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FASTlab Radiosynthesis of the ^{18}F-labelled HER2-binding Affibody Molecule [^{18}F]GE-226

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Abstract

An ^{18}F-labelled HER2 receptor binding radiotracer is a potential tool to non-invasively identify HER2 positive tumour lesions in subjects with recurrent metastatic breast cancer. Having explored the manual radiochemistry to conjugate the Affibody molecule $Z_{HER2:2891}$ with [^{18}F]4-fluorobenzaldehyde, we have developed and optimized a full protocol for the automated GE FASTlab^{TM} synthesiser. Our chemometric model predicted the best radiochemical purity for a short conjugation time (2.8 min), a low temperature (65^{o}C), and a medium Affibody molecule precursor amount (5.5 mg). Under these optimised conditions [^{18}F]GE-226 was produced after solid-phase extraction purification with activity yield of 30% ± 7 (n = 18) and a radiochemical purity of 94% ± 2 (n = 18). The synthesis and purification was complete after 43 minutes and provided apparent molar activities of 12-30 GBq/μmol (n = 12) at the end of synthesis.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jlcr.3789

Introduction

Approximately 20-25 % of breast cancers express the human epidermal growth factor receptor (HER2) which is associated with malignant disease.[1] Currently, the standard practice to determine HER2 status in tumours is by tissue biopsy. This technique is impractical to assess multiple metastatic lesions and is generally restricted to a single lesion for assessment, therefore non-invasive *in vivo* imaging of the HER2 biomarker could improve clinical management of patients with recurrent metastatic disease. Our work is focussed around the fluorine-18 radiolabelling of the $Z_{HER2:2891}$ HER2-binding Affibody molecule.[2] This versatile class of high-affinity binder has already been subjected to ^{18}F conjugation by other laboratories. Successful studies have been carried out using the Affibody molecule labelled with fluorine-18 using *N*-[2-(4-[^{18}F]fluorobenzamido)ethyl]maleimide (^{18}F-FBEM) for the maleimide-thiol based protocol,[3-10] 4-[^{18}F]fluorobenzaldehyde ([^{18}F]FBA) for aminooxy/aldehyde coupling,[11, 12] or the [^{18}F]AlF/NOTA approach.[13, 14] We have evaluated various approaches to conjugate $Z_{HER2:2891}$ with fluorine-18.[15] The study concluded that [^{18}F]FBA conjugation to a *C*-terminal Cys-maleimide-aminooxy function was the preferred method both with regard to *in vivo* data and prospective automation. We have consequently translated the manual protocol to the GE FASTlab™ synthesiser. We report here the development of an automated and robust synthesis of [^{18}F]GE-226 for the commercial FASTlab synthesiser with single-use disposable cassettes and on-cassette solid phase extraction (SPE) purification.

Experimental

Materials and Methods

The aminoxy-modified Affibody molecule labelling precursor was prepared as described previously.[15] No-carrier-added aqueous [^{18}F]fluoride was obtained from the $^{18}O(p,n)^{18}F$ reaction (PETtrace cyclotron, GE Medical Systems) by the irradiation of an isotopically enriched [^{18}O]H_2O target using a 16.4 MeV proton beam. The Sep-Pak Accell™ Plus QMA carbonate Plus Light, Ion exchange Oasis® MCX Light and Sep-Pak® Plus Long tC2 cartridges were purchased from Waters. Analytical HPLC was carried out using a Gilson 322 pump with a UV/ViS 156 detector and a gamma detector (Bioscan Flow-count, PMT probe: Thermo MS3083). Radioactivity measurements were performed using a Capintec CRC-15R ion chamber. Radiochemical yields are reported as non-decay-corrected. The apparent molar activities were estimated based on the starting amounts of peptide precursor.

FASTlab synthesis of [^{18}F]GE-226

All radiochemistry was performed on the FASTlab synthesiser with single-use cassettes. The FASTlab sequence developed and optimised for the synthesis of [^{18}F]GE-226 was programmed in a step-by-step time-dependent sequence of events, e.g. syringe plunger movement, nitrogen pressure, vacuum pressure and temperature regulation. A 4-trimethylammonium benzaldehyde precursor (4-TMAB)[16] was used to prepare [^{18}F]FBA which was then conjugated to the aminoxy functionalised $Z_{HER2:2891}$ HER2-binding Affibody molecule utilising aniline as a catalyst (Scheme 1). Optimisation of the conjugation reaction between [^{18}F]FBA and the aminoxy functionalised $Z_{HER2:2891}$ HER2-binding Affibody molecule was carried out by varying the amount of precursor (3.5 to 7.0 mg), reaction temperature (60 to 80 °C), reaction time (2.8 to 20 min), amount of aniline catalyst (8.2 to 35 mg) and level of starting radioactivity (26 to 108 GBq) using a multivariate experimental approach. Multivariate analysis was performed by Unscrambler (v. 9.8, Camo Inc). The software was used to develop Design of Experiment (DoE) templates and the statistical correlation between X- and Y-variables was determined by partial least squares regression (PLS). Following the DoE approach allowed the number of experiments required to be reduced as much as possible, whilst still having sufficient resolution to determine the design space for the various responses and to make an assessment of how important the different variables were in the values of the responses observed. In these initial experiments the precursor was re-constituted manually to pH 2.7 in a citrate/phosphate buffer. Non-decay corrected yields were determined by RP-18 reverse phase thin-layer chromatography (silica gel 60 RP-18 on alumina sheets Merck KGaA) eluting with water/70% acetonitrile (labelled Affibody molecule at origin).

