Original Article

Radiosynthesis of [18F]-flumazenil Using an Isotopic Approach

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Abstract

Background: Fluorine-18 (^{18}F) flumazenil (FMZ) has been synthesized using various precursors, and its role has been explored in imaging Gamma-aminobutyric acid-A receptors. **Aim and Objective:** The main objective was to synthesize (^{18}F) FMZ using isotopic substitution. **Materials and Methods:** Around 18 ± 2 GBq was added to the module, dried, and radiolabeling was standardized with 3.0 mg of the FMZ precursor at various temperatures (110°C –160°C) for 10–30 min. The product was finally eluted with 20% ethanol (in phosphate buffer). The final product was characterized by high-performance liquid chromatography (HPLC). The stability was evaluated in water, saline, and phosphate-buffered saline for 4 h. **Results:** The radiolabelling efficiency of cartridge-based purification was 16 ± 4% (n = 10) with a radiochemical purity of 96.5 ± 1.8%, whereas in HPLC-based purification, the yield was 10 ± 4% (n = 5) with a radiochemical purity of 97.3 ± 1.4%. The specific activity was 120 ± 20 GBq/μmol. **Conclusions:** (^{18}F) FMZ was successfully synthesized using an isotopic approach and could be used as an alternative cheaper option for the synthesis.

Keywords: *Flumazenil, fluorine-18, gabba receptors, isotopic exchange*

Introduction

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. GABA's inhibitory effects are mediated by two types of receptors, $GABA_A$ and $GABA_B$. $GABA_A$ is one of the most significant drug targets in the treatment of neuropsychiatric disorders such as epilepsy, insomnia, and anxiety. GABAergic neurotransmission is critical in neurodevelopmental disorders.[1-3]

The depletion or decrease in GABA receptors has been observed majorly in neurodegenerative disorders such as schizophrenia and dementia. These disorders are usually treated with benzodiazepine drugs. Among various benzodiazepine drugs, flumazenil (FMZ) (ethyl 8-fluoro-5 and 6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5a][1,4] benzodiazepine-3-carboxylate) has the highest affinity and selectively binds to $GABA_A$ receptors (Ki~1nM) in comparison with other benzodiazepines and competitively inhibits the effects of benzodiazepines.[4,5]

The quantification of GABA receptors and treatment response can be assessed by positron emission tomography (PET). Therefore, FMZ has been labeled with radionuclides such as carbon-11 and fluorine-18 (^{18}F). (^{18}F) FMZ has been used widely due to its longer half-life (110 min) and can be transported to other sites as well. Various precursors have been used to synthesize (^{18}F) FMZ, such as nitromazenil, FMZ-BPin, and tosyloxyethylflumazenil.[6-8] The evolution of precursors throughout the years to increase the yield and increase the specific activity of final production. The use of (^{18}F) FMZ synthesized from nitromazenil precursor is limited by the presence of cold nitromazenil in the final preparation, which decreases its specific activity.[9,10] The other precursor, diaryl iodonium, had given high radiochemical yields, but their applicability in routine synthesis was challenging; therefore, it was not used widely.[11] In recent years many copper-mediated ^{18}F-flurotination of aryl boronic species have been used to produce (^{18}F) FMZ.[12]

Many times, the tracers are expensive due to the cost of consumables or precursors. This issue is mostly faced by developing countries where expensive precursors add extra cost to PET imaging. (^{18}F)FMZ was also reported to be labeled by isotopic exchange with (^{19}F)

How to cite this article: Thakur R, Kumar A, Joshi RK, Kumar P. Radiosynthesis of [18F]-flumazenil using an isotopic approach. Indian J Nucl Med 2024;39:286-91.

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Received: 13-06-2024
Revised: 07-08-2024
Accepted: 19-08-2024
Published: 18-11-2024

Access this article online

Website: https://journals.lww.com/ijnm

DOI: 10.4103/ijnm.ijnm_82_24

Quick Response Code:

FMZ using the tC18 cartridge purification method.[13] FMZ (as a precursor) is easily available and can be labeled with ^{18}F to produce (^{18}F) FMZ.[14] In our study, we are reporting the radiolabeling of FMZ using an isotopic exchange approach. We have studied the effect of temperature on labeling and compared two purification methods, i.e., cartridge and preparative high-performance liquid chromatography (HPLC).

