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Radiosynthesis of N^5-[^{18}F]fluoroacetylornithine (N^5-[^{18}F]FAO) for PET imaging of ornithine decarboxylase (ODC) in malignant tumors

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Polyamines are naturally occurring polycations derived from amino acids via decarboxylation by ornithine decarboxylase (ODC). Ornithine is a substrate for ODC; decarboxylation of ornithine is inhibited by difluoromethylornithine (DFMO) and its derivatives. Polyamine contents are increased in many epithelial cancers, including breast cancer, melanoma, and prostate cancer. In order to image and measure the levels of ODC expression in malignant tumors, we have synthesized a derivative of ornithine, N^5-[^{18}F]fluoroacetylornithine (N^5-[^{18}F]FAO), for use in positron emission tomography. The precursor compound N^2-Boc-N^5-bromoacetylornithine-*t*-butyl ester 2 was synthesized from 5-amino-2-(*tert*-butoxycarbonylamino)pentanoic acid, which was reacted with bromoacetyl chloride followed by esterification with *tert*-butyl-2,2,2-trichloroacetamidate. Fluorination of the precursor produced a fluoro-derivative, which was hydrolyzed in acid to obtain the desired compound, N^5-fluoroacetylornithine. The radiosynthesis of N^5-[^{18}F]FAO was accomplished by radiofluorination of 2 with *n*-Bu_4N[^{18}F], followed by high-performance liquid chromatography (HPLC) purification and then by acid hydrolysis. The radiochemical yield was 6–10% (decay corrected) with an average of 8% ($n = 10$) at the end of synthesis. The radiochemical purity was $>$99%, and specific activity was $>$1500 mCi/μmol. The synthesis time was 95–100 min from the end of bombardment.

Keywords: ornithine decarboxylase; ODC; N^5-Fluoroaceylornithine; ^{18}F; PET

Introduction

Ornithine decarboxylase (ODC) is the key enzyme that catalyzes the rate-limiting step in the biosynthesis of polyamines, which are positively charged aliphatic amines.[1,2] The decarboxylation of ornithine by ODC leads to the only route for *de novo* biosynthesis of the diamine putrescine, which is then further converted into spermidine and spermine by the concerted actions of down stream enzymes.[3,4] Polyamines are ubiquitous to all cells, and they play a critical role within living cells via their involvment in cell proliferation, translation, replication and transcription, and in cell differentiation.[5,6] Transcriptional and posttranscriptional regulations of ODC levels strictly control the intracellular polyamine pools. ODC is upregulated in various types of cancer, including breast cancer, melanoma, and neuroblastoma.[7–9] An elevated ODC activity is associated with malignancy resulting in an increased polyamine biosynthesis to support rapid cell proliferation. The modest reduction in ODC activity could lead to a significant decrease in tumor development.[4]

The specific inhibition of polyamine biosynthesis has been under comprehensive investigation and widely explored for designing ornithine analogues as drug targets in the treatment of cancer.[10–14] Previous studies have revealed the structural features of ODC inhibitors and substrates via mechanistic study of ODC inactivation by α-difluoromethylornithine (DFMO) and other ornithine derivatives.[5,14] The structural motif of inhibitors that alter the polyamine biosynthesis pathway has to contain the ornithine substructure with a substitution at either the α-carbon and/or the C_5-nitrogen.

DFMO was developed as an active inhibitor of ODC, and it is currently in clinical trials as a chemopreventive drug to suppress surrogate end-point biomarkers of carcinogenesis in patient populations at elevated risk for the development of specific epithelial cancers, including, colon, esophageal, breast, cutaneous, and prostate malignancies.[15] Many ornithine derivatives other than DFMO, including N^5-chloroacetyl and N^5-iodoacetyl derivatives, have been reported as inhibitors of ODC.[16,17] Both docking simulation and kinetic studies showed that N^5-chloroacetyl and N^5-iodoacetyl derivatives of ornithine are active ODC inhibitors. Therefore, a radiolabeled fluoro analog of the N^5-substituted ornithine should be an effective agent for non-invasive imaging of ODC in malignant tumors with positron emission tomography (PET).

Although much work has been carried out on the development of ODC inhibitors, no attempt has been made to enable the detection of ODC expression in malignant tumors. ODC expression is increased with increase in malignancy grade, and may be used for detection, prognosis, and selection of individual targeted combined therapies that include ODC inhibitors. In this article, we report for the first time the synthesis and

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radiosynthesis of an [^{18}F]-labeled ornithine derivative, N^5-fluoroacetylornithine (N^5-[^{18}F]FAO).

