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Development of an automated, GMP compliant FASTlab™ radiosynthesis of [^{18}F]GE-179 for the clinical study of activated NMDA receptors

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Abstract

N-(2-chloro-5-(*S*-2-[^{18}F]fluoroethyl)thiophenyl)-*N*'-(3-thiomethylphenyl)-*N*'-methylguanidine, ([^{18}F]**GE-179**), has been identified as a promising positron emission tomography (PET) ligand for the intra-channel phencyclidine (PCP) binding site of the *N*-methyl-*D*-aspartate (NMDA) receptor. The radiosynthesis of [^{18}F]**GE-179** at low radioactivity levels has been previously reported.[1] However, the manufacture of a GMP compliant product at high radioactivity levels was required for clinical studies. We describe the development of a process using the GE FASTlab™ radiosynthesis platform coupled with HPLC purification. The radiosynthesis is a two-step process, involving the nucleophilic fluorination of ethylene ditosylate, **11**, followed by alkylation to the deprotonated thiol precursor, *N*-(2-chloro-5-thiophenol)-*N*'-(3-thiomethylphenyl)-*N*'-methyl guanidine, **8**.

The crude product was purified by semi-preparative HPLC to give the formulated product in an activity yield (AY) of 7 ± 2% (n = 15) with a total synthesis time of 120 minutes. The radioactive concentration (RAC) and radiochemical purity (RCP) were 328 ± 77 MBq/mL and 96.5 ± 1% respectively and the total chemical content was 2 ± 1 µg. The final formulation volume was 14 mL.

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The previously described radiosynthesis of [^{18}F]**GE-179** was successfully modified to deliver an process on the FASTlab^{TM} that allows the manufacture of a GMP quality product from high starting radioactivitity (up to 80 GBq) and delivers a product suitable for clinical use.

Background

N-Methyl-D-aspartate (NMDA) receptors are ligand-gated heteromeric ion channels that regulate fast neurotransmission by allowing prolonged influx of Ca^{2+}, Na^{+} and K^{+} ions, into the synapse. The NMDA receptor is part of the glutaminergic excitatory pathway involved in many neurological processes including synaptic transmission and plasticity.[2, 3] Abnormalities in the level of activation of NMDA receptors are known to be involved in the pathophysiology of many diseases, (e.g. epilepsy, neuropathic pain). It is believed that an increased level of activation is important to these disease states rather than the up-regulation of the receptor population.[4-9] A tracer that can image activated channels has potential utility in epilepsy, TBI and other central nervous system (CNS) disorders.

Our group have previously reported the design and evaluation of [^{18}F]**GE-179**,[1] which has been identified as a lead candidate to progress to clinical studies to assess its potential as a PET tracer for imaging activated NMDA receptors.[10-14] The reported precursor synthesis and radiosynthesis were appropriate to support a biological screening program for the identification of the lead compound, however, the manufacture of a GMP quality product was required for clinical studies.[15] The original radiosynthesis[1] was suitable for the preparation of [^{18}F]**GE-179** for pre-clinical studies but was not suitable for delivering a GMP quality product for clinical studies with high starting radioactivity (up to 80 GBq). In order to deliver an automated process capable of delivering a GMP quality product, a number of steps needed to be introduced or updated. These included the development of an [^{18}F]fluoride cartridge trapping and elution regime, and the replacement of the insoluble base with a soluble base to deprotonate the thiol precursor, **8**. Furthermore, the original radiosynthesis[1] was a 2-pot process with two semi-preparative HPLC purifications to purify both [^{18}F]**9** and [^{18}F]**GE-179**. As the FASTlab^{TM} only has one reaction vessel and capacity for one semi-preparative HPLC purification step, the original radiosynthesis was not compatible and required modifications in order to automate the radiosynthesis of [^{18}F]**GE-179**.

In this work, we describe the development of an improved, scalable precursor synthesis and the development of a GMP compliant automated radiosynthesis of [^{18}F]**GE-179** on the FASTlab^{TM} synthesiser using a single use, disposable cassette coupled with semi-preparative HPLC purification.

Methods

Materials and analysis

All reagents and solvents (unless stated otherwise) were purchased from Alfa Aesar (Massachusetts, U.S), TCI Europe (Zwijndrecht, Belgium), VWR International (Lutterworth, UK) or Sigma-Aldrich (Gillingham, UK) and used without further purification. FASTlab^{TM} cassette and consumables were supplied by Clinical Supplies, GE Healthcare (Oslo, Norway). Saline for injection (0.9% w/v) and dehydrated ethanol BP were purchased from Hameln Pharmaceuticals (Gloucester, UK) and Martindale Pharmaceuticals (High Wycombe, UK) respectively. QMA-carbonate-Sep Pak (p/n 186004051) and tC18 SPE (p/n WAT036810) cartridges were purchased from Waters (Elstree, Hertfordshire, UK). Millex GS, 0.22 µm filter unit (P/N SLG5V255F) was purchased from Sigma-Aldrich (Gillingham, UK).

HPLC conditions for radiochemistry

Semi-preparative radio-HPLC was carried out on a Knauer Smartline 1000 system equipped with a Bioscan Flowcount FC3200 sodium iodide/PMT gamma detector (Lablogic, Sheffield, UK) and a Knauer Smartline UV detector.

Analytical HPLC analysis was carried out on an Agilent 1100 series system equipped with a Bioscan Flowcount FC3200 Iodide/PMT gamma detector and Laura 3 software (Lablogic, Sheffield, UK).

Analytical conditions for synthetic chemistry

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were obtained on either a Bruker Avance DPX-400 MHz or a Varian 500 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) with reference to an internal solvent. HPLC was obtained on an Agilent 1100 series HPLC system with UV detection. The analytical HPLC column used was an Xbridge shield Reverse Phase 18, particle size 2.5 µM with 4.6 mm id and a length of 50 mm. Analytical thin layer chromatography (TLC) was carried out using Merck

TLC Silica gel 60 F_{254} aluminum plates. Flash column chromatography was carried out using a Biotage Flash system using pre-packed silica columns or using a glass column packed with Merck Silica gel 60 (0.040-0.063 mm).

Synthesis of thiol precursor (8) and GE-179 reference standard (10)

Synthesis of *N*-(2-chloro-5-thiophenol)-*N*'-(3-thiomethylphenyl)-*N*'-methyl guanidine (8)

The synthesis of **8** and unlabelled standard GE-179 described in our original publication was somewhat challenging and not as scalable or robust as is ideal for a GMP manufacture.[1] In particular, the final debenzylation step was difficult to reproduce at multigram scales. Therefore, an alternative strategy was developed wherein the dimeric molecule **7** was prepared, negating the need for a protecting group that is difficult to remove. The final step of the synthesis was a reduction to cleave the disulfide to give the free thiol. The improved synthetic route is described in Scheme 1 and has been employed to synthesise multigram quantities of **8** for GMP radiosynthesis.

N-(3-(Methylthio)phenyl)cyanamide (2)

3-(Methylthio)aniline (99.8 g, 0.72 mol) was dissolved in diethyl ether (600 mL) in a flask equipped with a gas scrubber containing a sodium hypochlorite solution. The mixture was cooled to 0-5 °C. A solution of cyanogen bromide (38.1 g, 0.36 mol) dissolved in diethyl ether (500 mL) was added drop-wise to the reaction mixture, while maintaining the reaction temperature below 5 °C. Upon complete addition, the reaction mixture was slowly allowed to come to ambient temperature and stirred for a total of 18 h. A solution of hydrochloric acid (550 mL of a 0.028% v/v solution) was added to quench the reaction. The organic phase was washed with an additional portion of HCl (550 mL of a 0.028% v/v solution) and dried over $MgSO_4$, filtered and concentrated *in vacuo* at 20-25 ^oC to afford the product as a brown oil which solidified upon standing (53.0 g, 90%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.23 (d, 1H), 6.96 (dm, 1H), 6.87 (t, 1H), 6.75 (dt, 1H), 6.01 (broad, s, 1H), and 2.48 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.2, 137.7, 130.1 (1C), 121.7, 112.7, 111.9, 110.5, and 15.4. Purity by HPLC (Agilent 1100 series, X-Bridge column) 98.6 % area.