Robustness studies

The Unscrambler software version 9.8 by Camo was used to establish suitable statistical experimental designs and to build multiple linear regression models.

Starting activities of 42 to 71 GBq were used. Each batch of the [^{18}F]GE-226 for robustness testing was formulated in 25 mL phosphate buffered saline containing the radiostabiliser sodium p-aminobenzoic acid (Na-pABA, 2.8 mg/mL). Radiochemical purity (RCP) and chemical impurity values with UV detection at 280 nm were determined by HPLC immediately after synthesis and approximately 3 hr post formulation according to the method

shown in Table 1. Ethanol, acetonitrile and acetaldehyde amounts were determined using GC while aniline was determined by both GC and HPLC.

Quality control

Details of the HPLC method developed for determination of radiochemical and chemical purity of the radioactive samples are listed in Table 1. HPLC-UV/MS was used to identify chemical impurities in the decayed samples, details of which are shown in Table 2. The radiochemical purity was also determined by RP-18 reversed-phase thin-layer chromatography as described above.

Results and Discussion

Conjugation optimisation

A detailed description of the FASTlab synthesis of [^{18}F]GE-226 is shown in Figure 1. The [^{18}F]GE-226 FASTlab cassette is shown in Figure 2.

A series of experiments were carried out to determine the optimum conditions for the conjugation of [^{18}F]FBA and the aminoxy functionalised $Z_{HER2:2891}$ HER2-binding Affibody molecule. The amounts of precursor and aniline and the conjugation temperature and time were varied. The unpurified conjugation reaction mixtures were analysed by thin layer chromatography.

Multivariate modelling of the results indicated that both time and temperature had a negative effect on RCP. A short time (2.8 min) and a relatively low temperature (65°C) produced the highest RCP values see (Figure 3(c)). The [^{18}F]FBA recovery variable in Figure 3 was the proportion of the starting activity that was returned, as purified [^{18}F]FBA, to the reactor for the conjugation reaction with the precursor.

An amount of 5.5 mg of the aminoxy precursor was selected as the optimum precursor amount from the experiments as this gave acceptable RCP values and a [^{18}F]GE-226 yield which would allow the purification of [^{18}F]GE-226 using tC2 SPE cartridges. When 3.5 mg precursor was used, the RCP obtained (67%) was too low to allow adequate purification of the larger amount of unreacted [^{18}F]FBA and other radioactive impurities. Use of 7 mg precursor gave the highest RCP values but this amount was not chosen to minimise the amount of precursor used.

Purification

On-cassette SPE purification was desired as it is simpler, faster and potentially more reproducible than purification by HPLC. A SPE method was therefore developed to purify [^{18}F]GE-226 using reverse phase tC2 SPE cartridges integrated on the cassette to remove radioactive impurities such as unreacted [^{18}F]FBA, [^{18}F]Fluoride and some non-Affibody molecule related chemical impurities. Separation of Affibody molecule related impurities was not possible by SPE purification as these impurities were too structurally similar. However, [^{18}F]FBA was purified prior to the conjugation reaction to minimise the levels of these Affibody molecule related impurities and their level was acceptable.

The two tC2 SPE cartridges used to purify [^{18}F]GE-226 were preconditioned with ethanol, acid/water and water during the conjugation reaction. The conjugation reaction solution was then diluted with water and transferred through the tC2 cartridges to initiate the SPE purification step. Washes using a 19% ethanol / H_3PO_4 (v/v) solution were then used to remove unconjugated [^{18}F]FBA and other uncharacterised, radioactive impurities from the tC2 cartridges.

[^{18}F]GE-226 was eluted from the tC2 cartridges after purification using ca. 2 mL of 80:20 (v/v) ethanol/water solution followed by a 4 mL water rinse. Elution into 19 mL phosphate buffered saline containing Na-pABA as radiostabiliser resulted in a final ethanol concentration of ca. 5% (v/v).

Robustness testing

A robustness study was performed to challenge the [^{18}F]GE-226 synthesis and SPE purification on FASTlab with factors that could impact [^{18}F]GE-226 yield and purity anticipated at radiopharmaceutical manufacturing sites for potential clinical studies (e.g. FASTlab synthesizer hardware, environmental factors and variations for selected materials used).

