallization from methyl-*tert*-butyl ether (MTBE) which provided material of > 99% (by HPLC) chemical purity.

2.3 Synthesis of 6-[^{18}F]fluorodopamine

Briefly, [^{18}F]F-DA was produced in a two-step synthesis by thermolysis of a diaryliodonium [^{18}F]fluoride and subsequent deprotection of the resulting [^{18}F]fluoroarene (Scheme 1). The method was adapted for preparation on a Synthera® automated synthesizer (Version 1, IBA) using an Integrated Fluidic Processor™ (IFP) originally designed for nucleophilic production of [^{18}F]fluorodeoxyglucose (FDG) as shown in Figure 1.

[FIGURE 1.]

Aqueous [^{18}F]fluoride, produced in an IBA Cyclone® 18/9 cyclotron from ^{18}O-enriched water, was passed through a QMA cartridge (46 mg; ORTG, Inc. or Myja Scientific - pretreated with 2 mL of 1 M sodium bicarbonate and rinsed with 5 mL water) to capture the [^{18}F]fluoride. The [^{18}F]fluoride was eluted from the QMA resin with 620-650 μL of one of three phase-transfer reagent solutions: (1) 94:6 acetonitrile:water solution containing potassium carbonate (0.62 mg) and Kryptofix® 222 (6.2 mg), (2) 1.5 - 3.4 mg

tetraethylammonium bicarbonate (TEA HCO_3) dissolved in acetonitrile:water (95:5), or (3) 50-150 uL (1.5 - 3.4 mg) of tetrabutylammonium bicarbonate (TBA HCO_3) solution (ABX, provided as 0.075 M in water and ethanol for stabilization) diluted to 650 µL with acetonitrile. The solvents were removed under argon flow with reduced pressure and heating at 110 °C for 2.5 minutes. The diaryliodonium salt precursor (**1**, ALPdopamine™), dissolved in 10% anhydrous acetonitrile and toluene (15 mg in 1 mL), was added to the reactor for ion-exchange at room temperature followed by heating to 120 °C for 8 minutes to allow for thermolysis of the fluorinated complex. The solvent was removed under reduced pressure with argon flow (approx. 3 minutes) and deprotection of the fluorinated intermediate (**2**) was achieved by addition of 1 mL of 4 M hydrochloric acid and heating to 95 °C for 20 minutes to afford the crude 6-[^{18}F]fluorodopamine product (**3**). The solution was diluted by the addition of 4 mL of HPLC eluent (details below), then passed through a 0.45 µm filter and into a 5 mL HPLC loop. Purification was achieved by semi-preparative HPLC utilizing a Hamilton PRP-1 column (10 x 250 mm, 10 µm) with an eluent of 0.1% acetic acid and 0.02% ascorbic acid at a flow rate of 3 mL/min. The desired product was collected in 3.3 mL of eluent at a retention time of approximately 8-12 minutes. The retention time of the desired peak was shifted 3 minutes later when using the tetra-*n*-alkyl ammonium salts vs. Kryptofix 2.2.2. Peak collection was initiated based on monitoring with a radioactivity detector and collected for 1 minute 10 seconds. Neutralization of the final product was achieved by the addition of 2 mL of 50 mM ammonium acetate (pH 9.2), resulting in a final pH of 5.5 and an osmolarity of 60.4 mOsm/L.

2.4. Quality Control

Full quality control testing was designed to comply with the published FDA requirements for release.[10] In addition to the detailed testing described below, tests for pH, color and clarity, radionuclidic identity, filter integrity, bacterial endotoxins, and sterility were performed. The final product was confirmed to be clear and colorless with no visual evidence of cloudiness or particulate matter as per USP <823> and USP <631> Color and achromicity. After the product [^{18}F]F-DA was collected, the sterilizing filter was tested for filter integrity to give an indication of likelihood of product sterility by using a bubble point procedure, whereby the sterilizing filter was placed on a gas line with a pressure gauge and the outlet of the filter is placed under water. The gas pressure on the inlet to the filter was increased slowly until a steady stream of bubbles was observed at the filter outlet. The pressure when the bubble stream began had to be ≥ 50 psi to pass filter integrity. The pH of the final formulation of [^{18}F]F-DA was tested by spotting on a narrow range pH indicator strip and the color was compared to the color range chart provided by the manufacturer. The radionuclidic identity was determined on a small aliquot of the final product by observing the radioactivity half-life ($t_{½}$) using a Capintec CRC-15PET dose calibrator with measurements taken at intervals of every 10 minutes over a period of 20 min and the half-life was determined.

The final drug product was tested for the presence of bacterial endotoxins utilizing the Endosafe®-PTS™ unit to determine the endotoxin concentration in a sample. Sterility was tested using the direct inoculation method as is recommended by the USP, and the samples were observed over 14 days. Positive growth is indicated by cloudiness in the culture media.

2.4.1. Radiochemical Purity & Molar Activity

Radiochemical purity of 6-[^{18}F]fluorodopamine was confirmed by analytical HPLC using an Agilent Zorbax SB-Aq column (4.6 x 150 mm, 5 μm) with an eluent of 10% acetonitrile and 0.1 M monosodium phosphate monohydrate, 0.27 mM disodium ethylenediaminetetraacetic acid (EDTA) dihydrate, and 0.92 mM octanesulfonic acid (pH 3.5) at a flow rate of 1 mL/min with UV monitoring at 220 nm. Initially, a range of 6-fluorodopamine (ABX) standard concentrations was analyzed to confirm linearity of the UV absorbance curve and to determine the lowest detectable injected mass for this system. Subsequently, standards of known concentration (200 μg/mL) were run before and after each [^{18}F]F-DA sample to confirm system suitability and to calculate the amount of F-DA mass in the sample, if present. The area of any UV peak that corresponded to the retention time of the standard was used to extrapolate the mass present in the sample. The radioactivity of the sample was also measured, and together, these values were used to calculate the molar activity.