***N*-Methyl-*N'*-(3-(methylthio)phenyl)cyanamide (3)**

The cyanamide **2** (53 g, 0.32 mol) was dissolved in THF (400 mL) and cooled to 0 ºC. Sodium hydride, 14.9 g of a 60% dispersion in mineral oil, 0.37 mol) was washed with heptane (2 x 195 mL) and suspended in THF (145 mL). The NaH suspension was added to the reaction mixture portion wise while maintaining the reaction temperature below 20 ºC. The resulting mixture was cooled to 0-5 °C and methyl iodide (92.3 g, 0.65 mol, 40.2 mL) was added dropwise while maintaining the reaction temperature below 5 °C. The reaction mixture was slowly allowed to come to ambient temperature and stirred for a total of 18.5 h. THF was removed *in vacuo* at 20-25 °C to form a thick paste. The thick paste was suspended in diethyl ether (645 mL), stirred for 45 minutes and filtered. The filtrate was evaporated *in vacuo* at 20-25 °C. The crude material was purified using a Biotage flash system with a pre-packed silica column (400 g silica), using DCM as eluent. The product fractions were combined and concentrated *in vacuo* at 20-25 °C to afford the product as a pale-yellow oil (54.1 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (t, 1H), 6,96 (m, 1H), 6.95 (m, 1H), 6.82, (m, 1H), 3.31 (s, 3H), and 2.48 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.9 (1C), 129.8 (1C), 121.0 (1C), 115.6 (1C), 113.8 (1C), 112.6 (1C) 111.4 (1C), 36.9 (1C), and 15.5 (1C). Purity by HPLC (Agilent 1100 series, X-Bridge column) 98.7 % area.

5,5'-disulfanediylbis(2-chloroaniline) HCl salt (6)

Tin(II) chloride (205 g, 1.08 mol) was dissolved in concentratedhydrochloric acid (495 mL) and added 4-chloro-3-nitrobenzenesulfonyl chloride (25.1 g, 0.01 mol) The resulting mixture was refluxed for 4 h. The reaction mixture was then allowed to come to ambient temperature and stirred overnight. The product was isolated by filtration and dried using suction to afford the aminothiophenol **5** as a pale-yellow solid that was used without further purification. The aminothiophenol **5** was suspended in water (125 mL) and agitated at ambient temperature. An aqueous solution of potassium iodide (59.8 g, 0.36 mol) and iodine (61 g, 0.24 mol) in water (900 mL) was added to the mixture portionwise. The reaction was monitored by HPLC analysis. Upon complete conversion, the pH in the reaction mixture was adjusted to pH 12 by addition of sodium hydroxide (6 M). The mixture was extracted using diethyl ether (3 x 1000 mL) and the combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo* to approximately 1000 mL. HCl in diethyl ether (65 mL of a 2 M solution) was added dropwise. The precipitated solid was collected by filtration and further washed with diethyl ether (100 mL). The solid was dried *in vacuo* overnight to afford the product as an off-white solid (16.3 g, 86%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 7.35 (broad s, 4H), 7.23 (d, 2H), 7.05

(d, 2H), and 6.73 (dd, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ143.8, 134.9, 129.8, 117.3, 116.2, and 113.9. Purity by HPLC (Agilent 1100 series, X-Bridge column) 98.5 % area.

1,1'-(Disulfanediyl*bis*(6-chloro-1,3-phenylene))*bis*(3-methyl-3-(3-(methylthio)phenyl)guanidine) (7)

The cyanamide **3** (21.1 g, 0.12 mol) was heated to 140-145 °C in a reaction flask equipped with a mechanical stirrer. The aniline hydrochloride **6** (11.6 g, 0.03 mol) was added in portions to the melt.. Upon complete addition, the resulting melt was stirred for 5 h, in process HPLC analysis confirmed that the reaction had reached completion. The reaction melt was cooled to ambient temperature and the residue was re-dissolved in chloroform (1055 mL) and washed with hydrochloric acid (4 x 1055 mL of a 0.1% v/v solution). The pH of the combined aqueous phases was adjusted to pH 9 using sodium hydroxide (2M). The aqueous phase was then back-extracted with diethyl ether (4 x 950 mL), and the combined organic phases were dried over $MgSO_4$, filtered and evaporated to afford a yellow oil. The oil was purified by flash chromatographic purification, using silica gel eluting with chloroform added methanol (0-15%) afford the product as a foamy yellow solid (15.27 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (d, 2H), 7.29 (d, 2H), 7.17 (t, 2H), 7.13 (m, 2H), 7.11 (d, 2H), 7.06 (m, 2H), 7.04 (m, 2H), 3.89 (broad s, 4H), 3.34 (s, 6H), and 2.49 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ150.7, 147.7, 144.9, 140.6, 135.9, 130.5, 129.9, 127.3, 124.5, 124.3, 124.1, 123.3, 122.6, 38.8, and 15.6. Purity by HPLC (Agilent 1100 series, X-Bridge column) 95.2 % area.

***N*-(2-Chloro-5-mercaptophenyl)-*N*'- *N*'-methyl -N'-(3-thiomethylphenyl) guanidine (8)**

All solvents were degassed with argon prior to use. The disulfide **7** (5.1 g, 7.6 mmol) dissolved in ethanol (100 mL) was treated with sodium borohydride (954.7 mg, 25.2 mmol) by portion wise addition. The reaction progress was monitored by in-process HPLC analysis. Upon complete reaction, the reaction mixture was concentrated *in vacuo* at 20-25 °C to afford a foamy pale yellow solid. The solid was purified by filtration through celite using chloroform as eluent. The collected product fractions were acidified to pH 1 by addition of hydrogen chloride in diethyl ether (2 M) and concentrated *in vacuo* at 20-25 °C to afford the product as a foamy pale-brown solid (4.8 g, 95%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (broad s, 1H), 8.53 (broad s, 1H), 7.13 (t, 1H), 7.03 (d, 1H), 7.02 (m, 2H), 6.92 (s, 1H), 6.89 (d, 1H),

6.86 (d, 1H), 3.74 (s, 1H), 3.66 (s, 3H), and 2.46 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.7, 147.7, 144.9 , 140.6, 135.9, 130.5, 129.9, 127.3, 124.5,124.3, 124.1, 123.3, 122.6, 38.8, and 15.6. Purity by HPLC (Agilent 1100 series, X-Bridge column) 93.3 % area.

3-(2-Chloro-5-(2-fluoroethylthio)phenyl)-*N*'-methyl-*N*'-(3-(methylthio)phenyl)guanidine, GE-179, (10)

The route described in Scheme 2 was employed to synthesise **GE-179** for analytical reference standard or cold competition studies.

The disulfide **7** (3.0 g, 4.5 mmol) was dissolved in ethanol (120 mL) and 2-fluoroethyl tosylate, **9**, (2.1 g, 9.8 mmol) was added. The reaction mixture was heated to 60°C under an inert atmosphere and sodium borohydride (0.8 g, 22.3 mmol) was added portionwise. The resulting mixture was stirred for 45 minutes before being evaporated to dryness *in vacuo*. The crude product was purified by flash column chromatographic purification over silica gel, eluting with chloroform added methanol (2%) to afford the product as a clear oil that crystalised on standing (2.2g, 5.6 mmol, 62.9%).

^{1}H NMR (300MHz, $CDCl_3$): δ 7.32 (t, 1H), 7.30 (d, 1H), 7.21 (t, 1H), 7.14 (ddd, 1H), 7.09 (ddd, 1H), 7.07 (d, 1H), 6.95 (dd, 1H), 4.53 (dt, 2H), 3.90 (broad s, 2H), 3.41 (s, 3H), 3.18 (dt, 2H), and 2.51 (s, 3H). 13C NMR (75 MHz, CDCl3) δ_C 150.7, 147.7, 145.0, 140.6, 133.9, 130.4, 130.0, 126.6, 126.2, 124.7, 124.6, 124.4, 123.4, 81.6 38.8, 33.8, and 15.5. Purity by HPLC (Agilent 1100 series, X-Bridge column) 96.1 %.

Radiosynthesis of [^{18}F]GE-179

Preparation of reagents and FASTlab^{TM} cassette for the radiosynthesis of [^{18}F]GE-179

The reagents and consumables for the radiosynthesis of [^{18}F]**GE-179** were prepared as described in Table 1 and were contained in sealed vials or bottles in the cassette positions according to Figure 1. The tC18 Sep-Pak cartridge was pre-conditioned with ethanol (2 mL) followed by water (10 mL) before being placed on the FASTlab^{TM} cassette.

Production of [^{18}F]Fluoride

No carrier added [^{18}F]fluoride (1.5 mL, 40-80 GBq) was produced *via* an $^{18}O(p,n)^{18}F$ nuclear reaction on a GE PETtrace cyclotron by irradiation of an enriched [^{18}O]H_2O target using 16.4 MeV protons. The aqueous [^{18}F]fluoride solution was transferred through a Teflon line by helium overpressure of the target from the GE PETtrace cyclotron directly into the collection funnel at position 6 on the FASTlab™ cassette (Figure 1).