Reagent variables related to the [^{18}F]FBA synthesis were excluded as 26 previous experiments had shown the [^{18}F]FBA production to be sufficiently robust (30-47% yield, non-decay corrected, mean = 43% ± 4). The robustness study was therefore restricted to the conjugation reaction and the SPE purification with assessment of chemical purity, RCP and radiochemical yield (RCY).

Temperature was included as a variable to cover the possible range in manufacturing facilities of 18 to 34°C. The highest temperature (34°C) was included as the temperature within a hot

cell tends to be increased by the electrical equipment it contains. The lowest temperature limitation of the process came from the freezing temperature of the DMSO in the 4-trimethylammonium benzaldehyde (4-TMAB) precursor solution.

Previous testing of the amount of precursor had shown that the minimum acceptable amount of precursor was 5.3 mg. Therefore, the robustness study included the amount of precursor as a variable so that a 5.3 to 6.3 mg range was covered by the experiments.

As the aqueous-ethanolic solutions used during the SPE purification and elution steps were mixed using automated movements of syringe 2 of the FASTlab cassette and, because the ethanol % of these solutions was expected to be critical for reliable purification and elution, deliberate variations in the syringe S2 plunger position in mm were investigated to assess impact on purity and RCY.

The robustness study also included the tC2 SPE cartridge fill mass as a variable as the mass of fill material potentially affected the performance of the SPE cartridges during the SPE purification step. Multivariate modelling focused on the three responses RCY, RCP and total chemical impurities.

The main variation in RCY was due to the total tC2 SPE cartridge fill mass and syringe S2 position variables, both of which were important during the SPE purification stage. Higher RCY was associated with higher total tC2 SPE cartridge fill mass and lower offset syringe S2 value. Variables of starting activity, precursor mass and hot cell temperature did not give significant variation over the design space tested.

RCP values varied from ca. 90 to 95 % at approximately 3 hr post formulation. A time of 3 hr post formulation was chosen as this would allow sufficient time for QC testing and transport before clinical use. The main effects on RCP were found to be syringe S2 position and temperature with activity as a negative correlation. Variables of precursor mass and tC2 cartridge mass did not give a significant variation in RCP over the design space tested. All RCP values were acceptable over the design space ($\geq$ 90%) except one of the experiments which gave an RCP value of 89%, 3 hr post formulation. This experiment was carried out at a low hot cell temperature (18.9°C).

It can be concluded from the modelling results that, with the SPE purification conditions used, the synthesis should be carried out at hot cell temperatures between 20 to 34°C as RCP values > 90% are predicted to be obtained post formulation between t = 0 and 3 hr in this temperature range (see Figure 4).

The main variation in chemical impurities was due to the purification stage and the main variables were tC2 SPE fill mass and syringe S2 position. There was a positive correlation between the level of chemical impurities with precursor mass. (Figure 5) Variables of starting activity and hot cell temperature did not give a significant variation in the level of chemical impurities over the design space tested.

The results from the robustness study were used to calculate the amounts of chemical impurities which would be present in a 370 MBq injection at 3 hr post formulation. This was achieved by determining the volume of formulated product required to provide a 370 MBq injection at 3 hr and then calculating the amount of chemical impurities in this volume. The amounts of chemical impurities, estimated by HPLC, varied from 87 to 226 µg in 25 mL of which 60 to 86% were Affibody molecule related. HPLC-UV/MS analysis of the decayed formulation samples showed that the most abundant Affibody molecule related impurities were the aminoxy precursor, GE-226, oxidised GE-226 and the Affibody molecule acetate, acetaldehyde and formaldehyde conjugates.

A recent study in metastatic breast cancer patients with ^{68}Ga-ABY025 ($Z_{HER2:2891}$ Affibody molecule) indicated that higher contrast images were obtained with 427 µg $Z_{HER2:2891}$ Affibody molecule at 2 hr post injection.[17] Higher uptake in metastases in all locations and lower uptake in normal hepatic tissue were observed with the higher Affibody molecule dose.[17] It may therefore be beneficial to add in additional $Z_{HER2:2891}$ Affibody molecule to the [^{18}F]GE-226 formulation to optimize imaging for clinical applications.

Measurement of pH values (5.9 to 6.1) showed that all formulations were suitable for injection with respect to pH. The % ethanol values (4.6 to 5.9 % (v/v)) were acceptable for intravenous use. All other values for chemical impurities and residual solvents (acetonitrile, acetaldehyde, aniline and K222) were significantly below those which would have an impact on efficacy or safety of the [^{18}F]GE-226 injection (see Table 3).

Manufacturing Process

The non-decay corrected radiochemical yield for a number of productions is shown in Figure 6. Starting activities were 49-88 GBq. The average non-corrected radiochemical yield was 30% ± 7 (n = 18).