2.4.2. Volatile Organic Impurities

Volatile organic impurities in the final product were determined by analyzing a small aliquot (0.5 μL) of the [^{18}F]F-DA final formulation by gas chromatography (GC) using a Carbowax column (J & W 122-7032; 30m x 250 μm x 0.25 μm). The initial oven temperature was held at 60 °C for 1 minute then the temperature was ramped to 140 °C at a rate of 40 °C/min over 3 minutes, followed by an additional minute at 140 °C for a total of 5 minutes. The front inlet heater was held at 250 °C, the split ratio was 15:1, hydrogen flow was 40 mL/min, and the air flow was 400 mL/min. The GC peak retention times and areas were compared to standards of toluene (100 ppm (v/v)), acetonitrile (100 ppm), ethanol (1000 ppm), and ammonium acetate (1000 ppm). The amount of each volatile solvent was

calculated based on the ratio of peak areas for the sample vs. the standard. Standards were analyzed before and after the [^{18}F]F-DA sample to confirm system suitability.

2.4.3. Residual Phase Transfer Agent

To confirm that the toxic phase transfer reagent, Kryptofix® 222, had been sufficiently removed from the final formulation of [^{18}F]F-DA, a visual comparison, color spot test was performed.[23] Silica test strips were prepared in advance with an acidic iodoplatinate solution and spotted with 2 μL each of a 0, 20, and 50 μg/mL solution of Kryptofix® 222. Standard spots were visually compared to 2 μL spots of [^{18}F]F-DA final product to assess if they complied with the US Pharmacopoeia (USP) recommendation of < 50 μg/mL.[24]

Residual TEA HCO_3 was determined via ion-pair HPLC with indirect UV detection using 4-aminophenol HCl and 1-heptanesulfonic acid as background UV absorbing regents based on a method by Zou et al.[25] A series of injections (0.05 to 5 μg, 0.1 to 10 μL) of TEA HCO_3 were performed using an Agilent Zorbax XDB-C18 column (5 μm, 4.6 x 150 mm) and a flow rate of 1 mL/min with 20% methanol , 0.7 mM 4-aminophenol HCL, and 0.15 mM 1-heptanesulfonic acid as the eluent. The standards were prepared in a solution containing HPLC purification eluent and neutralization buffer (0.1% acetic acid, 0.02% ascorbic acid, 50 mM sodium bicarbonate). The area of the negative peak near 3.2 minutes (230 nm) was plotted vs. injected mass to create a linear curve. The equation of this line was used to determine the residual TEA HCO_3 mass in [^{18}F]F-DA samples based on the area of the same negative peak. In addition, a second experiment was performed to confirm the predictive power of this method by injecting eight known amounts of TEA HCO_3 (0.2 to 3.5 μg) and comparing the calculated mass value to the actual mass.

Residual TBA HCO_3 was determined by visual spot test on silica by over-spotting each sample with a MeOH/NH_4OH solution and developing in an I_2 chamber by adapting the

method of Kuntzsch et al.[26] Reference standards of 0, 50, 100, 150, and 200 µg/mL TBA HCO_3 were prepared in a solution of HPLC purification eluent and a neutralization buffer (0.1% acetic acid, 0.02% ascorbic acid, 50 mM sodium bicarbonate). Each standard was spotted (4 µL) on a glass-backed silica TLC plate. After drying, 10 µL of methanol/25% ammonium hydroxide solution (9:1) was applied on top of each spot. The plate was then placed in an iodine chamber to develop for one minute. Samples were visually compared to reference standards for interpolation of TBA HCO_3 concentration.

2.5. NB Cell Uptake

All cell culture reagents were purchased from Invitrogen and cells were cultured in humidified atmospheric conditions (37 °C, 5% CO_2). Two neuroblastoma cell lines were selected based on their differential NET expression and demonstrated uptake of norepinephrine. SH-EP cells - with virtually no expression of NET[27, 28] and non-detectable uptake of norepinephrine[29] - were kindly provided by Dr. Eva L. Feldman, M.D., Ph.D., University of Michigan (Ann Arbor, MI) and grown in high glucose Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), pyruvate and 2 mM glutamate. SK-N-BE(2)-C cells showed expression of adrenergic neuronal markers and significant uptake of norepinephrine[29] and were obtained from American Type Culture Collection (ATCC® CRL-2268™). These cells were grown in a 1:1 mixture of Eagle's MEM and F-12 medium containing 10% FBS and 2 mM *L*-glutamine. Cells were seeded approximately 16 hours prior to the experiment in 35 mm, 6-well plates (BD, Falcon) at a density of 8 x 10^5 cells/well. Wells were treated with 0.185 MBq (5 µCi) of 6-[^{18}F]fluorodopamine in 1 mL of media and incubated for 2, 10 and 30 minutes at 37 °C. The reaction was terminated by aspirating the media and rinsing the wells three times with ice-cold phosphate-buffered saline (PBS), pH

7.4. Cells were incubated for 5 minutes with 0.5 mL 1X cell lysis buffer (Cell Signaling) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. Plates were scraped and lysates were counted in a gamma counter. Each sample was normalized for protein concentration by Pierce BCA protein assay (Thermo Scientific). Error bars denote the standard deviation from three wells.

3. RESULTS

3.1. Synthesis of 6-[^{18}F]fluorodopamine

As [^{18}F]fluoride incorporation into electron-rich aromatic systems has historically been challenging, several radiolabeling reactions were performed and halted prior to deprotection to investigate the effect of the phase transfer agent on the conversion of precursor (1) to ^{18}F-fluorinated intermediate (2). The extent of [^{18}F]fluoride incorporation was analyzed by TLC on silica developed in 30:70 ethyl acetate:hexane and analyzed for radioactivity. Unreacted fluorine-18 remained at the origin on the plate while the fluorinated intermediate (2) migrated (R_f = 0.4 - 0.45). Utilizing a Kryptofix® 2.2.2 as the phase transfer agent (620 µL of a 94:6 acetonitrile:water solution containing 6.2 mg of K222 and 0.62 mg of K_2CO_3), [^{18}F]fluoride incorporation averaged 72.2 ± 3.7% (n = 4; Figure 2).