Quality Control

Quality control of [^{18}F]**GE-179** was carried out in accordance with the European Pharmacopeia.[16]

Visual Inspection and pH

Doses were visually examined to be clear, colourless and free of particulate matter. ColourpHast 2.0-9.0 pH-indicator strips (Fisher Scientific, Loughborough, UK) were used to confirm the pH by comparison with the scale provided.

Radionuclidic Identity

Radionuclidic identity was confirmed by taking three activity measurements at 5 minute intervals using the Capintec CRC-15R radioisotope dose calibrator. The half-life was calculated from the generated calibration and compared with the known half-life of [^{18}F]fluorine (109.8 mins).

Bioburden, Sterility and Endotoxin Testing

Bioburden and sterility was performed by international Laboratory Services (Shardlow, UK). Samples prior to aseptic dispensing were analysed for total yeast, mould and total aerobic microbial count. Post aseptic sample were incubated for 14 days and tested by EP/USP membrane filtration, absence of culture growth after 14 days was indicative of sterility. Endotoxin content was analysed using a Charles River Laboratories Endosafe Portable Testing System according to European Pharmacopeia guidelines.[16]

Aseptic Dispensing and Filter Integrity Testing

Aseptic dispensing was performed *via* a single-use arrangement of tubing, filter and needles provided as a sterile (gamma-irradiated) pre-assembled kit from Pall (Portsmouth, UK). Integrity testing was achieved by bubble point analysis performed in *situ* by Pall Palltronic Flowstar II integrity test instrument.

Residual solvent and Kryptofix®222 analysis

Residual solvent analysis was determined by using an Agilent gas chromatography (GC) system, equipped with headspace autoinjector, split/splitless inlet, flame ionisation detector (FID) and a Restek column (Stabilwax 30 m x 0.25 mm, 0.25 µm, G16 stationary phase). Kryptofix®222 levels were analysed using the established spot test method.[17, 18]

Specification for the release of formulated [^{18}F]GE-179

[^{18}F]**GE-179** for clinical use was analysed in accordance with European Pharmacopeia guidelines for radiopharmaceuticals.[16] Table 3 summarises the specification for the release of [^{18}F]**GE-179** for clinical use with a maximum injected dose of 10 mL.

Results and Discussion

Radiosynthesis of [^{18}F]GE-179

The radiosynthesis of [^{18}F]**GE-179** (Scheme 3) is a two-step process involving the nucleophilic fluorination of **11** to produce 2-[^{18}F]fluoroethyl tosylate, [^{18}F]**9**, which is a common intermediate for the addition of a [^{18}F]fluoroethyl group.[19-21] The second step is the alkylation of the thiolate, **12**. The crude product is purified by semi-preparative HPLC followed by reformulation on a tC18 Sep-Pak cartridge. In our original synthesis, a 2-pot-2-HPLC purification process was employed which was both not compatible with the FASTlab™ and low yielding. In this work, we have developed a one-pot-one-HPLC purification process which results in a higher yielding radiosynthesis.

Development of the automated FASTlab™ radiosynthesis of [^{18}F]GE-179

The radiosynthesis of [^{18}F]**GE-179** for pre-clinical studies has previously been reported.[1] In order to deliver [^{18}F]**GE-179** for clinical studies, a GMP compliant automated radiosynthesis was required. However, the original radiosynthetic route was not compatible with automation on FASTlab™ for three reasons:

1) [^{18}F]Fluoride needs to be introduced directly from the cyclotron. Therefore, an automated and reproducible [^{18}F]fluoride trapping and elution process at high radioactive levels was required
2) Cesium carbonate is not soluble in either anhydrous acetonitrile or ethanol. Therefore, an alternative soluble base to deprotonate **8** was required
3) A 2-pot-2-semi-preparative HPLC process is not compatible with the FASTlab™, due to the restricted number of cassette positions. Therefore, a one-pot-one-HPLC purification process was required

The original radiosynthesis involved drying a mixture of [^{18}F]fluoride in target [^{18}O]H_2O (200-300 µL), Kryptofix®222 (5 mg, 13 µmol) and potassium carbonate (1 mg, 7 µmol) in anhydrous acetonitrile (800 µL) and water (200 µL).[1] Automated processes require [^{18}F]fluoride to be trapped on a QMA-carbonate Sep-Pak cartridge followed by elution using a fixed volume of eluent (e.g. Kryptofix®222 and potassium carbonate solution). This allows for a fixed [^{18}F]fluoride drying process. Secondly, we previously used cesium carbonate as the base to deprotonate **8** to create the thiolate, **12**. This base is not suitable for use on the FASTlab™ as it is not soluble in either anhydrous acetonitrile or ethanol. Furthermore, the introduction of a second base, in addition to the QMA eluent, increased the number of reagent vials on the cassette and limited the number of vial positions for other reagents on the FASTlab™ cassette. Therefore, the Kryptofix®222 and potassium carbonate solution was investigated as the base for the deprotonation of **8**.

The QMA eluent vial for the commercial synthesis of [^{18}F]FDG on FASTlab™ contains Kryptofix®222 (44 mg, 117 µmol), and potassium carbonate (7.8 mg, 57 µmol) in acetonitrile (660 µL) and water (165 µL). To enable the use of this higher concentration FASTlab™ vial, we had to use a smaller volume (70 µL) which contained the same amount of Kryptofix®222 and base as the original radiosynthesis. This was diluted with water (500 µL) and used to

elute the trapped [^{18}F]fluoride from the QMA-carbonate Sep-Pak cartridge into the reaction vessel. After the [^{18}F]fluoride solution was evaporated to dryness, ethylene ditosylate in anhydrous acetonitrile was added and the reaction mixture was heated at 100 °C for 10 minutes. These conditions delivered crude [^{18}F]**9** with a radiochemical conversion of > 80% (Figure 2).

The Kryptofix®222 and potassium carbonate solution was found to be a suitable base for the deprotonation of **8** (Experiment 1, Table 2). Therfore, a series of experiments were performed with different amounts of eluent from the commercially available FASTlab™ eluent vial (Experiments 2-6, Table 4). Analysis of the crude reaction mixtures showed that a conversion of [^{18}F]**9** to [^{18}F]**GE-179** was >90% for the experiment using 350 µL of the FASTlab™ eluent and confirmed the FASTlab eluent to be a suitable base required to deprotonate the thiol precursor **8**, prior to the alkylation reaction.

Due to the position of the eluent vial (P2) and the QMA-carbonate Sep-Pak cartridge (P4) on the FASTlab™ cassette, the volume of eluent in the reagent vial (825 µL) was sufficient to deliver 70 µL of eluent to elute the [^{18}F]fluoride from the QMA-carbonate Sep-Pak cartridge, however, due tothe position of the thiol precursor **8** vial (P16) , this volume was not sufficient to deliver 350 µL of base to the thiol precursor vial at P16. The volume within the FASTlab™ manifold between P2 and P16 is *ca.* 500 µL , therefore, to deliver the required amount of base, the volume of the QMA eluent solution was increased to 1.5 mL while maintaining the same concentration of reagents [i.e. Kryptofix®222 (79 mg, 210 µmol), potassium carbonate (14.2 mg, 103 µmol) in anhydrous acetonitrile (1300 µL) and water (200 µL)].

Semi-preparative HPLC purification of crude [^{18}F]**GE-179** proved to be a challenge due to the presence of a close eluting impurity (Figure 3). It was postulated that the close eluting peak was the acetate, 13. Formation of the acetate was thought to derive from the reaction of acetonitrile with the hydroxyethylsulfide (Scheme 4), followed by partial hydrolysis of the imidate.(Scheme 4). Based on this hypothesis, the formation of impurity **14** could be avoided by replacing acetonitrile with another solvent (or removal of **11** by HPLC purification). Therefore, a series of experiments were performed to test this hypothesis.

In the first experiment, both the synthon labelling and alkylation reactions were performed in acetonitrile. In the second experiment, acetonitrile from the labelling reaction was removed by evaporation prior to the alkylation and ethanol was used as the solvent for the alkylation reaction and in the third experiment, HPLC purified [^{18}F]**9** in acetonitrile was added to **8**, which was also dissolved in acetonitrile.