The variation in end of synthesis RCP values for several productions at different radioactive concentrations (RAC) is shown in Figure 7. RCP values above the minimum required value

of 90% were obtained in each production. Increasing the RAC from 400 to 1100 MBq/mL had little or no effect on the RCP. These results show that starting activities up to ca. 88 GBq could be routinely used to produce up to 30 GBq of [^{18}F]GE-226 in a 25 mL formulation.

Quality control

Representative HPLC chromatograms used to determine the radiochemical and chemical purity of the formulated [^{18}F]GE-226 are shown in Figure 8.

Conclusion

We have successfully automated the manual radiolabelling production of [^{18}F]GE-226 by transferring to the FASTlab synthesiser. This automated process has been optimised and [^{18}F]GE-226 was produced after solid-phase extraction purification with a non-corrected radiochemical yield of 30% ± 7 (n = 18) and a radiochemical purity of 94% ± 2 (n = 18). The synthesis and purification was complete after 43 minutes and provided apparent molar activities of 12-30 GBq/μmol (n = 12). Our results demonstrate that this reliable automated manufacture for [^{18}F]GE-226 will allow the production of doses for clinical studies. The automated FASTlab process has already been successfully used to supply [^{18}F]GE-226 for a pre-clinical study to detect HER2 expression and pharmacodynamic response to HER2 modulating therapies.[18]

Acknowledgements

We thank Robert Forrest for assistance with the FASTlab preparations and Ole Henrik Hansteen and Inger Oddbjorg Fremstad for assistance with the analyses.

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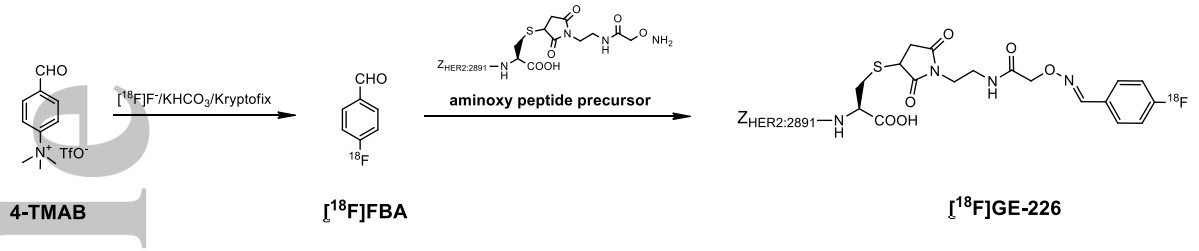

Scheme 1. Radiosynthesis of [18F]FBA and Affibody molecule conjugation reaction forming [18F]GE 226.