Materials and Methods

All the chemicals and reagents were procured from Sigma Aldrich (USA) and used without any further purification. The cold standard of FMZ was procured from Sigma-Aldrich, USA. The ^{18}F radioisotope was produced in our in-house 16.5MeV Cyclotron (PETtrace 860, GE Healthcare, Chicago, USA) by the proton bombardment on the enriched ^{18}O water using standard (^{18}O[p, n]^{18}F) reaction. The proton bombardment was done with the beam current of range of 30–65 μA for 10–30 min depending upon the requirement of (^{18}F)fluoride and delivered to the synthesizer module (Tracerlab FX2N, GE Healthcare, Chicago, USA) using the Helium (UHP-5.5) as a carrier gas. The stock solution of Kryptofix 2.2.2 (K222; 150 mg) and K_2CO_3 (30 mg) was dissolved in acetonitrile (9 mL), including sterile deionized water (1.0 mL). A solution of 0.5 M potassium dihydrogen phosphate (KH_2PO_4) buffer was prepared, and the pH was adjusted to 4.0 with concentrated phosphoric acid, and the eluting solution was 20% ethanol in 0.5 M KH_2PO_4. The pH was tested using pH paper (Fisher Scientific, New Hampshire, USA) and a pH meter (Mettler Toledo, Ohio, USA). The radiochemical purity was measured using HPLC and thin-layer chromatography methods. HPLC system (Dionex, California, USA) equipped with ultraviolet (UV)-Visible coupled and radioactivity detector was used. The quantitative analysis was done on a C18 column (5um 4.6 × 250, Shim-pack GWS, Shimadzu) using mobile phase composition of acetonitrile (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid), starting with 5% acetonitrile (0–5 min), 5% to 100% acetonitrile (5–20 min), then 100% acetonitrile (20–25 min) and again at 5% acetonitrile (25–30 min). The radionuclide purity was evaluated by the half-life method and energy (511 KeV) peak determination on the CAPRAC-t well counter (S. No.10395; New Jersey, USA). The residual solvents like DMF ethanol were measured using Gas chromatography (GC) (Scion 436 GC, Netherlands) with a flame ionization detector. The column was operated initially at 40°C for the first 3 min and then rose to 50°C/min for up to 8 min, and the final temperature was set at 240°C, and the column was BR-200 ms, 0.32 mm ID. The makeup gas consists of Nitrogen (28 mL/min), zero air (300 mL/min), and hydrogen gas (30 mL/min) flow at the rate of 2 mL/min. The dose was measured on a dose calibrator (Capintec CRC-25PET, New Jersey, USA).

Radiochemistry

a) Cartridge-based method (n = 10)

Briefly, 18 ± 2 GBq of (^{18}F) fluoride was transferred to the FX2N module, which was trapped using a preconditioned QMA sep-pak cartridge (ABX, Germany). (^{18}F) fluoride was eluted with 1.0 mL of the QMA eluent solution (from stock solution) filled in vial-1 of the module. The eluted ^{18}F-(K222) complex was dried under vacuum at 95°C for 6 min. The precursor FMZ (3 mg, 12 mmol) was dissolved in 1.0 mL of the acetonitrile and filled in vial 3 [Figure 1]. The dissolved precursor was added to the reactor vial, and radio-fluorination was carried out at 110°C–160°C for 10–30 min. Afterward, the reaction mixture was cooled to 50°C and diluted with acidic water (7 mL), filled in vial 5. The reaction mixture was transferred onto the C18 plus sep-pak cartridge through V14 to VX1+VX2, which was connected to V17 and onto the preconditioned C18 cartridge. The C18 plus cartridge was preconditioned with 5 mL of ethanol and 10 mL of water. The product, along with some impurities, was held in the cartridge, and the rest was passed to the waste. The C18 column was washed with 5 mL of water (vial-12) and 3 mL of 8% ethanol (vial-13). Two milliliters of 20% ethanol (in phosphate buffer) in vial-14 was used for eluting the purified product from the C18 cartridge in the collecting vial (prefilled with 10 mL of physiological saline).

b) HPLC-based purification method (n = 5).