[FIGURE 2.]

A second phase-transfer agent, tetraethylammonium bicarbonate (TEA HCO_3), was dissolved in 650 µL acetonitrile:water (95:5) at 1.5 - 3.4 mg per reaction. Conversion did not proceed reliably with less than 2 mg (5.1% and 32.4% for 1.5 and 1.75 mg, respectively), but

resulted in approximately 55% conversion for 2.0, 2.5, and 3.4 mg TEA HCO_3. A third phase-transfer agent, tetrabutylammonium bicarbonate (TBA HCO_3), was purchased as a 0.075M solution in water and ethanol (for stabilization). Varying amounts of this solution (50 - 150 µL,1.5 - 3.4 mg) were diluted to 650 µL with acetonitrile. This reagent resulted in at least 20% higher conversion of the intermediate over TEA HCO_3 with 74.3% to 84.9% for 2.0 to 3.4 mg.

The complete synthesis of [^{18}F]F-DA was achieved using an IBA Synthera® and a single nucleophilic fluorination cassette (IFP™) in 56-60 minutes, including HPLC purification. For syntheses using Kryptofix® 222/K_2CO_3 solution as a phase transfer agent, radiochemical purity of the crude [^{18}F]F-DA prior to HPLC purification was 53.1 ± 0.9% (n = 3), and the HPLC purified product was obtained in 22.9 ± 3.7% radiochemical yield at EOS (n = 19). Full syntheses using the tetraalkylammonium compounds resulted in similar, radiochemical yields (uncorrected) of [^{18}F]F-DA (Figure 3).

[FIGURE 3.]

Syntheses with TEA HCO_3 as the phase-transfer agent resulted in a purified [^{18}F]F-DA yield of 23.0 ± 3.8% (2.5 mg TEA HCO_3 , n = 9) and 22.5 ± 3.4% (3.4 mg TEA HCO_3, n = 8). Finally, the highest yields were achieved with TBA HCO_3 as the phase-transfer agent and resulted in a yield of 25.8 ± 2.6% (2-2.5 mg, n = 12) resulting in a significant improvement over Kryptofix® 222/K_2CO_3 (p = 0.025).

3.2. Quality Control

Full quality control testing was designed to comply with FDA-approved acceptance criteria for [^{18}F]F-DA previously reported for the electrophilic method in preparation for

human use[10] (see Table 1.) Standard tests for visual appearance, pH, radionuclidic identity, bacterial endotoxins, filter integrity, and sterility all passed without issue.

[TABLE 1.]

3.2.1. Chemical and Radiochemical Purity

The chemical and radiochemical purity of the final product was confirmed by analytical HPLC by comparison to a 6-fluorodopamine standard (ABX). Radiochemical purity was determined to be > 97% in all cases. In the samples analyzed for molar activity (n = 3), a UV peak correlating to the retention time of fluorodopamine showed an average mass concentration of 2.4 ± 1.1 μg/mL. These measurements were calculated on typical syntheses for the preparation of [^{18}F]F-DA for in vitro or in vivo experiments resulting in an average molar activity of 42.5 ± 28.7 TBq/mmol (1148 ± 776.2 Ci/mmol) with an average starting activity of 10.8 ± 0.48 GBq (293 ± 12.9 mCi) of fluorine-18. While no mass peak matching the specific retention time of fluorodopamine was present, a small peak within 0.2 min was observed. To be conservative in our determination of injected mass, this peak was attributed to fluorodopamine for the calculation of mass. Therefore, the calculated molar activity was likely much lower than the actual value, and in either case remained well above the lower limit permitted for injection.

3.2.2. Volatile Organic Impurities

The amount of each volatile solvent present in the final product was calculated based on the ratio of peak areas for the [^{18}F]F-DA sample vs. the standard values for analysis by gas chromatography (n = 3). All organic compounds visible on the gas chromatograph were significantly below allowable limits established by the United States Pharmacopoeia (USP).

Ammonium acetate was visible in the final product as a known excipient added as a diluent and buffer solution. Toluene was not detected in any of the samples (limit: ≤ 890 ppm), while trace amounts of ethanol (70 ± 90 ppm) and acetonitrile (10 ± 20 ppm) were seen if at all. USP limits for ethanol and acetonitrile are ≤ 5000 ppm and ≤ 410 ppm, respectively.

3.2.3. Residual Phase Transfer Agent

Assessment of the residual phase transfer agent was key to the optimization of this radiotracer for human use. Initial syntheses using Kryptofix® 222 resulted in levels of residual phase-transfer agent in the final product (> 200 µg/mL) which were significantly higher than the USP limit of 50 µg/mL. Multiple attempts to remove the Kryptofix® 222 from the final product while maintaining the one-pot reaction method proved unsuccessful. As a result, other phase transfer reagents were tested. The [^{18}F]F-DA product using TBA HCO_3 consistently contained < 50 µg/mL residual TBA HCO_3 when visually compared to reference standards (n = 8) based on the method of Kuntzsch et al.[26] (Figure 4.) This qualitative, visual test was quick and easy to perform making it amenable for quality control analysis during production for human use.

[FIGURE 4.]

Since the visual spot test was not effective for analysis of TEA HCO_3, an HPLC method employing a linear standard curve of injected masses between 0.05 and 2.5 µg was performed (R^2=0.998). The equation of this line was used to determine the residual TEA HCO_3 mass in [^{18}F]F-DA. By dividing the determined mass by the injected volume, the result was an average concentration of 48±7 ug/mL (n = 8, Figure 5).

[FIGURE 5.]

