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A fully automated synthesis of [^{18}F]-FEAU and [^{18}F]-FMAU using a novel dual reactor radiosynthesis module

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2′-Deoxy-2′-[^{18}F]fluoro-5-substituted-1-β-D-arabinofuranosyluracils, including 2′-deoxy-2′-[^{18}F]fluoro-5-methyl-1-β-D-arabinofuranosyluracil [^{18}F]FMAU and [^{18}F]FEAU are established radiolabeled probes to monitor cellular proliferation and herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene expression with positron emission tomography. For clinical applications, a fully automated CGMP-compliant radiosynthesis is necessary for production of these probes. However, due to multiple steps in the synthesis, no such automated synthetic protocols have been developed. We report here a fully automated synthesis of [^{18}F]-FEAU and [^{18}F]-FMAU on a prototype dual reactor module TRACERlab FX FN. The synthesis was performed by using a computer-programmed standard operating procedure, and the product was purified on a semipreparative high-performance liquid chromatography (HPLC) integrated with the synthesis module using 12% EtOH in 50 mM Na_2HPO_4. Finally, the percentage of alcohol was adjusted to 7% by adding Na_2HPO_4 and filtered through a Millipore filter to make dose for human. The radiochemical yield on the fluorination was 40 ± 10% ($n = 10$), and the overall yields were 4 ± 1% (d. c.), from the end of the bombardment; [^{18}F]FEAU ($n = 7$) and [^{18}F]FMAU ($n = 3$). The radiochemical purity was >99%, specific activity was 1200–1300 mCi/μmol. The synthesis time was 2.5 h. This automated synthesis should be suitable for production of [^{18}F]FIAU, [^{18}F]FFAU, [^{18}F]FCAU, [^{18}F]FBAU and other 5-substitued thymidine analogues.

Keywords: fluorine-18; nucleoside; PET; automated synthesis

Introduction

A number of radiolabeled 2′-deoxy-2′-fluoro-5-substituted-1-β-D-arabinofuranosyluracil derivatives have been recognized as efficient probes for imaging tumor proliferative activity[1–7] and herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene expression[8–20] with positron emission tomography (PET). Among these, 2′-deoxy-2′-[^{18}F]fluoro-5-methyl-1-β-D-arabinofuranosyl-uracil ([^{18}F]-FMAU), 2′-deoxy-2′-fluoro-5-[^{11}C]methyl-1-β-D-arabinofuranosyl-uracil ([^{11}C]-FMAU) and 2′-deoxy-2′-[^{18}F]fluoro-5-bromo-1-β-D-arabinofuranosyluracil ([^{18}F]-FBAU) are markers for DNA synthesis through phosphorylation by human and other mammalian nucleoside kinases including thymidine kinase TK1 and TK2,[3–5] and FMAU is currently undergoing clinical studies in two centers for imaging tumor proliferation in a variety of cancer types and DNA synthesis.[3–5,21] The other derivatives, such as 2′-deoxy-2′-[^{18}F]-fluoro-5-iodo-1-β-D-arabinofuranosyluracil ([^{18}F]-FIAU), 2′-deoxy-2′-[^{18}F]fluoro-5-fluoro-1-β-D-arabinofuranosyl-uracil ([^{18}F]-FFAU) and 2′-deoxy-2′-[^{18}F]-fluoro-5-chloro-1-β-D-arabinofuranosyl-uracil ([^{18}F]-FCAU) are excellent substrates for the viral kinases such as herpes simplex virus (HSV) type 1 and 2,[9,11] and FIAU is also a substrate for hepatitis B virus[22,23] and Epstein B virus (EBV) thymidine kinase.[24,25] These 2′-fluoro-5-substitued arabinosyluracil derivatives were synthesized and evaluated earlier as antiviral agents.[26–28] The radiochemical synthesis of FMAU with PET isotope ([^{11}C]) was reported by Conti *et al.*[29]. However, due to the short half-life of [^{11}C] ($t_{1/2} = 20$ min) there was a demand to develop [^{18}F]-labeled compounds; and the radiosynthesis of [^{18}F]-labeled FMAU and other 5-substituted thymidine analogues was reported by Alauddin *et al.*[30,31]. Following this synthesis another group of investigators also reported the [^{18}F]-labeled synthesis of these pyrimidine nucleoside analogues.[32]