The crude reaction material from each of these experiments were analysed by analytical HPLC and the chromatograms are shown in Figures 3, 4 and 5 respectively. Figure 3 shows the close eluting peak when using acetonitrile for the alkylation reaction, whereas Figure 4 shows the result of removing acetonitrile by evaporation and using ethanol as the alkylation solvent. Thus, performing the alkylation reaction in ethanol prevented the formation of the impurity, **14**. Figure 5 shows that by removing unreacted **11** by HPLC purification of [^{18}F]**9** and performing the alkylation reaction in acetonitrile also prevented the formation of **14**. Therefore, the data presented in Figures 3-5 support the hypothesis that the close eluting impurity is **14**. Figure 6 shows the synthesised reference standard **14**, co-elutes with the close eluting peak **14**, present in the crude reaction.

As a consequence of these results, a change to the composition of the eluent vial and removal of acetonitrile from the [^{18}F]**9** labelling reaction was required. Therefore, the eluent vial was prepared with ethanol instead of acetonitrile. Over 95% of the [^{18}F]fluoride was eluted from the QMA cartridge using the modified eluent vial and there was no impact on the radiochemical yield of [^{18}F]**9**. After formation of crude [^{18}F]**9** in acetonitrile, the reaction was evaporated to dryness by heating at 100 °C under a nitrogen flow and also by applying a vacuum to the reaction vessel. The subsequent alkylation reaction was performed in ethanol.

The FASTlab^{TM} only has one reaction vessel and the capacity for one HPLC purification step. Therefore, the automated process was modified so that both the labelling and alkylation reactions were performed in the same reaction vessel. Furthermore, as a single HPLC purification step was required for the purification of [^{18}F]**GE-179**, it was necessary to remove the the HPLC purification of [^{18}F]**9**.

A consequence of using crude [^{18}F]**9** was the presence of ethylene ditosylate, **11**, in the alkylation reaction which competes with the reaction of [^{18}F]**9** with **8**. When using HPLC purified [^{18}F]**9**, ethylene ditosylate, **11,** would not be present in the alkylation reaction and as

a result this competing reation would not be present. This would allow the use of lower amounts of **8**, however, in the case of using crude [^{18}F]**9**, it was necessary to increase the amount **8**. It was observed that when using 3 mg of **11** and 4 mg of **8**, the conversion of [^{18}F]**9** to [^{18}F]**GE-179** was *ca.* 40%. However, increasing the amount **8** to 10 mg increased the conversion of [^{18}F]**9** to [^{18}F]**GE-179** to *ca.* 80% (Figure 7).

Automated FASTlab™ radiosynthesis of [^{18}F]GE-179

The incoming [^{18}F]fluoride solution was trapped on a Waters QMA cartridge and eluted into the reaction vessel. The eluent was prepared in syringe 1 (P3) by diluting the QMA eluent solution with water (500 µL). The [^{18}F]fluoride solution was then evaporated to dryness with a combination of nitrogen flow and vacuum at 100 °C for 20 minutes.

Following the [^{18}F]fluoride evaporation, **11** (2.5 mg, 6.7 µmol) in anhydrous acetonitrile (1 mL) was added. The radiolabelling reaction was conducted at 86 °C for 15 minutes to produce [^{18}F]**9**. Subsequently, the crude reaction mixture was evaporated to dryness with a combination of nitrogen flow and vacuum at 80 °C for 15 minutes.

In parallel to the [^{18}F]**9** labelling reaction, the QMA eluent (350 µL) was added to the vial containing **8** (15 mg, 44 µmol) dissolved in ethanol (1.8 mL). The resulting ethanolic thiolate solution (1.5 mL, 10 mg, 30 µmol) was added to the reaction vessel containing dried crude [^{18}F]**9**. The alkylation reaction was carried out at 80 °C for 2 minutes followed by 100 °C for 10 minutes. Crude [^{18}F]**GE-179** was then diluted with water (2 mL) and 0.1 M hydrochloric acid (1 mL) and loaded onto a 10 mL HPLC loop. [^{18}F]**GE-179** was purified using reverse phase isocratic semi-preparative HPLC process on a Hichrom ACE C18 (100 x 10 mm, 5 µm) column with a flow rate of 3 mL/min with mobile phase A (0.8% Triethylamine (pH adjusted to *ca.* pH 7-7.3 with 85% H_3PO_4) and mobile phase B (100% acetontrile). The isocratic method was 60% (B) for 60 mins.

Purified [^{18}F]**GE-179** was collected into an external vial containing water (50 mL) and then trapped on to a tC18 cartridge on the FASTlab™ cassette. After trapping, the tC18 cartridge was washed with water (20 mL) and dried under a stream of nitrogen. Finally, [^{18}F]**GE-179** was eluted into an external formulation vial with ethanol (1 mL). The formulated product consisted of saline (0.9% w/v, 11.5 mL) and ascorbic acid (1 mg/mL in water, 1.5 mL) to

give a final formulation in a volume of 14 mL. Quality control was performed using a reverse phase isocratic analytical HPLC process on a Primesep B2, (250 x 4.6 mm, 5 µm) HPLC column at a flow rate of 1.6 mL/min with mobile phase A (200 mM Ammonium acetate) and mobile phase B (100% Acetonitrile). The isocratic method was 44% (B) for 35 mins.

Clinical production of [^{18}F]GE-179 using the FASTlab^{TM} synthesiser

The FASTlab^{TM} synthesiser was developed by GE Healthcare for the automated production of [^{18}F]fluorine radiotracers. The synthesiser was designed to aid PET radiopharmaceutical manufacturers to comply with the GMP requirements for PET radiopharmaceuticals.[22-33] The disposable, single-use cassette, removes the need to validate a cleaning protocol as the disposable fluid path removes any potential for cross contamination between productions.

The radiosynthesis of [^{18}F]**GE-179** described above resulted in a process that delivered an AY of 7 ± 2% (n = 15) with a radioactive concentration (RAC) of 289 ± 48 MBq/mL. The total synthesis time, including purification, was 120 minutes. Quality control was carried out in accordance with the European Pharmacopeia.[16] The formulated product was clear, colourless, pH neutral and free of particulate matter. The product successfully passed the yeast, mould count and total aerobic microbial count from the bioburden placed on the aseptic dispensing filter. Absence of culture growth following 14 days of incubation demonstrated the sample sterility post filtration. All samples were pyrogen free.

Analytical radio-HPLC established the radiochemical purity was 96 ± 1% and remained greater than 95% up to at least 6 hours. The molar activity and total chemical content was calculated to be 627 ± 416 GBq/µmol and 2 ± 1 µg respectively. The final formulation volume was 14 mL.

Residual solvent analysis showed the levels of acetonitrile to be within the permitted daily exposure (PDE) of < 410 ppm.[34] Formulation of the product in 0.9% saline (11.5 mL), ascorbic acid solution (1 mg/mL, 1.5 mL) and ethanol (1 mL) gave a formulation volume of 14 mL with an ethanol content of 7%. Ethanol content is acceptable up to a maximum of 10 % when added as an excipient.[34] The formulated product contained less than 50 µg/mL of

Kryptofix® 222 by visual comparison with positive and negative controls.[15, 16] The specifications for the release of [^{18}F]**GE-179** for clinical use are shown in Table 3.

Conclusion

An improved, robust and scalable precursor synthesis has been developed which enabled the preparation of multigram quantities of the thiol precursor to GMP standard. A successful transfer of the previously reported 2-pot-2-HPLC radiosynthesis of [^{18}F]**GE-179** to an automated process using FASTlab™ has been achieved. The new process has been implemented to GMP standard at up to 80 GBq starting activity and has enabled the delivery of [^{18}F]**GE-179** for clinical use. The radiosynthesis delivered an AY of 7 ± 2%, with an RAC of 289 ± 48 MBq/mL and molar activity of 627 ± 416 GBq/µmol. The RCP was 96 ± 1% and remained over 95% up to at least 6 hours. The total chemical content was 2 ± 1 µg in a formulation volume of 14 mL.