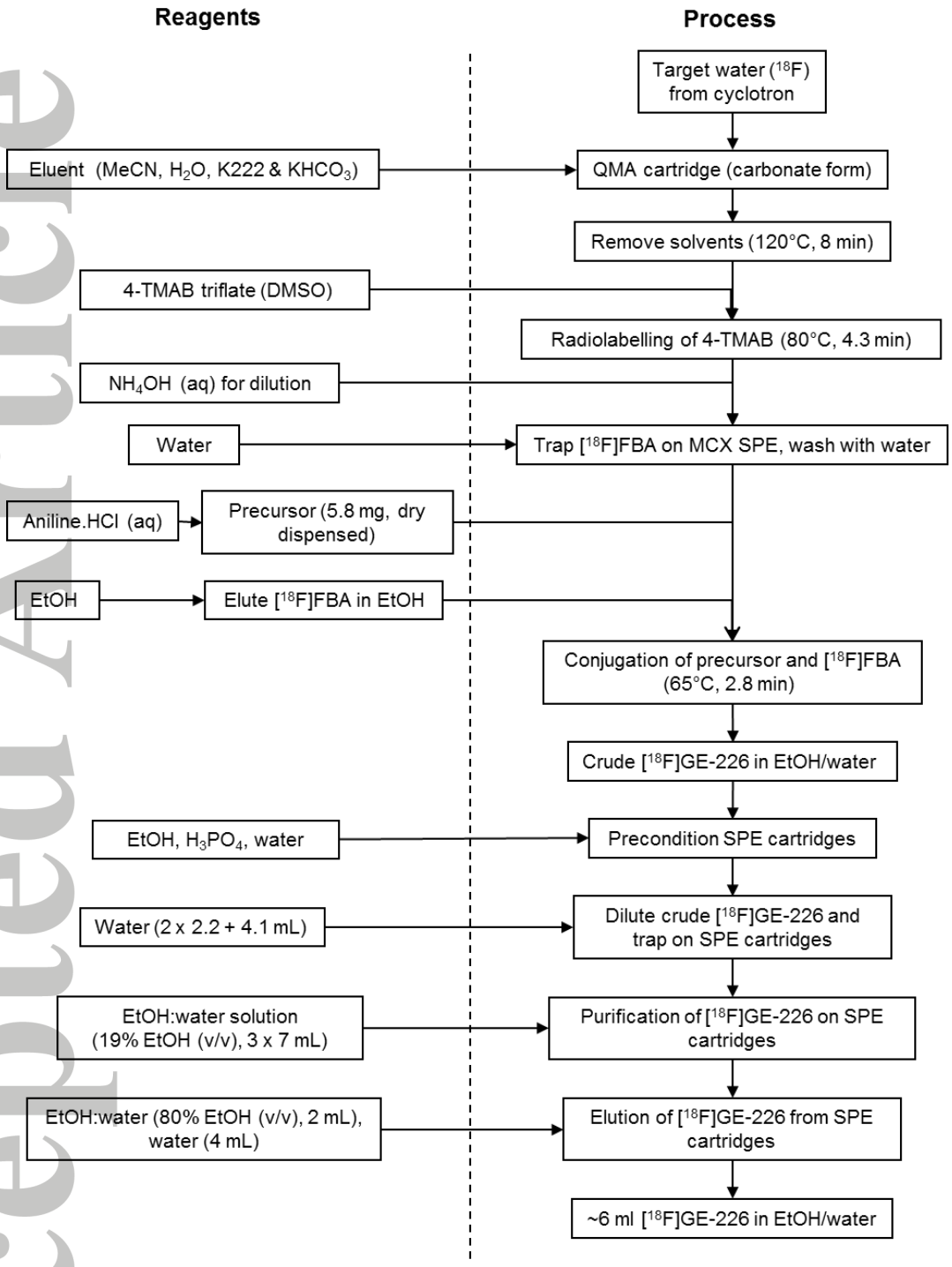

Figure 1. Detailed description of the optimised FASTlab synthesis of [^{18}F]GE-226.

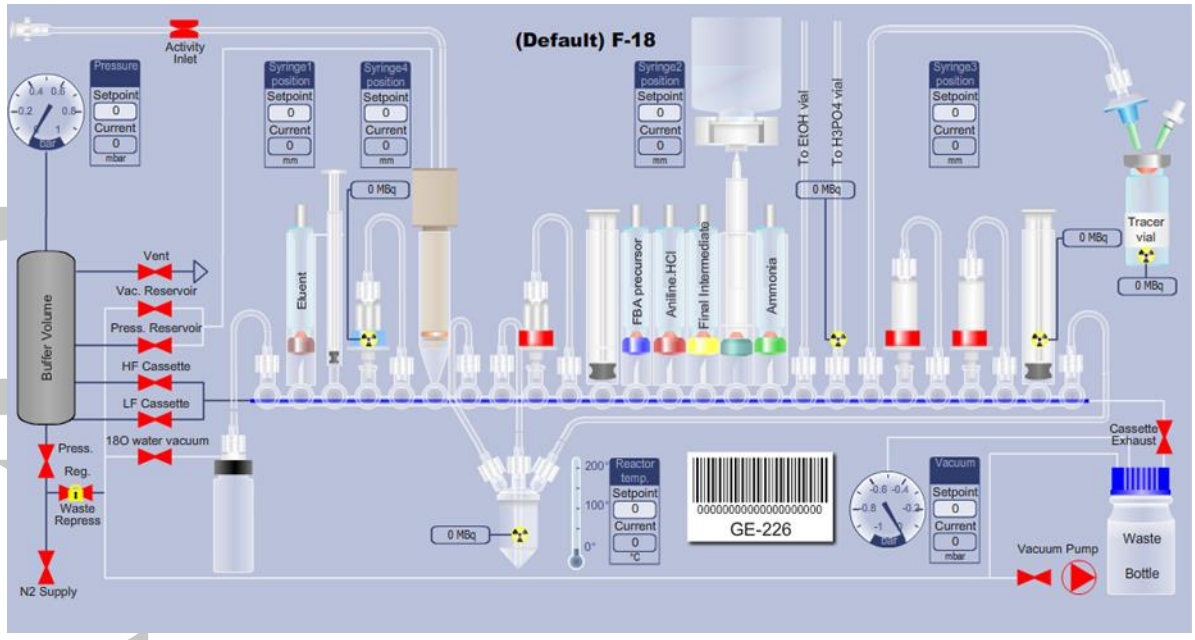

Figure 2. FASTlab cassette layout for radiosynthesis of [^{18}F]GE-226 including SPE purification.