Experimental

Reagents and instrumentation

All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. 5-Amino-2-(*tert*-butoxycarbonylamino)pentanoic acid was purchased from Chem-Impex International, Inc. (Wood Dale, IL). Solid-phase extraction cartridges (silica gel, 900 mg) were purchased from Alltech Associates (Deerfield, IL).

Thin layer chromatography (TLC) was performed on precoated Kieselgel 60 F254 (Merck, Darmstadt, Germany) glass plates. Proton and ^{19}F NMR spectra were recorded on a Bruker 300 MHz spectrometer with tetramethylsilane used as an internal reference and hexafluorobenzene as an external reference at The University of Texas M. D. Anderson Cancer Center. High-resolution mass spectra were obtained on a Bruker BioTOF II mass spectrometer at the University of Minnesota using electrospray ionization technique.

High-performance liquid chromatography (HPLC) was performed with an 1100 series pump (Agilent, Germany), with a built-in UV detector operated at 214 or 254 nm, and a radioactivity detector with single-channel analyzer (Bioscan, Washington DC) with a semi-preparative C_{18} reverse phase column (10 × 250 mm) and an analytical C_{18} column (4.6 × 250 mm) (Alltech, Econosil). An acetonitrile/water (MeCN/H_2O) solvent system (50% MeCN/H_2O) was used for purification of the radiolabeled product at a flow of 4 ml/min. Quality control analyses were performed on an analytical HPLC with the same solvents (50% MeCN/water) at a flow of 1 ml/min, and 5% MeCN/water at a flow of 1.0 ml/min.

Preparation of N^2-Boc-N^5-bromoacetylornithine 1

5-Amino-2-(*tert*-butoxycarbonylamino)pentanoic acid (2.50 g, 0.01 mol) and K_2CO_3 (2.85 g, 0.02 mol) were placed in a mixture of water and tetra hydrofuran (THF) (1:1, 80 mL). Bromoacetyl chloride (2.50 g, 0.01 mol) was added dropwise via a syringe, and the mixture was stirred at room temperature for 1.5 h. THF was removed under reduced pressure, the aqueous phase was extracted with ether (1 × 50 mL), and the ether extract was discarded. The aqueous layer was acidified to pH 1 using 4 N HCl and extracted with ethyl acetate (3 × 50 ml). The combined ethyl acetate extracts were dried over Na_2SO_4. The solvent was evaporated and the crude product was subjected to column chromatography, and then eluted with 10% methanol in dichloromethane. Appropriate fractions were combined and evaporated to dryness to obtain N^2-Boc-N^5-bromoacetylornithine **1** as a colorless oil in 40% yield. ^{1}H NMR ($CDCl_3$), δ: 6.70 (s, 1H, NH), 5.11 (d, 1H, J = 7.5 Hz, NH), 4.21 (m, 1H, C_2-H), 3.90 (s, 2H, CH_2Br), 3.35 (m, 2H, C_5-H), 1.90–1.6 (m, 4H, C_3- and C_4-H), 1.46 (s, 9H, Boc). High-resolution MS: [M+Na] for $C_{12}H_{21}BrN_2O_5Na$, calculated, 375.0532; found, 375.0474.

Preparation of N^2-Boc-N^5-bromoacetylornithine-*t*-butyl ester 2

To a solution of **1** (0.50 g, 1.42 mmol) in 20 ml of CH_2Cl_2 was added *tert*-butyl-2,2,2-trichloroacetamidate (1.10 g, 5.00 mmol). The solution was stirred at room temperature for 16 h, and then the solvent was removed under reduced pressure. The residue was chromatographed on a silica gel column using 20–50% ethyl acetate in hexane as a gradient elution to afford the product **2**, which was obtained as a thick oil in 86% yield. ^{1}H NMR ($CDCl_3$), δ: 6.70 (s, 1H, NH), 5.11 (d, 1H, J = 7.5 Hz, NH), 4.21 (m, 1H, C_2-H), 3.90 (s, 2H, CH_2Br), 3.35 (m, 2H, C_5-H), 1.90–1.6 (m, 4H, C_3- and C_4-H), 1.49 (s, 9H, *t*-butyl), 1.46 (s, 9H, Boc). High-resolution MS: [M+Na] for $C_{16}H_{29}BrN_2O_5Na$, calculated, 431.1158; found, 431.1166.