Abbreviations

Activity Yield; AY, COC: Cyclic Olefin Copolymer; FDG: Fluorodeoxyglucose; GMP: Good manufacturing practice;; HPLC: High Performance Liquid Chromatography; NMDA: N-Methyl-D-aspartate; NMR: Nuclear Magnetic Resonance; ODS: Octadecyl-silica; PET: Position Emission Tomography; QC: Quality control; QMA: Quaternary Methylammonium; RAC: Radioactive Concentration; RCP: Radiochemical Purity; RV: Reaction vessel; TLC: Thin Layer Chromatography ; TEA: Triethylamine; UV: Ultraviolet

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Table 1: FASTlab^{TM} reagents, components and vial composition

CASSETTE POSITION	SILICONE LINE LENGTH	COMPONENT
P1	14 cm	O-18 water Recovery vial
P2		Kryptofix®222 (79 mg, 210 µmol) and potassium carbonate (14.2 mg, 103 µmol) Solvent: [water (200 µL) and ethanol (1.3 mL)] (11 mm glass vial)
P3		Syringe 1
P4-P5	14 cm	QMA-carbonate Sep-Pak cartridge
P6		O-18 water Collection Funnel
P7	14 cm	COC Reaction vessel
P8	14 cm	COC Reaction vessel centre
P9		Empty
P10		Empty
P11		Syringe 2
P12		Ethylene ditosylate, **11**, (4.0 mg, 11 µmol) in anhydrous acetonitrile (1.6 mL) (11 mm glass vial)
P13		Ethanol (4.0 mL) (13 mm glass vial)
P14		0.1 M HCl (4.0 mL) (13 mm glass vial)
P15		Water bag spike and water bag (100 mL)
P16		Thiol precursor **8** (15 mg, 40 µmol) in ethanol (1.8 mL) (13 mm glass vial)
P17	42 cm	Line to HPLC

Table 2: Effect of the amount of FASTlab^{TM} eluent on the conversion of [^{18}F]**9** to [^{18}F]**GE-179**

	Volume of eluent (µL)	**Kryptofix®222**	**K_2CO_3**	**8**	**K_2CO_3 : 8**	**Conversion of [^{18}F]9 to [^{18}F]GE-179 (%)**
1	50	2.7 mg, 7 µmol	0.5 mg, 3.6 µmol	10 mg, 30 µmol	1:8	12
2	150	8 mg, 21.2 µmol	1.4 mg, 10.3 µmol	10 mg, 30 µmol	1:3	70
3	150	8 mg, 21.2 µmol	1.4 mg, 10.3 µmol	10 mg, 30 µmol	1:3	69
4	350	18.7 mg, 49.7µmol	3.3 mg, 24.0 µmol	10 mg, 30 µmol	1:1	89
5	350	18.7 mg, 49.7 µmol	3.3 mg, 24.0 µmol	10 mg, 30 µmol	1:1	91
6	700	37.3 mg, 99 µmol	6.6 mg, 48.0 µmol	10 mg, 30 µmol	1.6:1	90

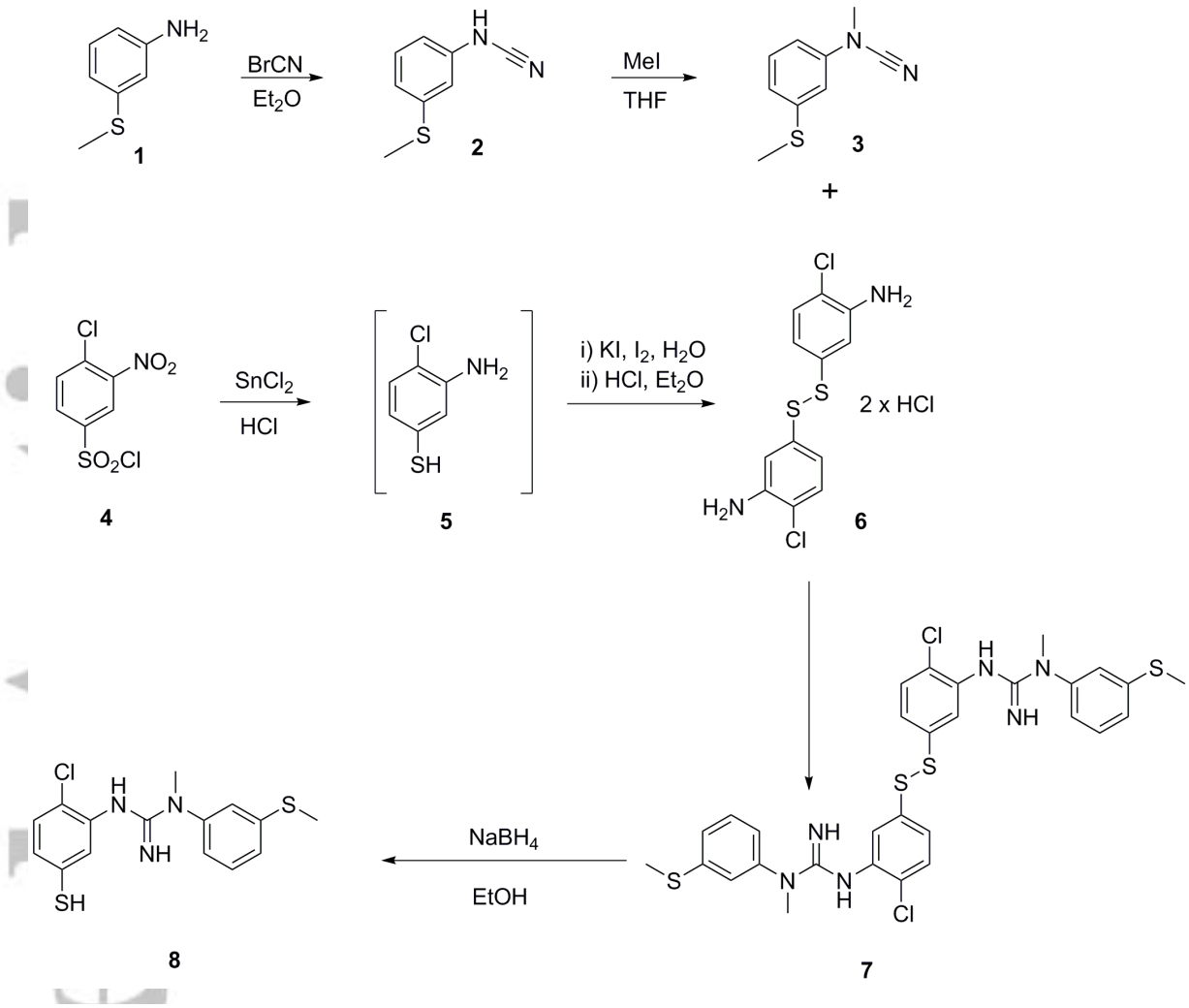
Table 3: Specification for the release of formulated [^{18}F]**GE-179***

Parameter	**Acceptance criteria**
Appearance	Clear, colourless and particulate free
pH	4-7
Bacterial endotoxin	< 17.5 EU/mL
Amount of [^{19}F]GE179	< 16 µg/dose
Amount of any individual unknown impurity	< 1.5 µg/dose
Amount of total chemical impurities	< 5 µg/dose
Kryptofix® 222	≤ 50 µg/mL
Residual solvent: Acetonitrile	≤ 410 ppm
Residual solvent: Ethanol	≤ 10 %
RCP	≥ 95%
Sterility	Pass
Filter Integrity	≥ 3.45 bar

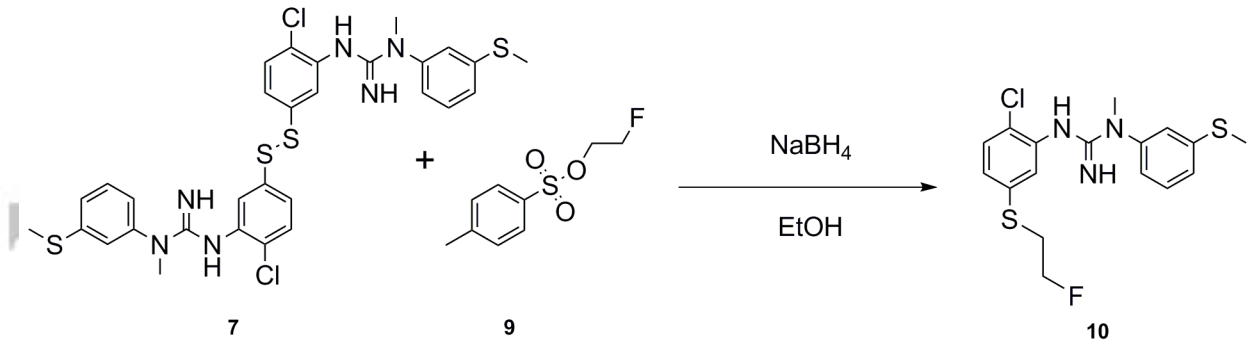
* Based on a maximum patient injection of 10 mL

Table 4: Summary of results from the clinical preparations of [^{18}F]**GE-179** at Hammersmith Imanet