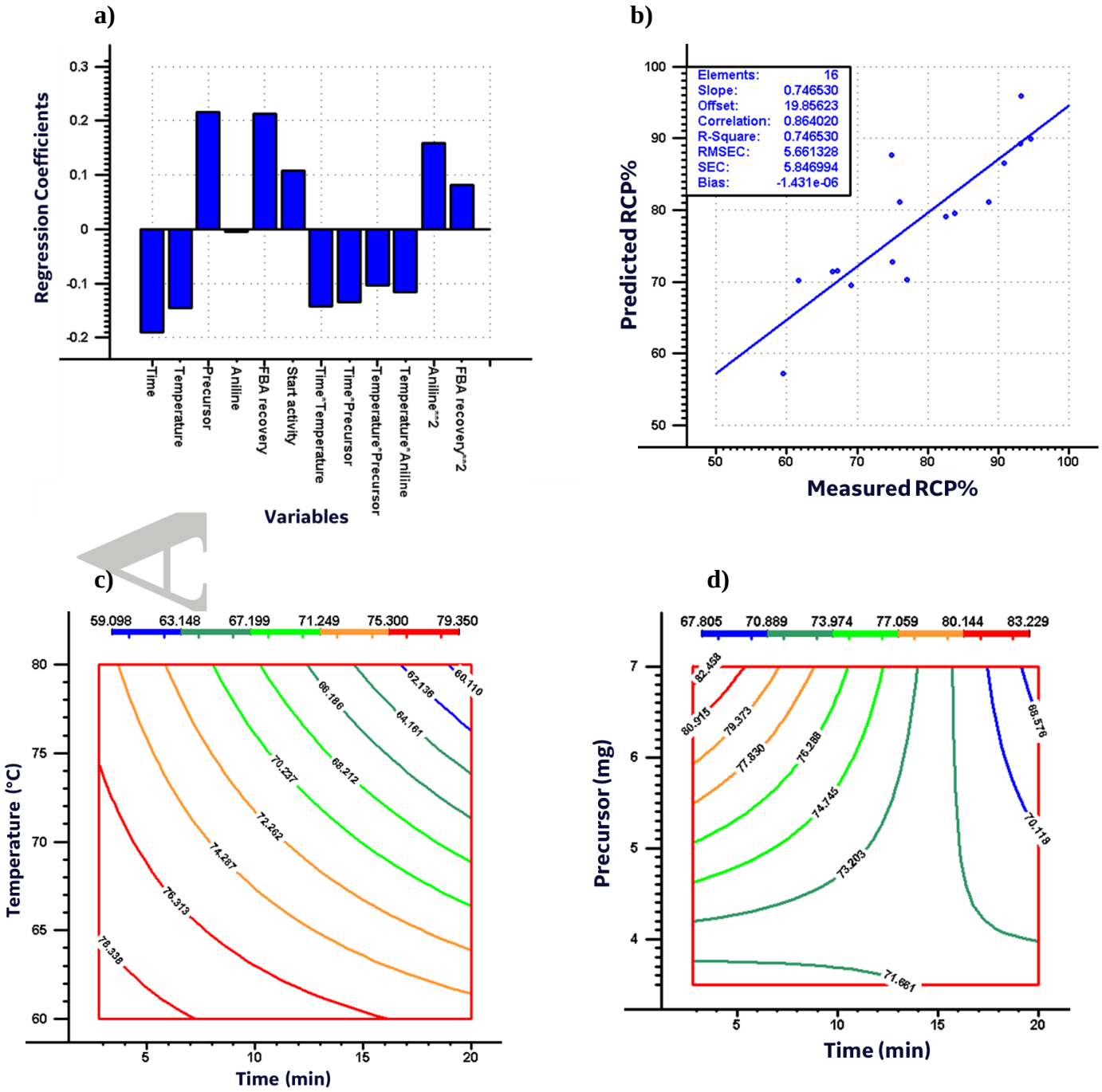

Figure 3. Chemometrics modelling data: a) regression coefficients for time, temperature, precursor amount, aniline amount, [^{18}F]FBA recovery and starting activity (+ interaction and squared terms); b) co-relation of measured RCP and predicted values; c) RCP response surface for time and temperature; d) RCP response surface for time and precursor amount.