Preparation of N^2-Boc-N^5-fluoroacetylornithine-*t*-butyl ester 3

To a solution of N^2-Boc-N^5-bromoacetylornithine-*t*-butyl ester **2** (0.10 g, 0.24 mmol) in DMSO (0.3 ml) was added *n*-Bu_4NF solution (1 M in THF, 0.48 ml). The reaction mixture was heated at 80°C for 20 min, then volatile solvent (THF) was removed by a stream of air and the crude product chromatographed on a silica gel column using 20–50% ethyl acetate in hexane as a gradient elution. The fractions that showed a peak at 7.7 min in HPLC (50% acetonitrile/water, 1 ml/min) were combined and the solvent was removed under vacuum to afford product **3** as a colorless oil in 47% yield. ^{1}H NMR ($CDCl_3$), δ: 6.46 (s, 1H, NH), 5.12 (d, 1H, J = 7.5 Hz, NH), 4.81 (d, 2H, J = 47.4 Hz, CH_2F), 4.20 (m, 1H, C_2-H), 3.4 (m, 2H, C_5-H), 1.90–1.6 (m, 4H, C_3- and C_4-H), 1.49 (s, 9H, *t*-butyl), 1.46 (s, 9H, Boc). ^{19}F NMR ($CDCl_3$) (decoupled), δ: (s, −224.6) High-resolution MS: [M+Na] for $C_{16}H_{29}FN_2O_5Na$, calculated, 371.1958; found, 371.1940.

Preparation of N^5-fluoroacetylornithine 4

N^2-Boc-N^5-fluoroacetylornithine-*t*-butyl ester **3** was dissolved in TFA (0.3 ml) and heated at 50°C for 10 min, when HPLC showed complete hydrolysis. Trifluoroacetic acid (TFA) was removed under a stream of air, and the residue was precipitated from methanol/diethylether and filtered to afford the final product **4** as a white solid in quantitative yield. Based on integration of the vertically expanded ^{1}H NMR spectrum, the purity of **4** was >97%. ^{1}H NMR (DMSO-d^6), δ: 8.25 (m, 3H, NH), 4.79 (d, 2H, J = 47.1 Hz, CH_2F), 3.92 (m, 1H, C_2-H), 3.13 (m, 2H, C_5-H), 1.80–1.40 (m, 4H, C_3- and C_4-H). ^{19}F NMR ($CDCl_3$) (decoupled), δ: (s, −225.0). High-resolution MS: [M+1] for $C_7H_{13}FN_2O_3$, calculated, 193.1881; found, 193.1620.

Preparation of N^2-Boc-N^5-[^{18}F]FAO-*t*-butyl ester [^{18}F]-3

The aqueous [^{18}F]fluoride produced from the cyclotron by the reaction of ^{18}O(*p*, *n*)[^{18}F] was trapped on an ion-exchange cartridge (Chromafix 30-PS-HCO_3; ABX, Germany) and eluted with an aqueous solution of *n*-Bu_4NHCO_3 (400 µl, 1% by wt) into a V-vial. Water was removed by an azeotropic evaporation at 80°C with acetonitrile (1.0 ml) under a stream of argon. A solution of **2** (2–3 mg) in DMSO (0.3 ml) was added to the dried *n*-$Bu_4N^{18}F$. The reaction mixture was heated at 80°C for 20 min. The crude reaction mixture was passed through a silica Sep-Pak cartridge followed by elution with two portions of ethyl acetate (EtOAc) (2.5 ml, total), which was evaporated at 50°C under a stream of argon. The residue was diluted with acetonitrile (1.0 ml) and purified by HPLC using a semi-preparative column using 50% MeCN/water. Appropriate fraction (radioactive) was collected, and the solvent was evaporated under reduced pressure. An aliquot of the intermediate product [^{18}F]-**3** was further analyzed on an analytical HPLC to verify its identity by co-injection with the cold standard **3**. The product [^{18}F]-**3** was transferred to a V-vial using dichloromethane, which was

evaporated under a stream of argon, and then hydrolysis was performed, as described in the following step.

Preparation of N^5-[^{18}F]fluoroacetyl)ornithine (N^5-[^{18}F]FAO) 4

N^2-Boc-N^5-[^{18}F]FAO-*t*-butyl ester [^{18}F]-**3** was dissolved in TFA (0.1 ml) and the solution was heated at 50°C for 10 min. TFA was removed under a stream of argon, and then the residue was neutralized with 1 M $NaHCO_3$ solution (0.1 ml). The final product was diluted with saline and analyzed by using HPLC, and co-injected with the standard non-radioactive compound to confirm its identity.