To confirm the accuracy of the predicted values from the standard curve of TEA HCO_3 injections, eight injections of known mass were performed over the range of the curve. Each calculated value was compared to the known mass injected and resulted in an average error of 4.8% (Figure 5). Of note, samples within the range of mass seen in [^{18}F]F-DA samples (0.5 - 1.5 µg injected) showed an error of only 1.3%.

[FIGURE 6.]

3.3. NB Cell Uptake

Since NB tumors are known to have high expression of NET[3], it was hypothesized that uptake of [^{18}F]F-DA would be significant in these tumor models. To test this in vitro, we selected two NB cell lines (SH-EP and SK-N-BE(2)-C) confirmed to have varying uptake of norepinephrine[29], and [^{18}F]F-DA accumulation was measured as a preliminary determination of the effectiveness of this tracer in this tumor type. We assessed transporter selectivity by comparing uptake in the SH-EP model, known as a NB cell line with minimal NET expression[28], to that in SK-N-BE(2)-C, which has higher expression of NET. Even at two minutes, accumulation of [^{18}F]F-DA in the SK-N-BE(2)-C cell lines was significantly higher than the control SH-EP cells, as is seen in Figure 7 ($p < 0.0001$). This distinction continued over the course of 30 minutes resulting in a >4-fold difference in accumulation in SK-N-BE(2)C vs SH-EP.

[FIGURE 7.]

4. DISCUSSION

Recent successes in improving the delineation of neuroendocrine tumors with PET imaging and ^{18}F-labeled catecholamines and analogs targeting NET[30-33] have created a demand for more efficient and routine syntheses of these tracers. We previously reported a reliable synthesis method for producing [^{18}F]F-DA that was compatible with multiple automated systems.[34] However, we sought to improve upon this method by developing a precursor and synthesis that would not require the use of corrosive acids such as HI or HBr and that would eliminate the need for organic solvents in the purification. In addition, we wanted to design a route that would allow a one-pot reaction on a single Synthera® system rather than a two-pot synthesis in which the synthesizers are connected in series. Further, we wanted to validate a full quality control method suitable for pediatric imaging applications. Building on our work optimizing the synthesis of [^{18}F]MFBG[35], we took a methodical approach to streamline and validate the synthesis of [^{18}F]F-DA and subsequent quality control testing.

Our previous method demonstrated that up to 50% of the [^{18}F]fluoride radioactivity delivered to the reactor adhered to the glass in the presence of toluene. To overcome this, the vial was pretreated with a base wash and thoroughly rinsed prior to use. This was effective in reducing the retention of activity by 15-20%, however, addition of this step was not easily amenable to clinical production. Previously, we demonstrated that the reaction of diaryliodonium salts with [^{18}F]fluoride in 100% acetonitrile resulted in poor yields of intermediate[34] (2), however, acetonitrile dramatically reduced the amount of [^{18}F]fluoride adsorbed to the vial. Based upon these observations and on a series of fluorination experiments performed in various solvents (see Supplemental Information), we determined that adding a small amount of acetonitrile (5-10%) was enough to keep the fluorine

solubilized without inhibiting the reaction. Previously, the precursor (1) was dissolved in acetonitrile to permit ion-exchange of the diaryliodonium triflate to the diaryliodonium [^{18}F]fluoride, and this solvent was removed prior to the thermolysis reaction in toluene. Transition to this new solvent system allowed solubilization of the precursor directly in the thermolysis solvent thereby eliminating one reagent needed for the synthesis. The subsequent elimination of an intermediate silica SepPak purification allowed the entire synthesis to be performed with only four reagents - phase-transfer solution, precursor (1) in 10% acetonitrile/toluene, 4 M HCl, and HPLC eluent for dilution - thus, allowing use of a single Synthera® IFP™.

In our prior work, purification of crude [^{18}F]F-DA (3) was achieved utilizing a C-18 column and an eluent of 20% ethanol and 55 mM citric acid. While the purification was effective, varying amounts of unreacted [^{18}F]fluoride were retained in the column, which prohibited an accurate assessment of the crude purity. In addition, the final product required significant dilution to reduce the ethanol concentration to acceptable levels for injection into pediatric patients. A transition to the Hamilton PRP-1 column with an aqueous eluent (0.1% acetic acid, 0.02% ascorbic acid) eliminated the need for dilution and reduced the amount of unreacted [^{18}F]fluoride retained in the column, including unreacted [^{18}F]fluoride salts in the same amount of time.

The optimized one-pot synthesis resulted in activity yields slightly lower than that previously reported for the two-pot process (35 ± 4%).[34] However, the operationally simpler one-pot process increases feasibility, maintains reproducibility, and produces quantities of the radiotracer sufficient for clinical studies.

Phase-transfer reagent modification did require optimization; when Kryptofix® 2.2.2 was used as the phase-transfer reagent, the lack of intermediate purification gave rise to incomplete removal of the kryptand. Quality control results showed high levels of residual

Kryptofix® 222, significantly above the release limit established by the USP (50 µg/mL per injection). Our investigation concluded the source of increased Kryptofix® 222 in the final product solution was due to co-elution of [^{18}F]F-DA with Kryptofix® 222; any attempts to remove residual Kryptofix® 222 also removed the desired product. Due to the lower-toxicity of alternate phase-transfer catalysts, TBA HCO_3 and TEA HCO_3 [36-40], we assessed what effect these regents had in the thermolysis reaction to produce (#, Scheme 1). No significant effect on [^{18}F]fluoride ion reactivity or overall yield of [^{18}F]F-DA was observed using tetra-*n*-alkyl ammonium salts as phase-transfer reagents. It is worth noting that use of TBA HCO_3 did result in a three-minute delay in the elution of the final product from the semi-preparative HPLC column. This likely results from interaction of the tetraalkylammonium salts with the non-polar polyvinyl styrene/divinyl benzene matrix of the Hamilton PRP-1column. When tetraalkylammonium ions are used in the buffer, these cations interact with the hydrophobic support in proportion to the length of the alkyl substituents (*i.e.* greater chain length, greater column surface interaction). The interaction of the tetrabutylammonium ions with the stationary phase likely leads to a surface modification which causes the PRP-1 column to behave more like a standard reverse phase column and leads to greater retention of the desired fluorodopamine product.