The radiosynthesis of these pyrimidine nucleoside analogues with [^{18}F] is a modification of the original synthesis reported earlier.[26–28] The radiosynthesis involves radiofluorination of 2-trifluoromethanesulfonyl-1,3,5-tri-*O*-benzoyl ribofuranose to 2-[^{18}F]-fluoro-1,3,5-tri-*O*-benzoyl arabinofuranose derivative followed by conversion to 1-bromo-2-[^{18}F]-fluoro-1,3,5-tri-*O*-benzoyl derivative, then coupling of the 1-bromo-2-[^{18}F]fluoro-2,3,-di-*O*-benzoyl-arabinofuranose with 2,4-*bis*-trimethylsilyluracil derivatives. Finally, hydrolysis of the protecting groups from the sugar moiety and high-performance liquid chromatography (HPLC) purification produces the desired products. An ideal radiosynthesis procedure involves a single step radiolabeling of a precursor compound, followed by hydrolysis of protecting

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groups, if necessary, and purification of the crude mixture. However, such an ideal method has not been successful when applied to the radiolabeling of 2′-fluoro-arabino substituted pyrimidine nucleosides. Multiple steps are required after radiolabeling of the sugar moiety. As a result, this method is difficult, and many radiochemists have failed in routine production of these radiotracers. Therefore, we have been producing these compounds on a routine basis by fluorination of the sugar in an automated synthesis module, such as an FDG synthesis module, and then the subsequent steps are performed on a home built semi-automated system operated by a switch box.[33] A similar semi-automated radiosynthesis method of [^{18}F]-FEAU has been published recently by others.[34] For clinical applications, an efficient fully automated CGMP-compliant radiosynthesis system for production of these probes is necessary. To our knowledge, no automated synthesis of [^{18}F]-FEAU or [^{18}F]-FMAU using a commercial synthesis module has been reported yet. In this article we report for the first time a fully automated synthesis of [^{18}F]-FEAU and [^{18}F]-FMAU using a prototype dual reactor radiosynthesis module, TRACERlab FX FN (GE Healthcare), which can produce radiotracers for clinical applications in a CGMP compliant way.

Results and discussion

Figure 1 represents the schematic diagram of the automated synthesis module and Figure 2 represents the scheme for the synthesis of the pyrimidine nucleoside analogues, [^{18}F]-FEAU, [^{18}F]-FMAU and other 5-substituted compounds. The chemistry and the synthetic steps are identical as reported earlier.[30,31] The difference between this automated synthesis and the previously developed semi-automated synthesis[33] is that a computer operated fully automated synthesis compared to a partial automated and partial manual synthesis. For the fully automated synthesis we used a modified dual reactor TRACERlab FX FN, which is completely different from the semi-automated system.