Results and discussion

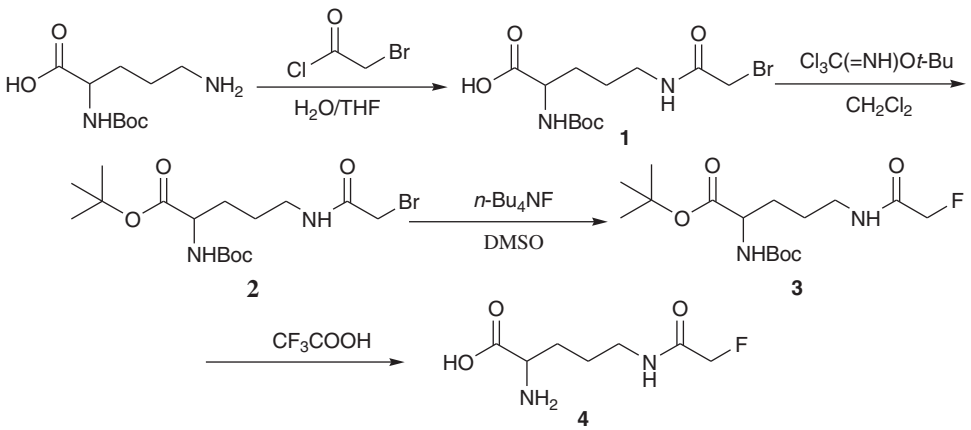
The synthetic scheme of N^5-[^{18}F]fluoroacetylornithine (N^5-FAO) is shown in Scheme 1.

A precursor compound **2** was prepared in two steps for fluorination and radiofluorination. Compound **1** was prepared from the commercially available N^2-Boc-protected ornithine, 5-amino-2-(*tert*-butoxycarbonylamino)pentanoic acid in 40% yield. The challenging part in this step of reactions was the solubility of the reactants, N^2-Boc-protected ornithine and K_2CO_3; as a result, a co-solvent of THF and water had to be used in this step. However, with 40% yield, we had enough material to continue the synthesis of the target compound. Compound **2** was prepared from **1** by esterification with *tert*-butyl-2,2,2-trichloroacetamidate in high yield (86%). Because of the presence of carbamate (Boc-protection) functionality, which is acid labile, the protection of the carboxylic acid function required acid-free conditions. In addition, the ester should be easily hydrolyzable under mild acidic conditions in a shorter time, especially for radiosynthesis. The use of a *t*-butyl group to protect carboxylic acid functionality has been reported to be an effective approach for protection and subsequent de-protection.[18] In our experiment, the use of *N*,*N*-dimethylformamide di-*tert*-butylacetal led to the formation of the desired product, *tert*-butyl-5-(bromoacetamido)-2-(*tert*-butoxycarbonylamino)pentanoate **2**, in poor yield (<5%). Therefore, we had to explore an alternative method, and the use of *tert*-butyl-2,2,2-trichloroacetamidate[19] afforded the desired product **2** in 86% yield.

The non-radioactive compound **3** was synthesized by nucleophilic fluorination of **2** using Bu_4NF in moderate yield (47%). Compound **3** was fully characterized by using NMR and high-resolution mass spectrometry (MS). The ^{1}H NMR spectrum of this compound showed a peak at 4.81 as a doublet with coupling constant of 47.4 Hz, a typical H-F germinal coupling. ^{19}F NMR also showed a signal at −224.6 ppm with similar coupling constants. The final target compound **4** was prepared by hydrolysis of compound **3** using trifluoroactic acid (neat) at 50°C for 10 min. When hydrolysis was performed at a higher temperature, the reaction was not clean: an unidentified by-product was observed in the product. Thus, temperature seems to be an important parameter in the hydrolysis of the compound **3**. Compound **4** was fully characterized by using ^{1}H and ^{19}F NMR spectroscopy and high-resolution MS. Both ^{1}H and ^{19}F NMR spectra of **4** were consistent with the desired compound.