Methods for determining residual amounts of TEA HCO_3 or TBA HCO_3 in the final product were validated and they demonstrated that approximately 0.05 mg/mL remained for either of the agents. While the USP does not provide a recommended release limit for either compound, the European Pharmacopoeia does have an established release limit of 2.6 mg/V (V = injected volume) for TBA HCO_3.[41] Moving forward, syntheses will be performed with no more than 2.0 mg of TBA HCO_3 so that even if all of the reagent were to be carried through to the final product, this recommended release limit could not be reached with a maximum injection of 10 mL.

An average mass concentration of [^{18}F]F-DA in the final product was determined to be approximately 2.5 µg/mL which is at least 100-fold less than the average mass of 3.4-5.5 mg/dose reported via the F_2 method currently in clinical use[10] and more than 1000 times less than the injectable mass limit of 6 mg/dose. This significant improvement in molar activity will allow clinicians to administer doses based on dosimetry and image quality rather than being restricted to lower activities due to a physiologically relevant mass of fluorodopamine approaching the injectable limit.

[^{18}F]F-DA has not been specifically tested in NB patients, thus, we sought confirmation of specific accumulation in NB cell lines in vitro. Over the course of 30 minutes, [^{18}F]F-DA uptake in the high NET expressing cells was an average of 7-fold higher than that of the low expressing control cells. While some accumulation was observed in the SH-EP cells, suggesting involvement of other transport mechanisms, the 7-fold higher accumulation in NET-positive SK-N-BE(2)-C cells provides sufficient evidence to pursue [^{18}F]F-DA as a tracer for NB.

5. CONCLUSIONS

ALPdopamine™ is an effective scaffold for the clinical production of [^{18}F]F-DA. Mild deprotection conditions and a simplified method resulted in greater feasibility and reproducibility in production. Improvements to the synthesis include (1) milder deprotection with HCl, (2) use of a single Synthera®, (3) less retention of [^{18}F]fluoride to the reactor vial, and (4) eliminating the use of organic solvents in purification. These optimizations contributed to consistent activity yields of > 25% with the introduction of TBA HCO_3 as the phase transfer agent. Extensive quality control testing confirmed that [^{18}F]F-DA prepared via this method met the published release criteria for human use. This new procedure has

subsequently been approved for use in an imaging trial of pediatric patients (IND #138638). Preliminary in vitro studies validate uptake by NB cell lines expressing NET and suggest that [^{18}F]F-DA is viable as a PET tracer for NB patients.

Disclaimer

Kiel D. Neumann was a consultant for Ground Fluor Pharmaceuticals, Inc., Lincoln, Nebraska during this work and is a shareholder. Stephen G. DiMagno holds a patent for the nucleophilic fluorination of aromatic ring systems, which includes the nucleophilic synthesis of [^{18}F]fluorodopamine via a diaryliodonium salt precursor (US Patent 8,604,213 B2, Dec. 10, 2013) and is a consultant for and a shareholder in Ground Fluor Pharmaceuticals, Inc. Other authors declare no conflict of interest.

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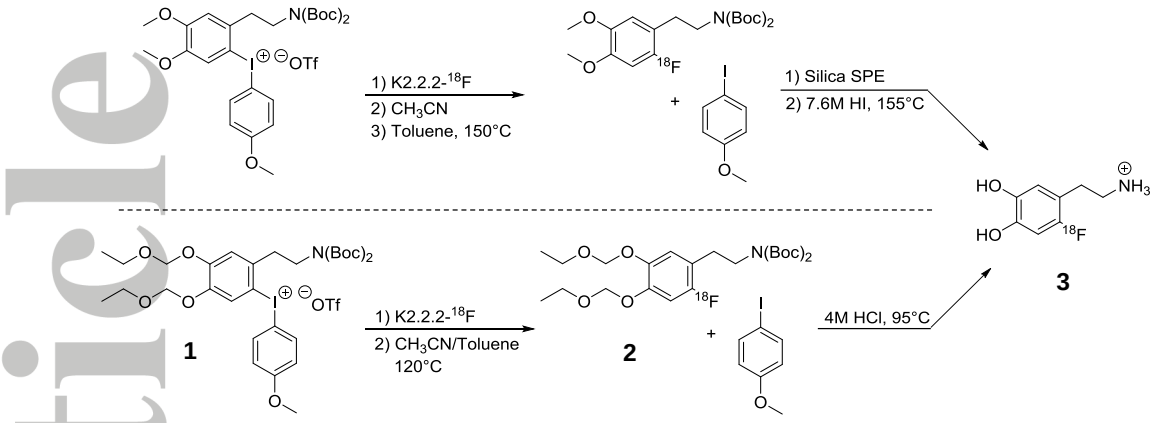
SCHEME 1.



Scheme 1. Synthetic procedures for [^{18}F]F-DA (**3**): previously reported method[19] (top), method presented in this work (bottom). Initial ion exchange at the hypervalent iodine center of the diaryliodonium precursor (**1)** is conducted in 10% acetonitrile and toluene to afford the diaryliodonium-[^{18}F]fluoride complex followed by thermolysis to produce protected intermediate (**2)**. Deprotection of the radiolabeled intermediate (**2**) in HCl produces (**3**).

FIGURE 1.