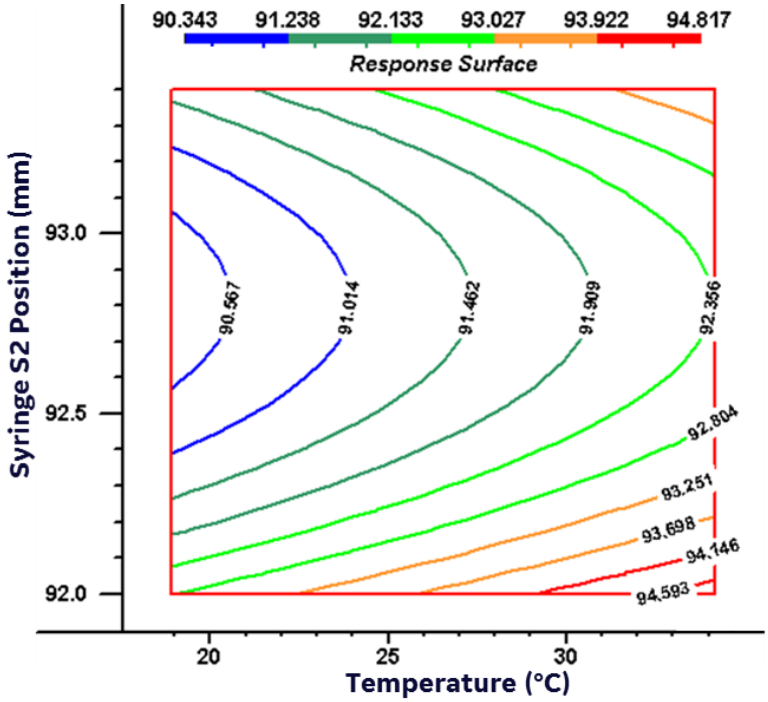

Figure 4. The dependence of RCP % (3 hr post formulation) on temperature and the syringe S2 position (mm) at start of synthesis activity of 70 GBq.

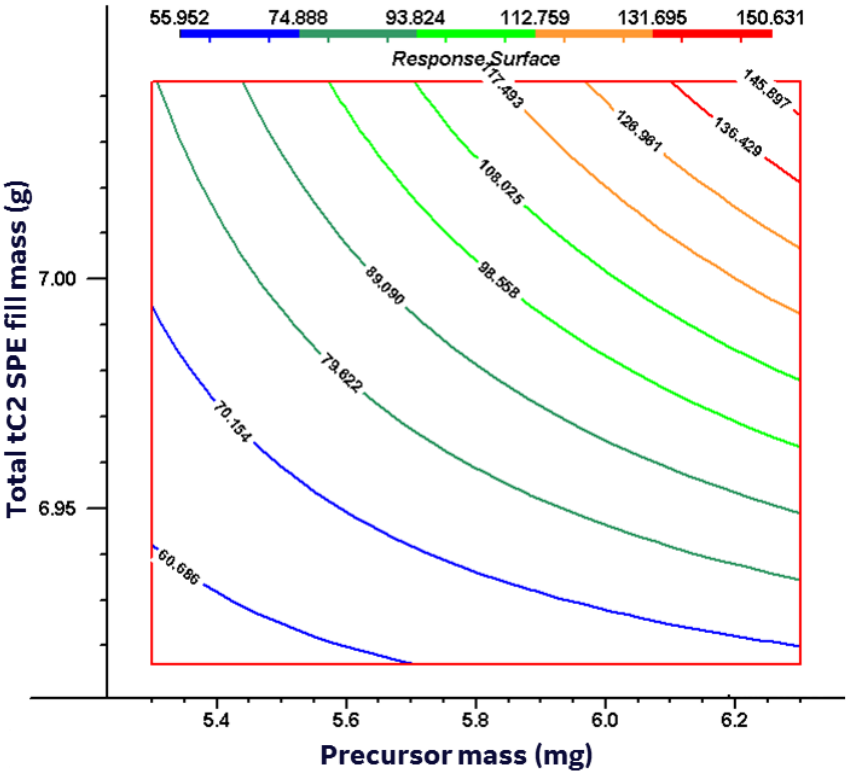

Figure 5. The dependence of total chemical impurity mass (µg) on the precursor mass (mg) and the total tC2 SPE fill mass (g).

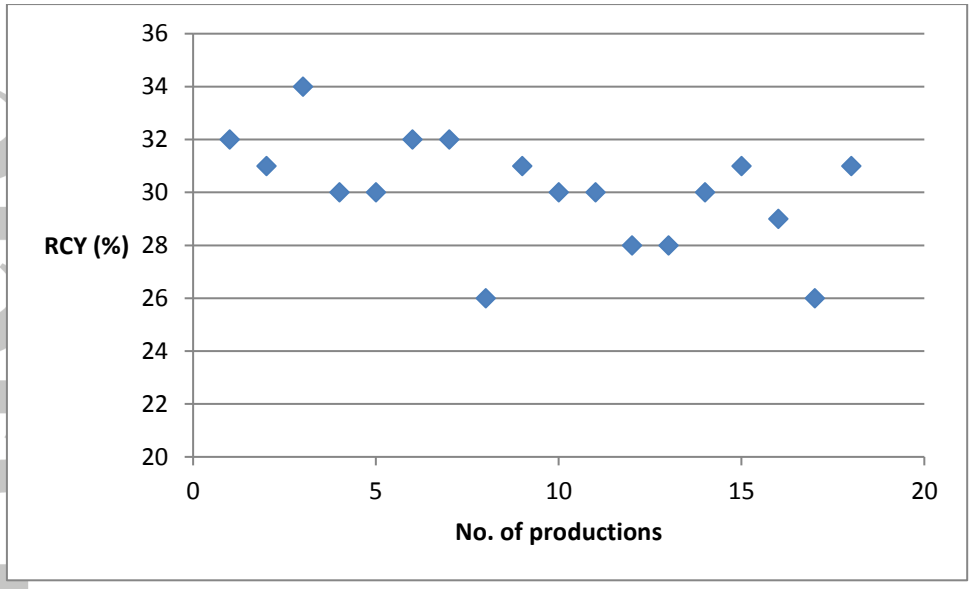

Figure 6. Radiochemical yield (% RCY) (non-decay corrected) of [^{18}F]GE-226 for a number of FASTlab productions.