The radiolabeled compound [^{18}F]-**3** was synthesized by no-carrier-added nucleophilic fluorination using $Bu_4N^{18}F$ in 6–10% decay-corrected yield with an average of 8%. Compared with the yield in the non-radioactive fluorination (47%), the radiochemical yield was quite low (8%). This significant difference between the chemical yield and radiochemical yield may have been due to the difference in concentration of the fluoride ion, 2-equivalent (non-radioactive) versus tracer level (radioactive). In order to improve the radiochemical yield, we performed the radiolabeling experiments at two different temperatures, 80 and 85°C, and heated for 30 min but no significant difference in yield was observed. We also performed labeling experiments using $K^{18}F$/kryptofix 2.2.2, which produced lower yields than those in $Bu_4N^{18}F$ reactions. Changing the reaction solvent from DMSO to MeCN did not improve the yield (Table 1). To improve the radiochemical yield, further optimization of the reaction conditions will be required.

The crude reaction mixture after radiofluorination was passed through a silica Sep-Pak cartridge to remove the un-reacted fluoride and then eluted with EtOAc, which was evaporated at 50°C. Evaporation of the EtOAc at higher temperatures results in significant loss of the product because of decomposition or partial hydrolysis. It is important to note that HPLC purification of the product, [^{18}F]-**3** was performed prior to hydrolysis of the protecting groups, because the reaction solvent DMSO cannot be separated from the product after hydrolysis. It was easy to remove the DMSO and other impurities, such as the protected bromoornithine (the precursor) by using HPLC purification before hydrolysis. Thus, HPLC purification of the crude product



Scheme 1. Scheme for synthesis and radiosynthesis of N^5-FAO.

produced the pure compound [^{18}F]-**3**. Figure 1 shows an example of HPLC purification of the crude product [^{18}F]-**3**. The purity of [^{18}F]-**3** was checked by HPLC on an analytical column, and its identity was confirmed by a co-injection of the radioactive product with an authentic non-radioactive standard compound (Figure 2).

Compound [^{18}F]-**4** was obtained by hydrolysis of [^{18}F]-**3** after purification and solvent evaporation. Hydrolysis was performed with TFA at 50°C for 10 min, when HPLC showed no remaining [^{18}F]-**3**. Excess TFA was removed under a stream of argon; the product was neutralized with $NaHCO_3$ solution, and diluted with saline. The final product [^{18}F]-**4** was further analyzed by using HPLC and co-injected with a non-radioactive standard (Figure 3), which verified the identity and purity of the product. The yield in this step was quantitative. The average radiochemical yield in this synthesis was 8% (decay corrected, $n = 10$) with radiochemical purity of >99%, and specific activity of >1500 mCi/μmol. The synthesis time was 95–100 min from the end of bombardment.

Table 1. Radiofluorination of compound **2** under various reaction conditions

Solvent	Time (min)	Fluoride	Temperature (°C)	Average % of crude yield [^{18}F]3	No. runs
DMSO	20	$Bu_4N^{18}F$	80	11.6	8
DMSO	25	$Bu_4N^{18}F$	80	8.3	1
DMSO	20	$Bu_4N^{18}F$	85	7.7	1
DMSO	20	$K^{18}F$/kryptofix	80	5.2	2
MeCN	20	$Bu_4N^{18}F$	80	2.6	2

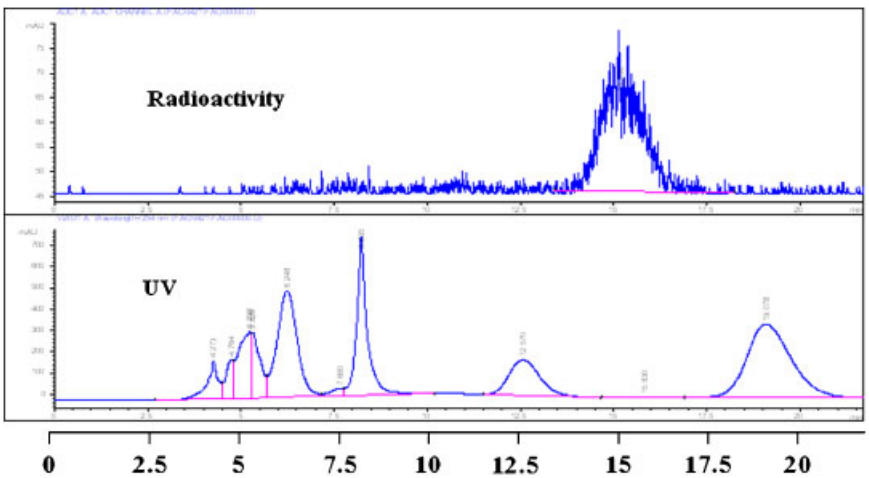


Figure 1. HPLC purification of N^2-Boc-N^5-[^{18}F]FAO-*t*-butyl ester: semi-preparative C_{18} column, 50% MeCN/water, flow: 3 ml/min.