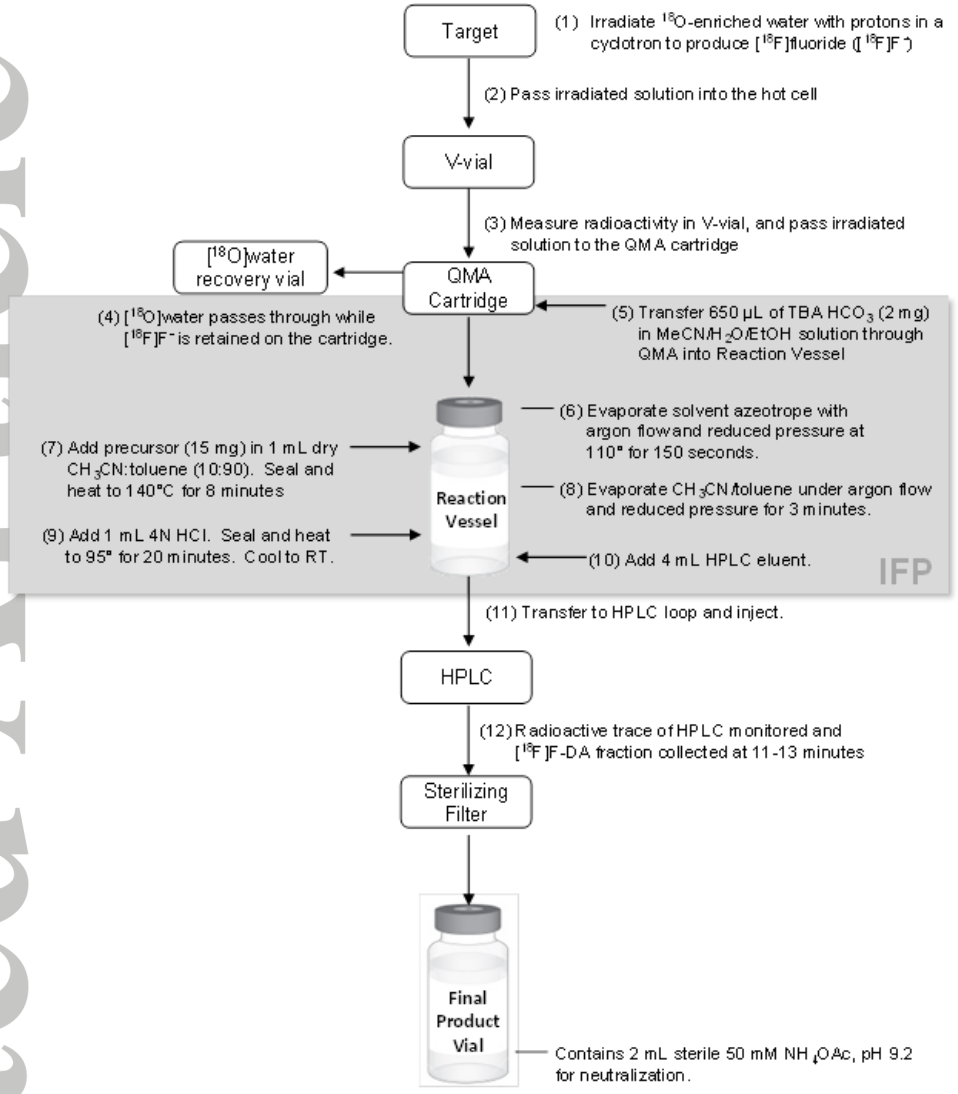


Figure 1. Synthetic steps for production of [^{18}F]F-DA using an IBA Synthera and a nucleophilic fluorination IFP.

FIGURE 2.

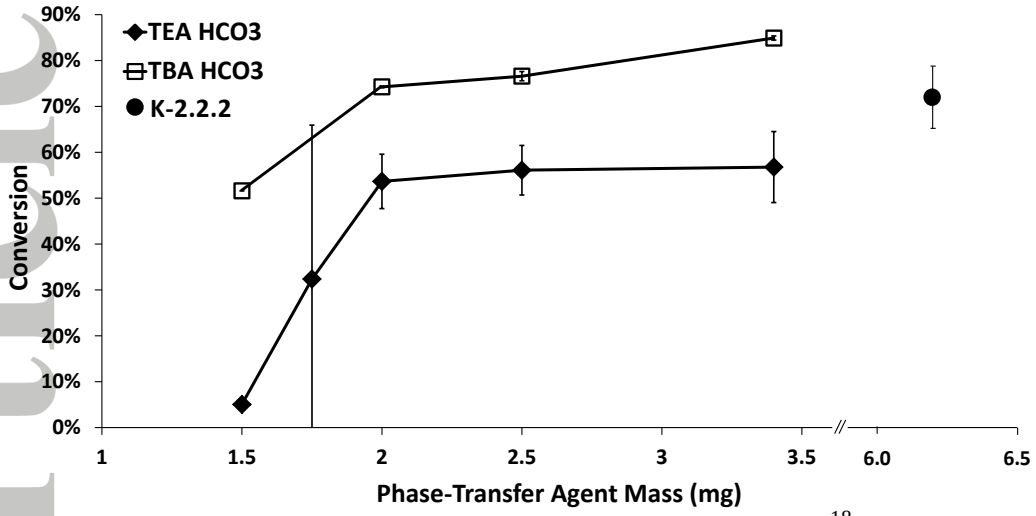


Figure 2. Percent conversion to fluorinated intermediate (protected [^{18}F]F-DA (**2**)) as a function of mass of phase-transfer agent. (n = 2-4 for each time point except 1.5 mg).

FIGURE 3.