The overall radiochemical yield in this automated synthesis was $4 \pm 1\%$ (d. c.) from the end of bombardment in ten runs. This yield is lower than that reported by others on a semi-automated system (5 ± 1),[34] and much lower than our own synthesis yields (15–20%).[33] We could estimate the radiochemical yields in the radiofluorination step on the sugar triflate **1** to produce **2**, which was in the range of 30–50% with an average of $40\% \pm 10$. However, we could not estimate the yields in the bromination of **2** to produce **3** and coupling between **3** and **4**, rather, we could calculate the overall yield of the final product **6**, which was $4\% \pm 1$ (d. c.) from the end of bombardment. The radiochemical yields in the fluorination step and coupling between the fluorosugar and the protected base were significantly lower compared with the manual synthesis and semi-automated synthesis. The main reason of the overall lower yield is the inefficient coupling between **3** and **4**, which is due to the differences in automation components between the fully automated synthesis module and the semi-automated or manual synthesis. One of the reasons is that a significant amount of the solvent from the reactor is evaporated during fluorination (80°C for 15 min), and coupling between the base and sugar (100°C for 1 h), and the evaporated solvent enters into the Teflon tubing. The solvent is condensed and remains as liquid in the tubing, as a result a part of the reaction mixture from the surface of the reactor adheres on the wall as white solid, which does not react. On the other hand, in the manual/semi-automated system the entire reaction mixture remains in solution in the V-vial in a closed system without any solvent loss. During our synthesis in this box using a low-level radioactivity, we used to shake the reactor manually to dissolve the reactants in the reaction mixture and kept them in solution. As a result, we observed similar coupling efficiency as our semi-automated synthesis. In the fully automated system, the manual shaking was not possible. A small magnetic bar provided with the module is not sufficient to agitate and wash the reagents that get solidified and adheres on the wall of the reactor. A larger magnetic bar may help to agitate and bring back the reagents into solution; however, more studies to be done for optimization of these steps and yields in both fluorination reaction and coupling reaction.

In this automated synthesis system we used both the commercial precursor compounds, such as sugar triflate **1** and the 2,4-*bis*-trimethylsilyl-5-ethyluracil **4** (ABX), as well as prepared in house. Precursors from both the sources were suitable for the synthesis; however, the 2,4-*bis*-trimethylsilyl-5-ethyluracil **4** from ABX produced a small amount of white precipitate after dissolving in the reaction solvent (dichloroethane). The white precipitate is 5-ethyluracil produced from the 2,4-*bis*-trimethylsilyl-5-ethyluracil **4** by decomposition. This low-level decomposition of the *bis*-trimethylsilyl-ether **4** did not reduce the yield in the coupling reaction significantly, as this reagent was used in 4- to 6-fold excess than the sugar triflate **1**. In the radio fluorination step, we optimized the amount of sugar triflate **1** by using as low as 5 mg/synthesis and high as 10 mg. The radiochemical yields were comparable in both low and high quantities of triflate; therefore, we preferred to use 5 mg of the sugar triflate in the synthesis. It should be noted that if higher quantity of the sugar friflate **1** is used for radiolabeling, the pyrimidine base **4** should also be higher (4- to 6-fold) to get high coupling efficiency (55–65%). Although the use of excess triflate may help to produce slightly higher yield in the fluorination step, the problem arises during purification of the product by HPLC. Using high-level of triflate and larger quantity of the pyrimidine base, a significant amount of the pyrimidine (5-EU or thymine) is eluted as a tail in the desired product during HPLC purification. Therefore, it is necessary to reduce the amount of triflate **1** at the fluorination step to avoid 5-EU in the desired product. We found that 5 mg/synthesis is optimal, because it produces a reasonably high yield (30–50%) of the fluorosugar **2** and reduces the amount of the 5-EU in the final product.

The lower yields observed with the fully automated synthesis module should be resolved either by using a smaller reactor or some mechanical mixing/shaking device in a large reactor to keep the reaction mixture uniform in solution. Additional studies will be required to optimize this step. At this stage, we have demonstrated that the dual reactor module TRACERlab FX FN is suitable for automated production of these nucleosides for clinical application, although the yields are slightly lower.

Experimental

Reagents and instrumentation

All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), and used without further purification. Solid phase extraction cartridges (silica gel, 900 mg) were purchased from Alltech Associates (Deerfield, IL, USA). Ion exchange cartridges were purchased from ABX (Germany).

2-Trifluoromethanesulfonyl-1,3,5-tri-*O*-benzoyl-α-D-ribofuranose (precursor) **1** and *bis*-2,4-trimethylsilyl-5-ethyluracil **4** were prepared in house or purchased from ABX (Germany). Nonradioactive compounds FEAU and FMAU were prepared in house for HPLC standards.