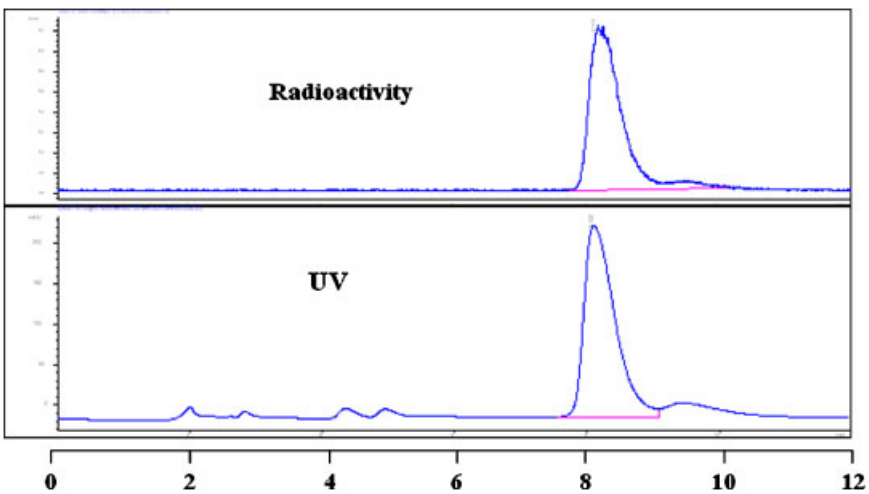


Figure 2. HPLC chromatogram of N^2-Boc-N^5-[^{18}F]fluoroacetylornithin-*t*-butyl ester, co-injected with the non-radioactive standard. Analytical C_{18} column, 50% MeCN/water, flow: 1 ml/min.

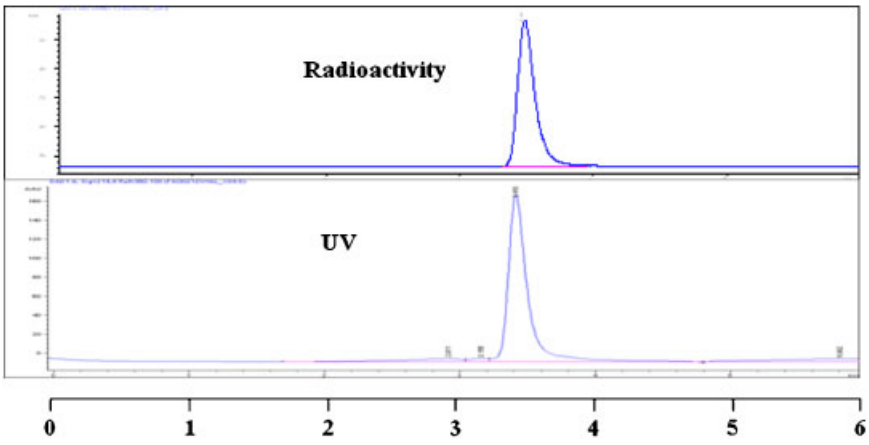


Figure 3. HPLC chromatogram of N^5-[^{18}F]FAO, co-injected with the standard N^5-FAO; analytical C_{18} column, 5% MeCN/water, flow: 1 ml/min.

It is worth noting that the sequence of protection of the functional groups of ornithine is of extreme importance. For instance, the ornithine methylester readily undergoes cyclization under basic conditions to form 3-aminopiperidin-2-one. Opening the cyclized compound (lactone ring) is far from straightforward and requires harsh conditions in addition to a lengthy reaction time, which is unsuitable for the synthesis of [^{18}F]-radiotracers. In our approach, the cyclization was prevented by the selective protection of the functional groups of the ornithine derivative. The synthesis was accomplished in four steps and good overall yield.

Conclusions

N^5-FAO and its radiolabeled analog N^5-[^{18}F]FAO have been synthesized for the first time in good yield, high purity, and high specific activity. This method should be applicable for radiosynthesis of other α-substituted derivatives of ornithine, including methyl, fluoromethyl, and difluoromethyl derivatives. Based on the previous biological studies reported in the literature for ODC substrate characteristics, N^5-[^{18}F]FAO should be a suitable agent for PET imaging of ODC expression in malignant tumors.

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