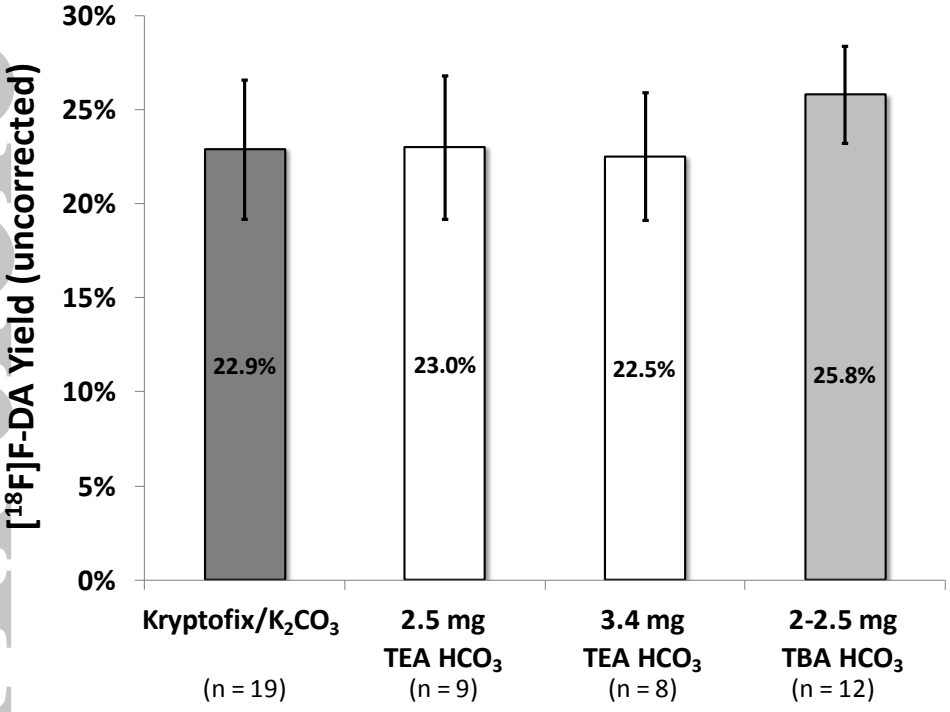


Figure 3. Comparison of [^{18}F]F-DA yield based on phase transfer agent.

FIGURE 4.

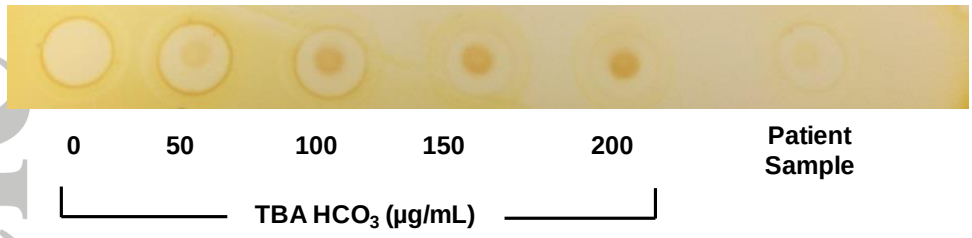


Figure 4. Visual spot test for determination of TBA HCO_3 concentration

FIGURE 5.

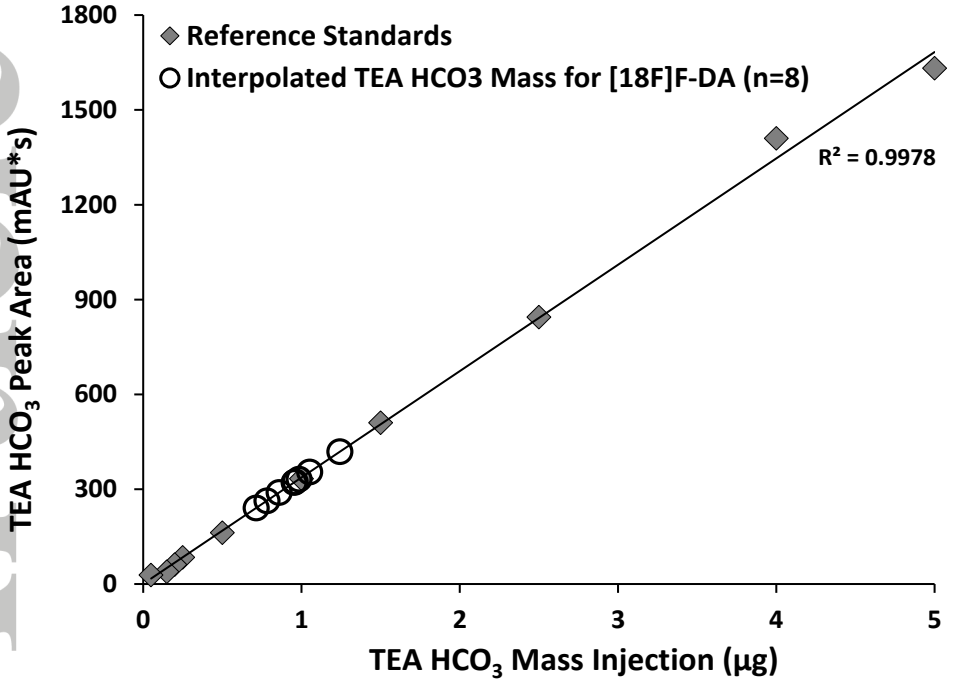


Figure 5. Standard curve for determination of TEA HCO_3 mass in [^{18}F]F-DA samples. The line represents the linear regression for the reference standards. The equation of this line was used with the TEA HCO_3 peak area for each [^{18}F]F-DA sample to calculate the TEA HCO_3 mass injected.

FIGURE 6.