HPLC was performed on a pump (integrated with the synthesis module) with UV detector operated at 254 nm, and a built in radioactivity detector (GE Healthcare, Germany) using a semipreparative C_{18} reverse phase column (GE Health care, $16 \times 250\,mm^2$, Germany) and an analytical C_{18} column (Alltech, $4.6 \times 250\,mm^2$, (Deerfield, IL, USA)). A solution of 12% ethanol in aqueous Na_2HPO_4 (50 mM, pH 6.5) was used for purification of [^{18}F]-FEAU, and 10% ethanol in aqueous Na_2HPO_4 (50 mM, pH 6.5) was used for purification of [^{18}F]-FMAU. A solution of 10% MeCN in water and an 8% MeCN in water was used for quality control analysis of [^{18}F]-FEAU and [^{18}F]-FMAU, respectively, on analytical HPLC.

The dual reactor automated synthesis module TRACERlab FX FN was provided by GE Healthcare (Germany) as a part of academic–industrial collaboration. The synthesis module is composed of nine reagent reservoirs operated by electric valves and an HPLC system (Figure 1). Two reactors are connected to the reagent reservoirs and vacuum pump. The valves from V1 to V9 control the solvent and reagent containing reservoirs 1–9. Reservoirs 2, 3 and 4 are directly connected with reactor 1 (R1), and reservoirs 5, 6, 7, 8 and 9 are connected with reactor 2 (R2). Reservoirs 3–9 are connected with a nitrogen or argon gas line. Reservoir 1 is connected with reactor 1 through several control valves. Both reactors R1 and R2 are connected with vacuum pump, and reactor 2 is also connected with the injection port of the HPLC system. Beside these valves 1–9, there are other valves, each control the appropriate operations as designated and necessary such as transferring reagents or solvents, injection of the crude product to the HPLC, collection of fraction during HPLC purification and transfer of the final product from the collection flask to a receiving vial. Valves 34, 35 and 36 are spare valves and they are connected with the respective reservoirs.

Methods

The automated syntheses were performed according to the synthetic scheme (Figure 2). All reagents were stored in the reservoirs sequentially with the appropriate reagents and solvents (Figure 1) under nitrogen before receive the [^{18}F]-fluoride from the target of the cyclotron. After receiving the radioactivity in the synthesis module, the automated synthesis was started using the computer-programmed standard operating procedure (time-list). Radioactivity in ^{18}O-water was transferred from the receiving vial (Figure 1 left bottom) to the ion exchange cartridge to trap the [^{18}F]-radioactivity, and then eluted with K_2CO_3/kryptofix solution (1.2 mL; K_2CO_3 2.75 mg/mL water, and kryptofix 12 mg/mL MeCN) from reservoir 1 into the reactor one (R1) through V1, V10, V11 and V13. Water and solvent were evaporated from the radioactive fluoride by heating at 95°C and in combination with nitrogen flow and vacuum. The residual water was removed by azeotropic evaporation with MeCN transferred through V2, under vacuum and nitrogen flow. To the dry fluoride (reactor 1) sugar triflate (compound **1**) in MeCN (5 mg/mL, 1 mL) was transferred through V3 and heated for 15 min at 85°C. After cooling the reactor (R1) to 40°C, the reaction mixture was transferred to reactor two (R2) through a silica gel sep-pack cartridge. The cartridge was washed with 2.5 mL EtOAc through V4, and collected into reactor 2. Solvent was evaporated and HBr/AcOH in dichloroethane (0.2 mL in 0.7 mL) was transferred to reactor 2 through V5, and heated for 10 min at 80°C. The reactor 2 was cooled to 55°C, solvent and excess reagents were evaporated by vacuum under nitrogen, and then toluene was added through V6 to evaporate any residual AcOH. Reagent **4** (2,4-*bis*-trimethylsilyl-5-ethyluracil)