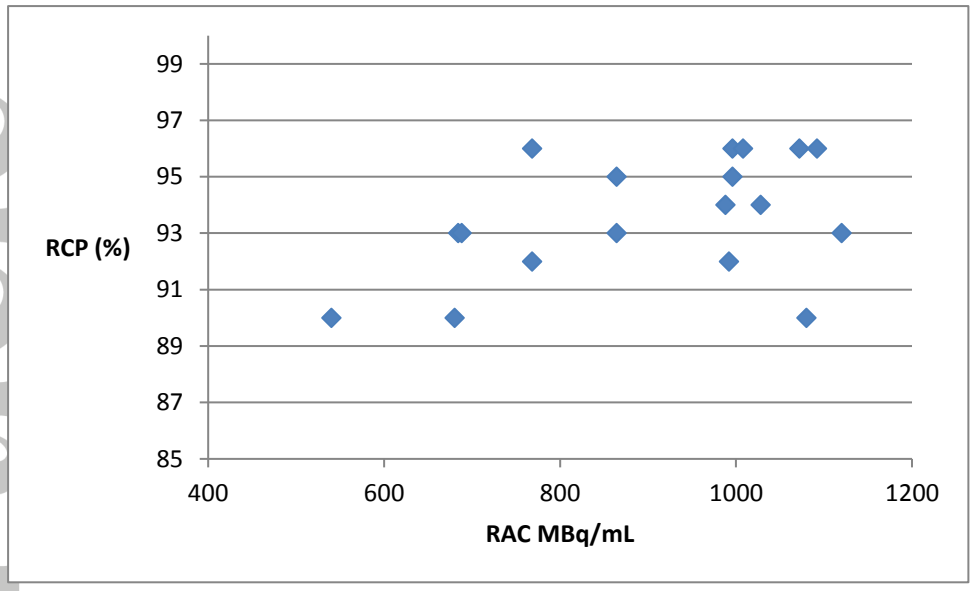

Figure 7. Radiochemical purity (% RCP) versus radioactive concentration (RAC, MBq/mL) of [18F]GE-226 for a number of FASTlab productions. The specification requirement is RCP not less than 90% at EOS.

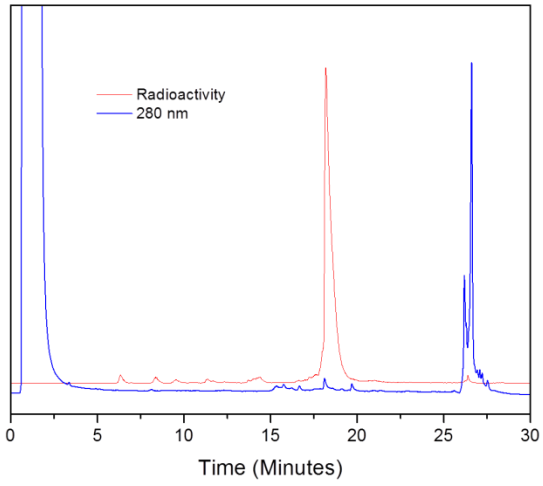

Figure 8. Representative HPLC radiochromatogram of formulated [^{18}F]GE-226 (tR = 18.5 min).

Table 1: HPLC conditions for determination of radiochemical purity and chemical impurities by UV detection	
Parameter	**Description**
Mobile phase	10 – 30 % B in 25 min
Mobile phase A	30mM ammonium formate pH 4
Mobile phase B	MeCN
Column	Phenomenex Kinetex C18, 2.6µm, 50 x 4.6 mm
Detector	UV 280 nm and radiometric detector
Guard Column	Phenomenex KrudKatcher
Flow rate	1.0 mL / min
Run time	31 min

Table 2: HPLC-UV/MS conditions for identification of chemical impurities in decayed samples	
Parameter	**Description**
Mobile phase	0 – 1 min 10% B, 1 -15min 10 – 40% B, 15 – 20 min 40 – 70% B
Mobile phase A	0.1% (v/v) formic acid, 20 mM ammonium acetate, 10% acetonitrile (v/v) in H_2O
Mobile phase B	0.1% (v/v) formic acid, 20 mM ammonium acetate in 85.9% (v/v) acetonitrile
Column	Phenomenex Kinetex C18, 2.6µm, 100 x 4.6 mm
Detector	UV 215 nm, mass spectrometry, mass range m/z 100 – 2000
Guard Column	Phenomenex KrudKatcher
Flow rate	1.0 mL / min
Run time	30 min