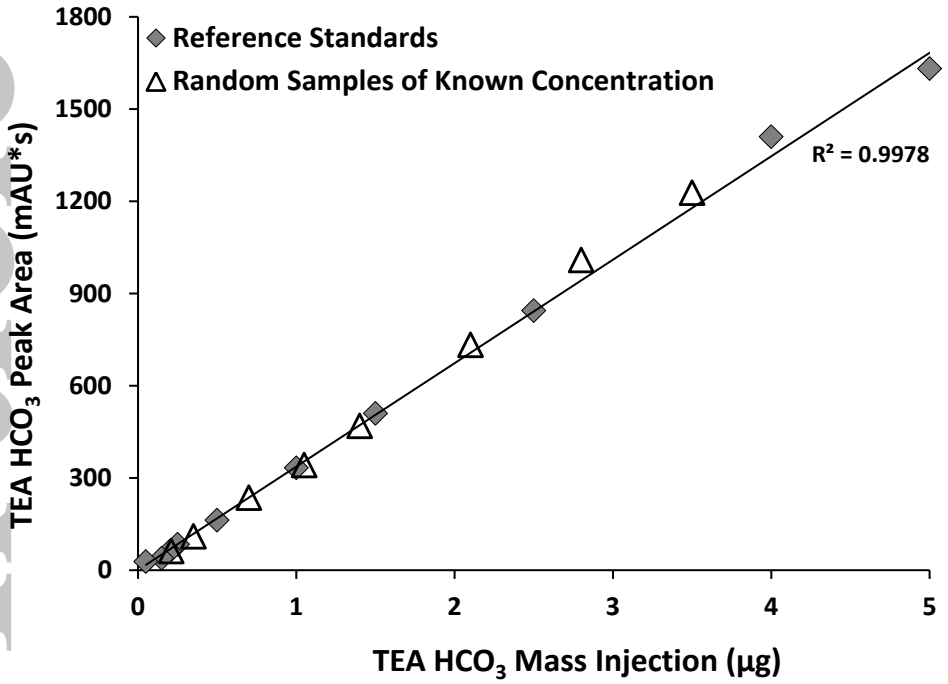


Figure 6. Predictive power of the standard curve for determination of TEA HCO_3 mass in [^{18}F]F-DA samples. The line represents the linear regression for the reference standards. The equation of this line was used with the TEA HCO_3 peak area for each of eight randomly selected but known concentrations to determine the accuracy of the predicted TEA HCO_3 mass.

FIGURE 7.

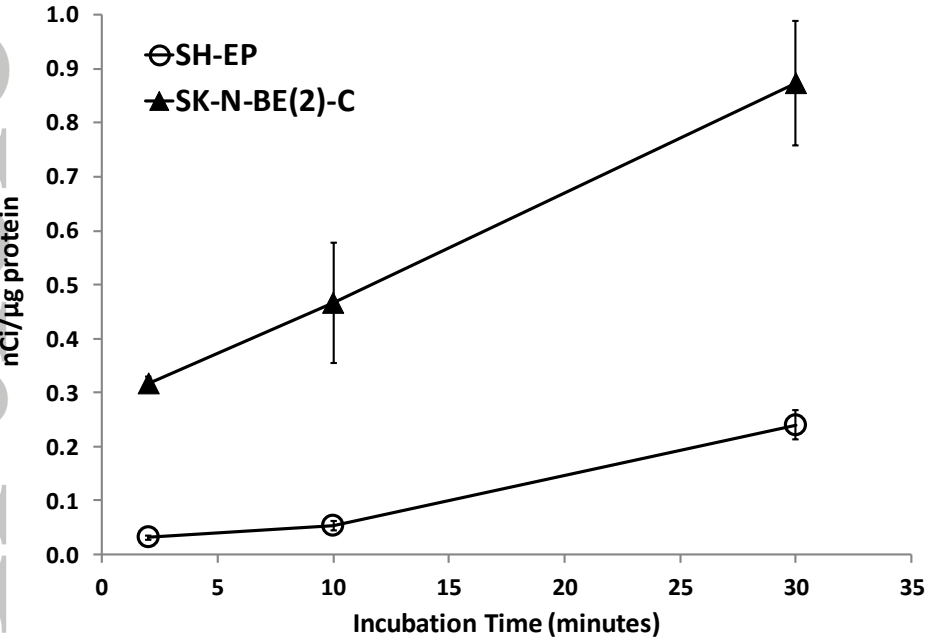


Figure 7. Cell association of [^{18}F]F-DA with SH-EP and SK-N-BE(2)-C NB cell lines over 30 minutes of incubation. Data are normalized to mass of protein in sample and are presented as mean ± standard deviation (n = 3).

Table 1. Comparison of quality control results of the current procedure for [^{18}F]F-DA production compared to the NIH elecrophilic production and the published acceptance criteria for human use.

TEST	ACCEPTANCE CRITERIA	NIH F-DA METHOD*	CURRENT F-DA METHOD**
Visual Appearance	Clear and Colorless	NR	Clear and colorless
pH	4.5 – 7.5	6.4 ± 0.5	5.0 – 5.5
Radiochemical Purity (HPLC)	> 90% [^{18}F]F-DA, RT within 15% of standard	> 99%	96.8 ± 0.1%
Radionuclidic Identity	Observed $t_{½}$ is 105 to 115 minutes (110 ± 5%)	Pass	108.8 ± 1.0 min
Mass Limit	≤ 6.0 mg [^{18}F]F-DA per injected dose	4.49 ± 1.04 mg	0.024 ± 0.011 mg (max, 10 mL dose)
Specific Activity	≥ 0.100 Ci/mmol at time of injection	1.359 ± 0.038 Ci/mmol (EOS)	1148 ± 776.2 Ci/mmol (EOS)
Volatile Organic Impurities	Chloroform ≤ 6 ppm	< LOD	N/A
	Ethyl acetate ≤ 5000 ppm	< LOD	N/A
	Heptane ≤ 5000 ppm	< LOD	N/A
	Ethanol ≤ 5000 ppm	271 ± 74 ppm	7 ± 9 ppm
	Acetonitrile ≤ 41 ppm	N/A	1 ± 2 ppm
	Toluene ≤ 89 ppm	N/A	< LOD
Bacterial Endotoxin (LAL)	≤ 2.5 EU/kg body weight, and ≤ 175 EU per subject	< 1.6 EU/kg	N/A
	≤ 5 EU/mL	N/A	< 2.50 EU/mL
Filter Integrity	≥ 50 psi breaking pressure	Pass	> 60 psi
Sterility	No growth observed in 7 days (BACTEC™)	Pass	N/A
	No growth observed over 14 days	N/A	Not turbid
Radionuclidic Purity	≥ 99.5% ^{18}F	NR	> 99.9%

* Average of 3 reported syntheses[8]
** Average of 3 clinical validation syntheses for IND submission.
NR = not reported; LOD = limit of detection