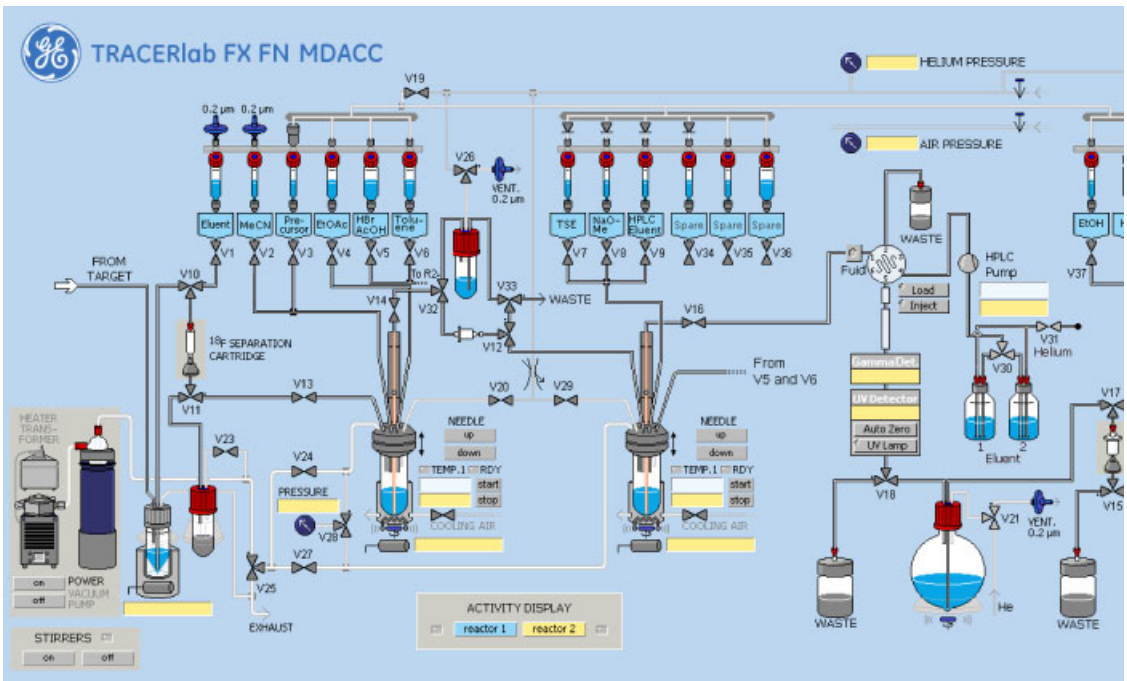

Figure 1. Schematic diagram (screen picture) of the automated synthesis module TRACERlab FX FN.