Starting Activity (MBq)	**Activity Yield (AY) (%)**	**Formulated product (MBq)**	**RAC (MBq/mL)**	**GE-179 only (µg)**	**Total Chemical Content (µg)**	**RCP (%)**	**Molar Activity (GBq/µmol)**
59108	7.4	4385.1	313.2	0.9	0.9	95.3	1177.9
27956	13.7	3816.6	272.6	0.6	2.1	97.8	498.6
57123	7.0	4018.6	287.0	2.0	4.6	96.1	194.7
47862	10.3	4931.1	352.2	0.7	1.8	95.6	601.3
65514	6.2	4038.7	288.5	0.6	0.6	96.5	1409.0
69650	6.2	4328.7	309.2	0.5	3.2	96.7	308.0
61663	5.6	3426.3	244.7	0.7	2.3	97.2	293.8
70765	5.0	3569.3	255.0	0.2	1.0	97.1	788.3
78492	5.8	4573.0	326.6	0.5	1.0	95.7	1043.5
47823	5.4	2568.1	183.4	1.1	1.8	97.9	318.9
63197	6.7	4223.6	301.7	0.6	1.4	95.3	689.4
66495	8.2	5455.5	389.7	2.7	3.1	96.1	358.5
53319	7.4	3966.6	283.3	2.2	3.5	95.8	230.2
52180	7.2	3735.5	266.8	1.7	3.4	95.9	234.5
66479	5.4	3582.4	255.9	0.6	0.6	96.1	1265.1



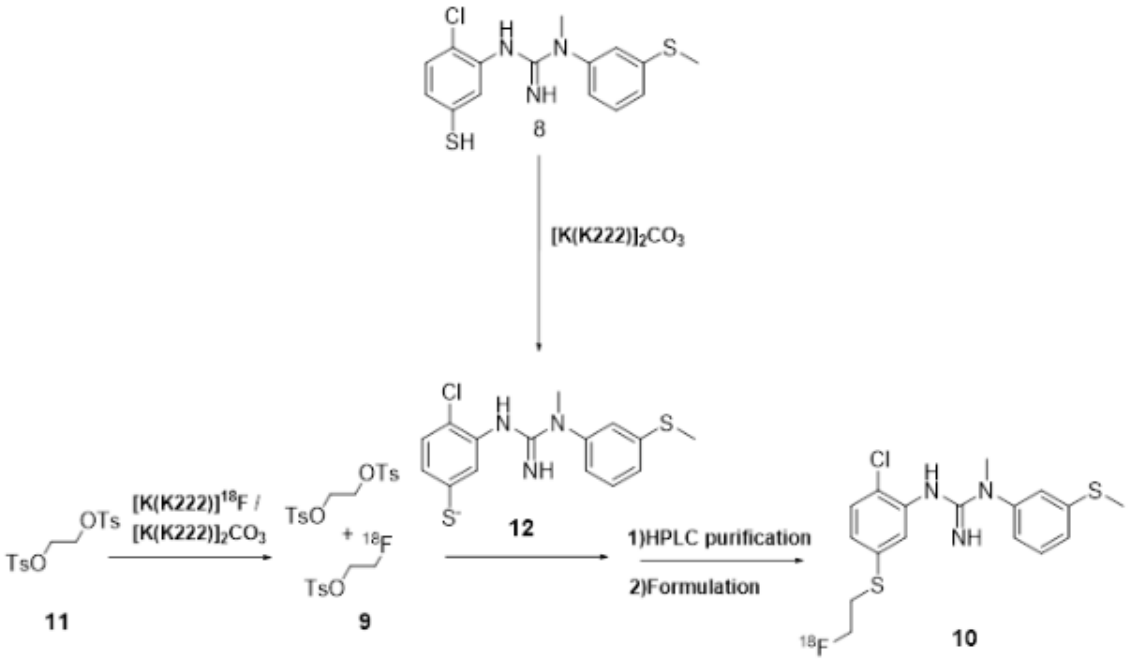
Scheme 1: Synthesis of *N*-(2-chloro-5-thiophenol)-*N*'-(3-thiomethylphenyl)-*N*'-methyl guanidine **(8)**



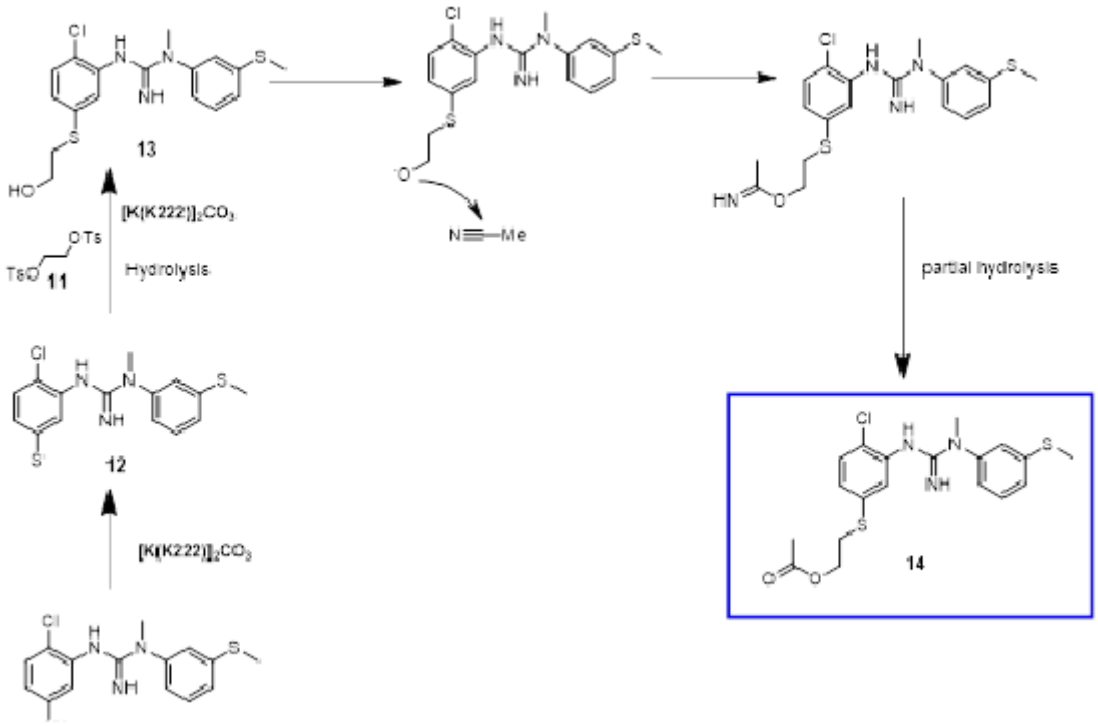
7 9 10

GE-179

Scheme 2: Synthesis of *N*-(2-chloro-5-(2-fluoroethylthio)phenyl)-*N'*-methyl-*N'*-(3-(methylthio)phenyl)guanidine, **GE-179**.