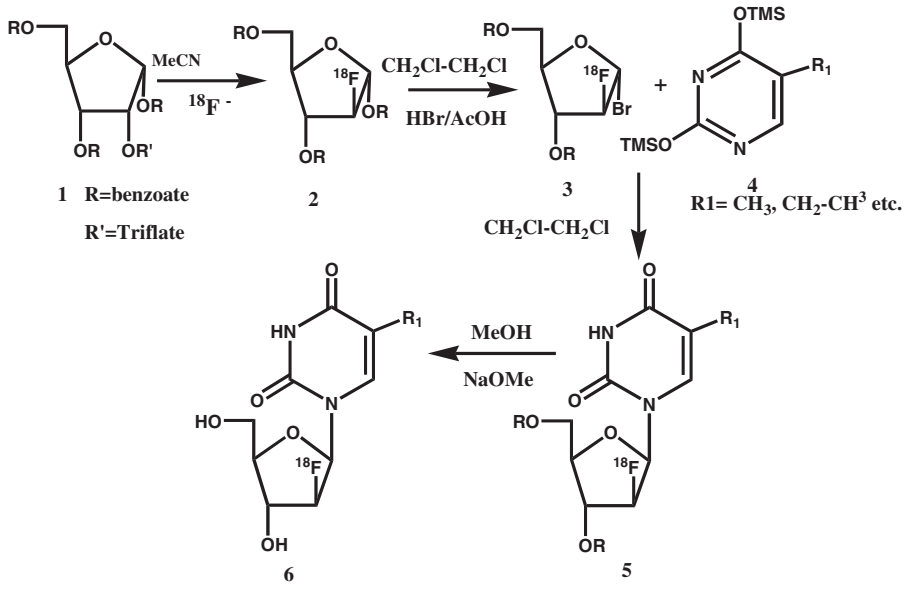

Figure 2. Synthetic scheme of 2′-deoxy-2′-[^{18}F]fluoro-5-substituted-1-β-D-arabino-furanosyluracil ([^{18}F]FEAU, [^{18}F]FMAU, etc.).

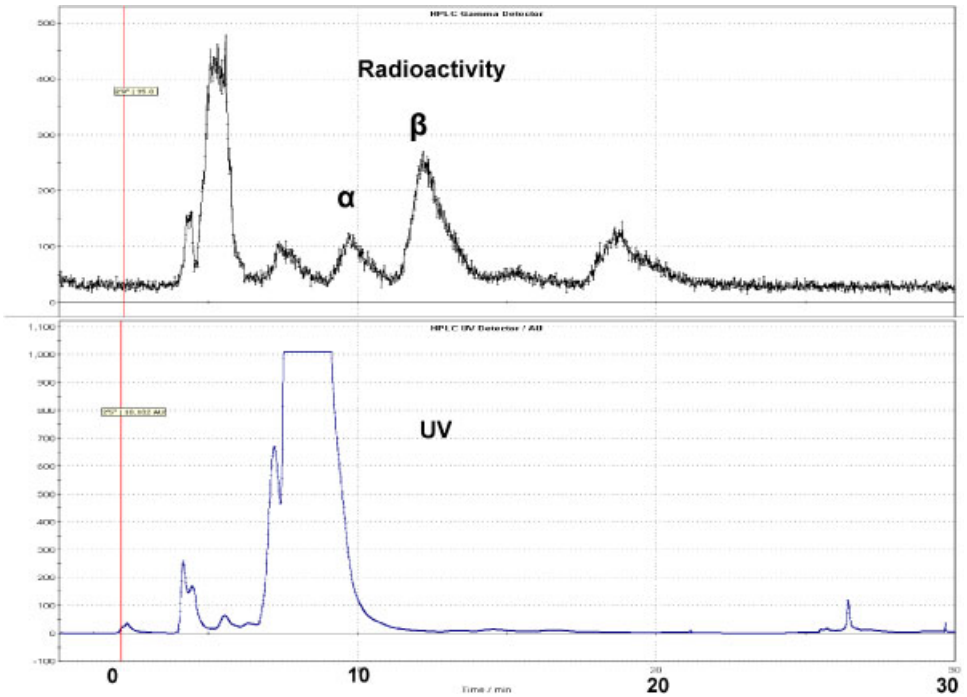

Figure 3. HPLC chromatogram for purification of [^{18}F]-FEAU on a semipreparative column; solvent 12% ethanol in aqueous Na_2HPO_4 (50 mM, pH 6.5), flow 10 mL/min. Two coupled anomeric products α and β are shown, β being [^{18}F]-FEAU.

was then transferred to the reactor 2 through V7, and the reaction mixture was heated for 1 h at 100°C. The reactor 2 was cooled to 40°C, evaporated solvent, NaOMe (0.5 M, 0.2 mL) in MeOH (0.8 mL) was added through V8, and then reactor 2 was heated for 7 min at 80°C. Reactor 2 was finally cooled to 40°C and the solvent was evaporated, and this ended the synthesis.

HPLC purification: The automated synthesis module was set at the manual operation mode, HPLC solvent was transferred to reactor 2 through V9 and the solution was stirred for a minute and then injected to the HPLC column through V16. The column was eluted with 12% EtOH/50 mM Na_2HPO_4 (pH 6.5). Figure 3 represents a semipreparative HPLC chromatogram. The appropriate fraction was collected into the collection flask and then transferred to the receiving vial. A solution of Na_2HPO_4 (75% v/v of the collected product) was added to reduce the percent of ethanol (<7%), and the product was filtered through a Millipore filter. The product was co-injected with an authentic unlabeled sample onto an analytical column to confirm its identity and radiochemical purity (Figure 4).

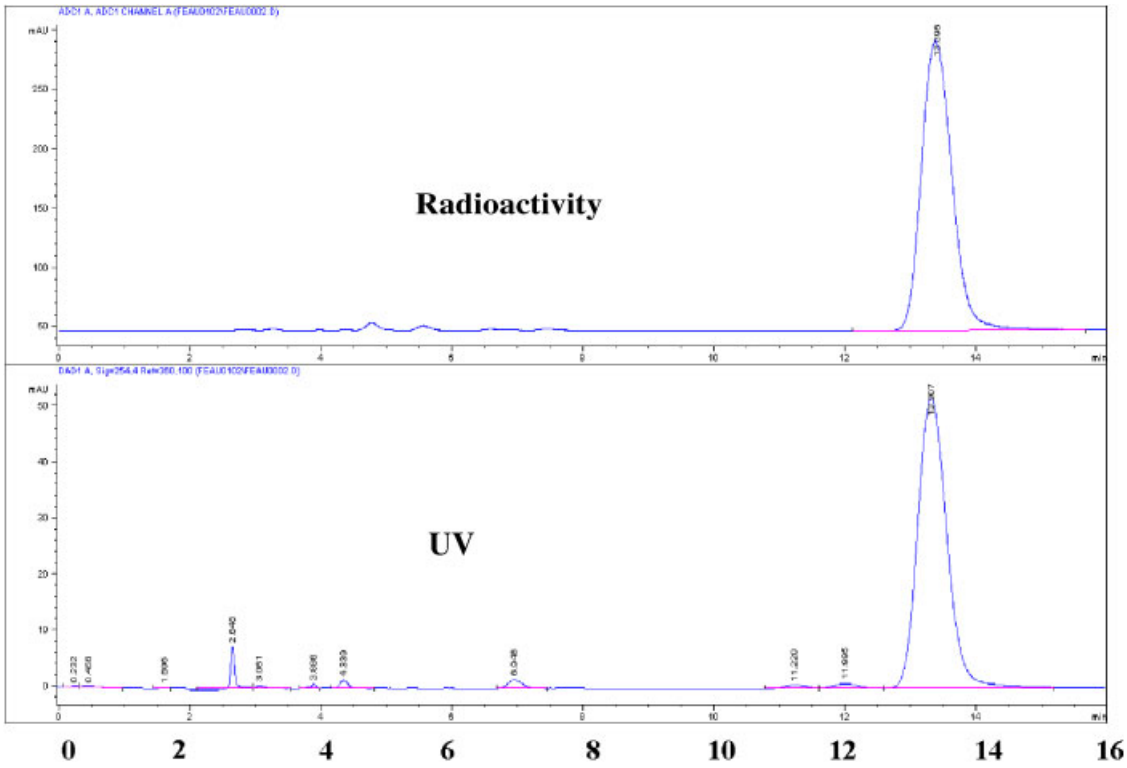

Figure 4. HPLC chromatogram of [^{18}F]-FEAU, co-injected with standard FEAU: analytical C_{18} column; 10% MeCN/H_2O; flow 1.0 mL/min.

Conclusion

A fully automated synthesis of [^{18}F]-FEAU and [^{18}F]-FMAU has been achieved for the first time in reasonable yields and high purity using a prototype dual-reactor synthesis module TRACERlab FX FN for clinical applications. The synthesis module can be applied for the production of other 2′-[^{18}F]fluoro-2′-deoxy-arabino-5-substituted pyrimidine nucleoside analogues.

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