Scheme 3: Radiosynthesis of [^{18}F]**GE-179**



Scheme 4: Postulated mechanism of the formation of **13** and **14**

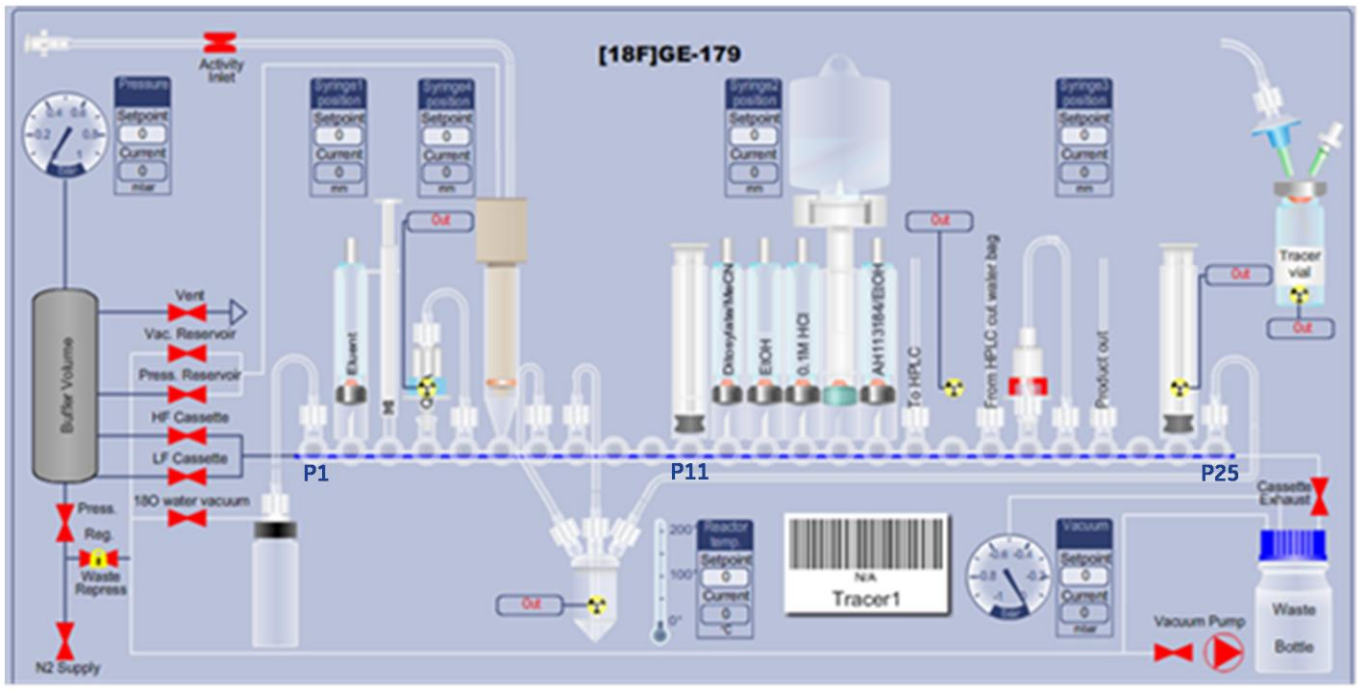


Figure 1: FASTlab^{TM} cassette for [^{18}F]**GE-179** radiosynthesis

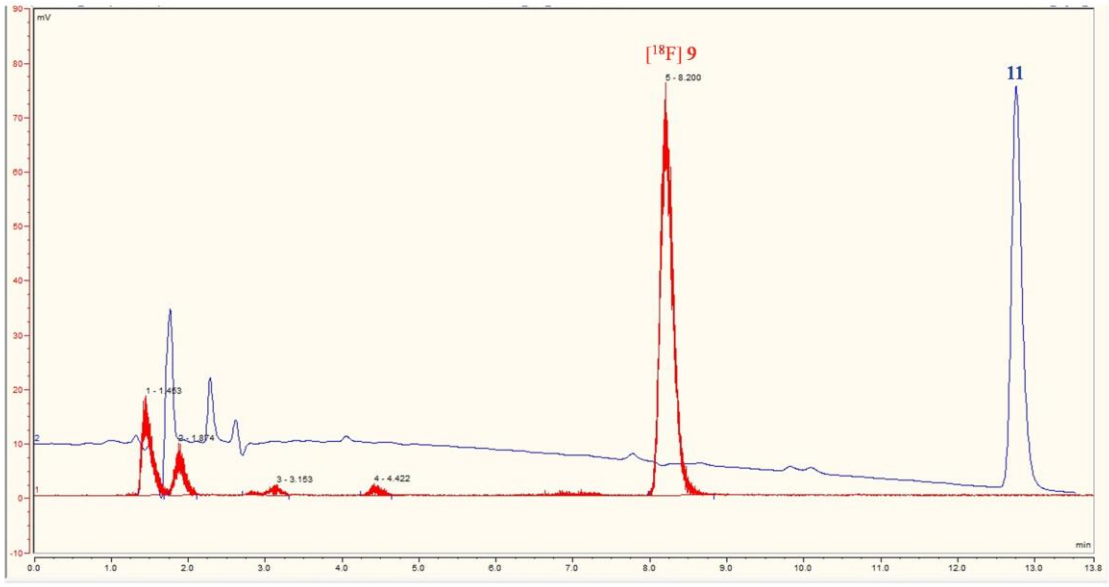


Figure 2: Analytical HPLC chromatogram of crude [^{18}F]**9** (radiochemical in red, UV in blue)

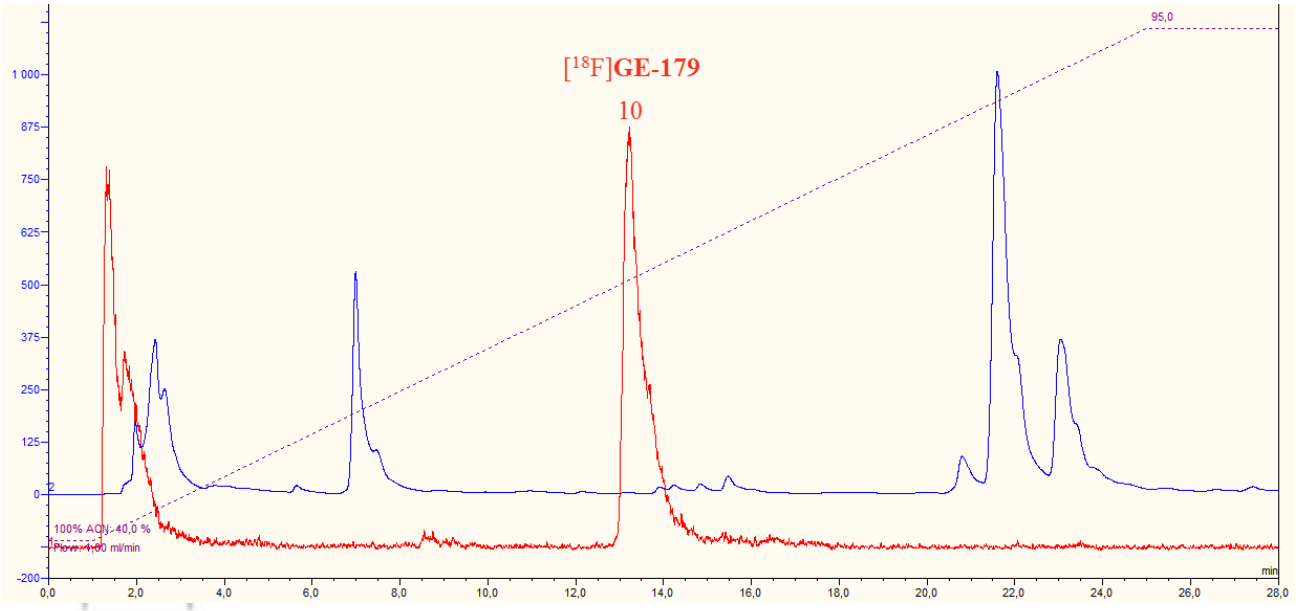


Figure 3: Analytical HPLC chromatogram of crude [^{18}F]**GE-179** (radiochemical in red, UV in blue) showing the close eluting impurity, **13**, when the alkylation reaction was performed using unpurified [^{18}F]**9** in acetonitrile.

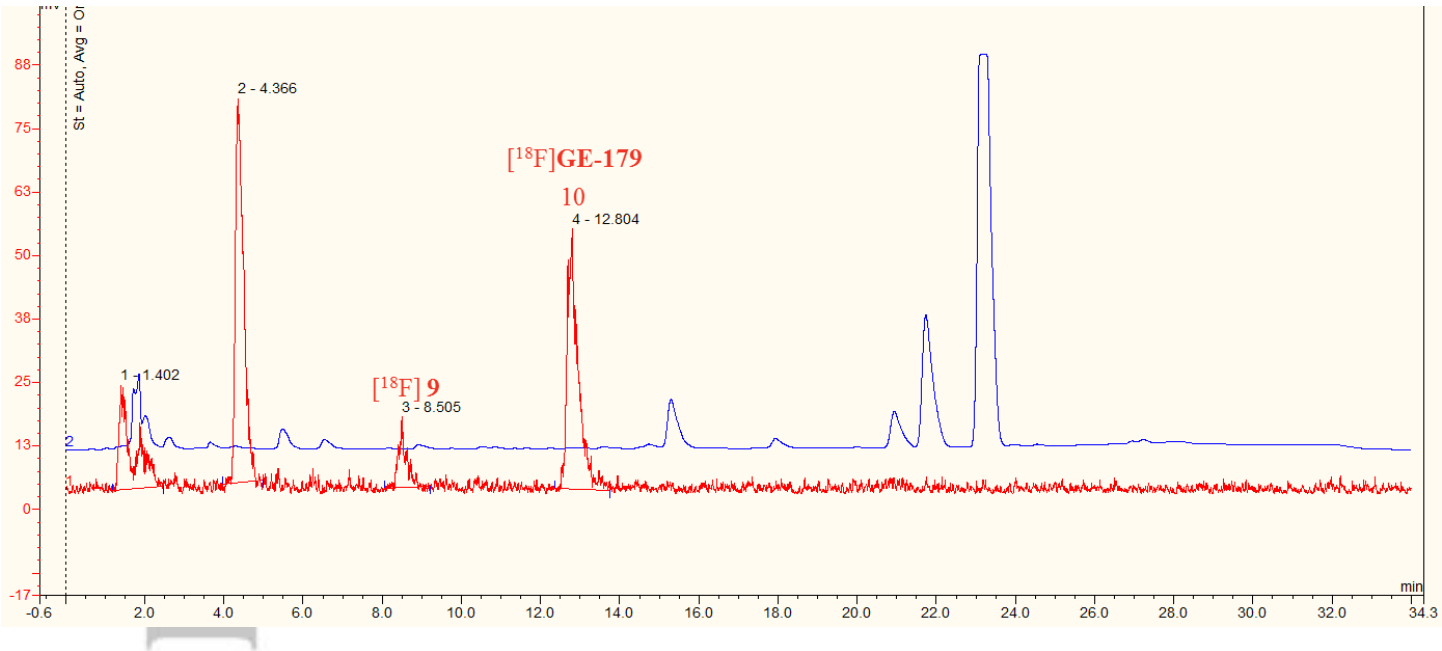


Figure 4: Analytical HPLC chromatogram of crude [^{18}F]**GE-179** (radiochemical in red, UV in blue) showing that the close eluting impurity was not present when the alkylation reaction was performed using unpurified [^{18}F]**9** in ethanol.

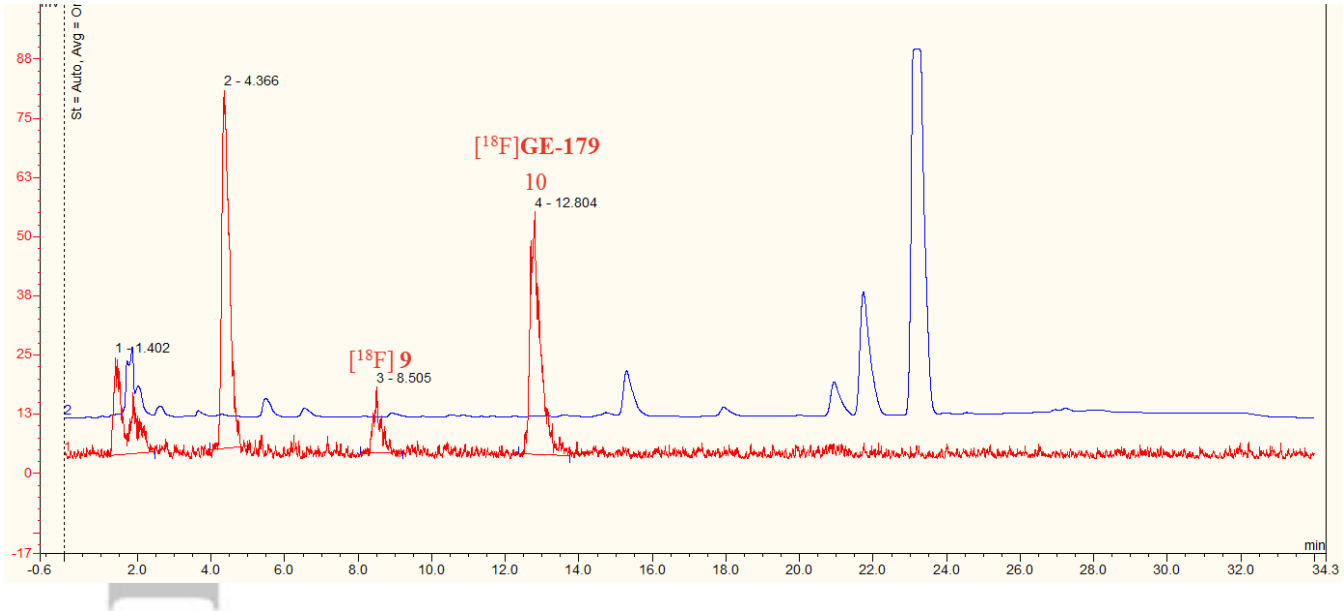


Figure 5: Analytical HPLC chromatogram of crude [^{18}F]**GE-179** (radiochemical in red, UV in blue) when the alkylation reaction was performed using HPLC purified [^{18}F]**9** in acetonitrile.

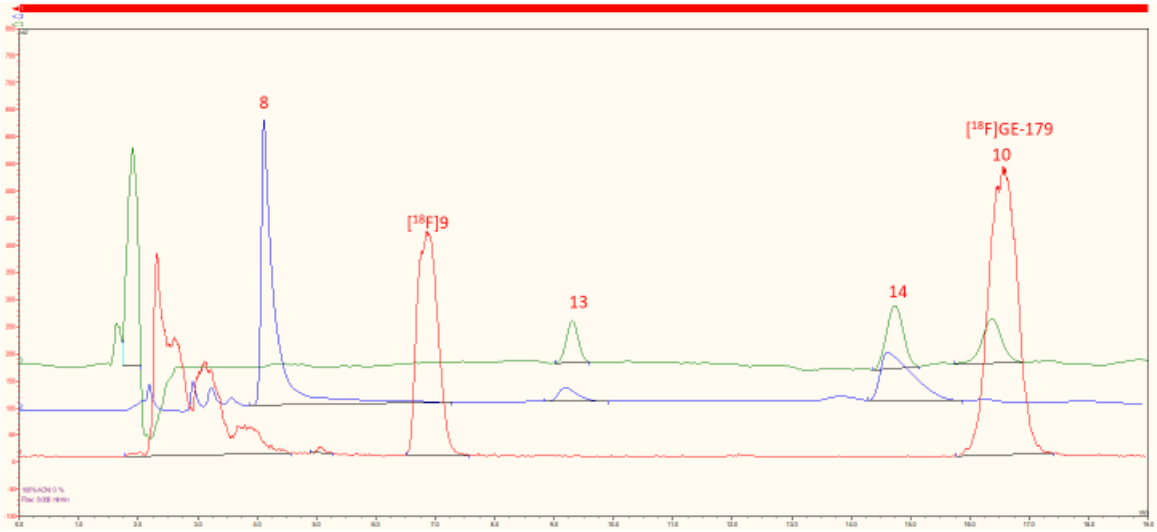


Figure 6: Analytical HPLC chromatogram of crude [^{18}F]**GE-179** (radiochemical in red, UV in blue) showing the impurities, **13** and **14.** UV trace in green shows the synthesised reference standards, **10**, **13** and **14**.

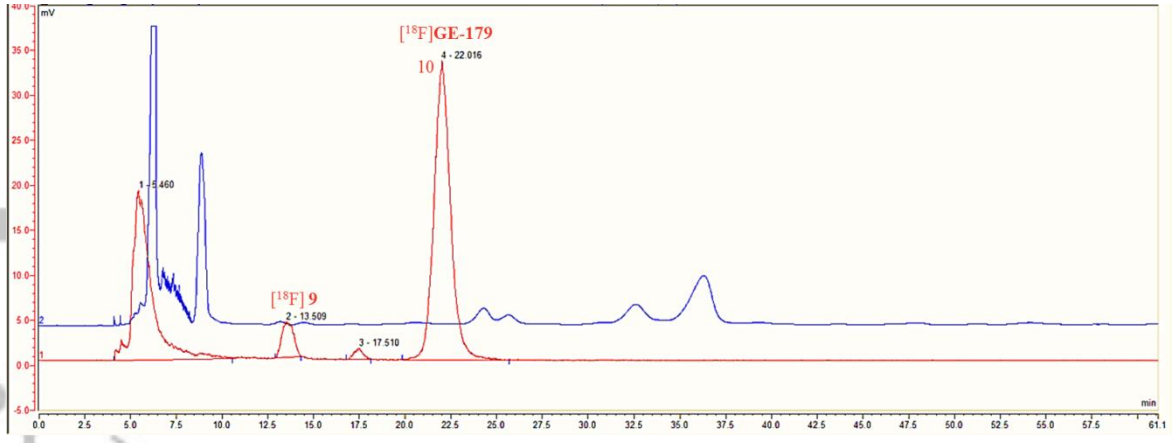


Figure 7: Semi-preparative radio-HPLC chromatogram of crude [^{18}F]**GE-179** showing > 80% conversion of [^{18}F]**9** to [^{18}F]**GE-179**.