The labeling reaction for both precursors was carried out in the same module (FX2N Tracerlab). The VX2 L tubing line was connected to VZ1, and the V17 tubing line was connected to the round bottom flask [Figure 1 and Table 1]. The reaction of (^{18}F)-K222 with precursor was done under the same condition as described above. The labeling with ^{18}F was carried out at 160°C for 30 min, followed by dilution with 3 mL of acidic water. The crude mixture was loaded (through tube-2) onto the preparative HPLC column (C18 prep column, 100 Å, 10 μm, 10 mm×250 mm, WATERS, India) and eluent (0.01 M phosphoric acid/acetonitrile [4/1]-500 mL) were passed through the column at the rate of 5 mL/min. The product was passed to the round bottom flask and then loaded onto the preconditioned

Table 1: Contents of the vials in the FX2N Tracerlab module

Vials	Reagents
Vial-1 (eluent)	15 mg of K222 with 3 mg of K_3CO_3 in 1000 μL of acetonitrile/water (9/1)
Vial-3 (precursor)	FMZ precursor (5.0 mg) was dissolved in 1 mL of acetonitrile
Vial-5	Acidic water - 7 mL
Vial-12	Water - 5 mL
Vial-13	8% ethanol - 3 mL
Vial-14	20% ethanol (in phosphate buffer)
HPLC eluent 1	0.01 M phosphoric acid/ acetonitrile (4/1) - 500 mL were passed through the column at the rate of 5 mL/min

HPLC: High-performance liquid chromatography, FMZ: Flumazenil, K222: Kryptofix-222

C18 plus column. The C18 column was washed with 5 mL of water (vial-12) and 3 mL of 8% ethanol (vial-13). Two milliliters of 20% ethanol (in phosphate buffer) in vial-14 was used for eluting the purified product from the C18 cartridge in the collecting vial (prefilled with 10 mL of physiological saline). The quality control parameters like radiochemical purity, stability, residual solvents, and sterility were performed on the final formulation as per protocol published elsewhere.[15] The specific activity was calculated by HPLC by calculating the amount of FMZ (area under the curve) labeled with ^{18}F and compared to AUCs of the serially diluted standards.

In vitro stability assay

The *in vitro* stability assay was evaluated in water, saline, and phosphate-buffered saline (PBS). 100 µL of the radiotracer was added to the 900 µL of water, saline, and PBS, and the mixture was incubated at 37°C in the water bath. The HPLC was done at 1 and 4 h.

Results and Discussion

We have standardized and synthesized (^{18}F) FMZ using cold FMZ. In this study, we have extensively studied the synthesis of (^{18}F) FMZ by isotopic method and purified using cartridge-based and HPLC methods. 15 mg/3 mg of K222/K_2CO_3 was used in all the experiments to elute the (^{18}F) fluoride from the QMA column, which resulted in 98 ± 1% efficiency. Around 3.0 mg of precursor was dissolved in 1 mL of acetonitrile and was reacted with dried (^{18}F) fluoride. (^{18}F) fluoride incorporation efficiency was around 55 ± 5% and final efficiency was 16 ± 4% [n = 10; Table 2] at 110°C for 10 min. The retention time of the (^{18}F) FMZ was 15.1 ± 1.5 min and the standard (UV/Vis) peak was observed at 14.8 ± 0.6 [Figure 2a and b]. The radiochemical purity of the product was 96.5 ± 1.8% (n = 5). The labeling reaction carried out at higher temperatures (>110°C) showed the degradation (~30%) in the radiochemical purity of the (^{18}F) FMZ. The labeling

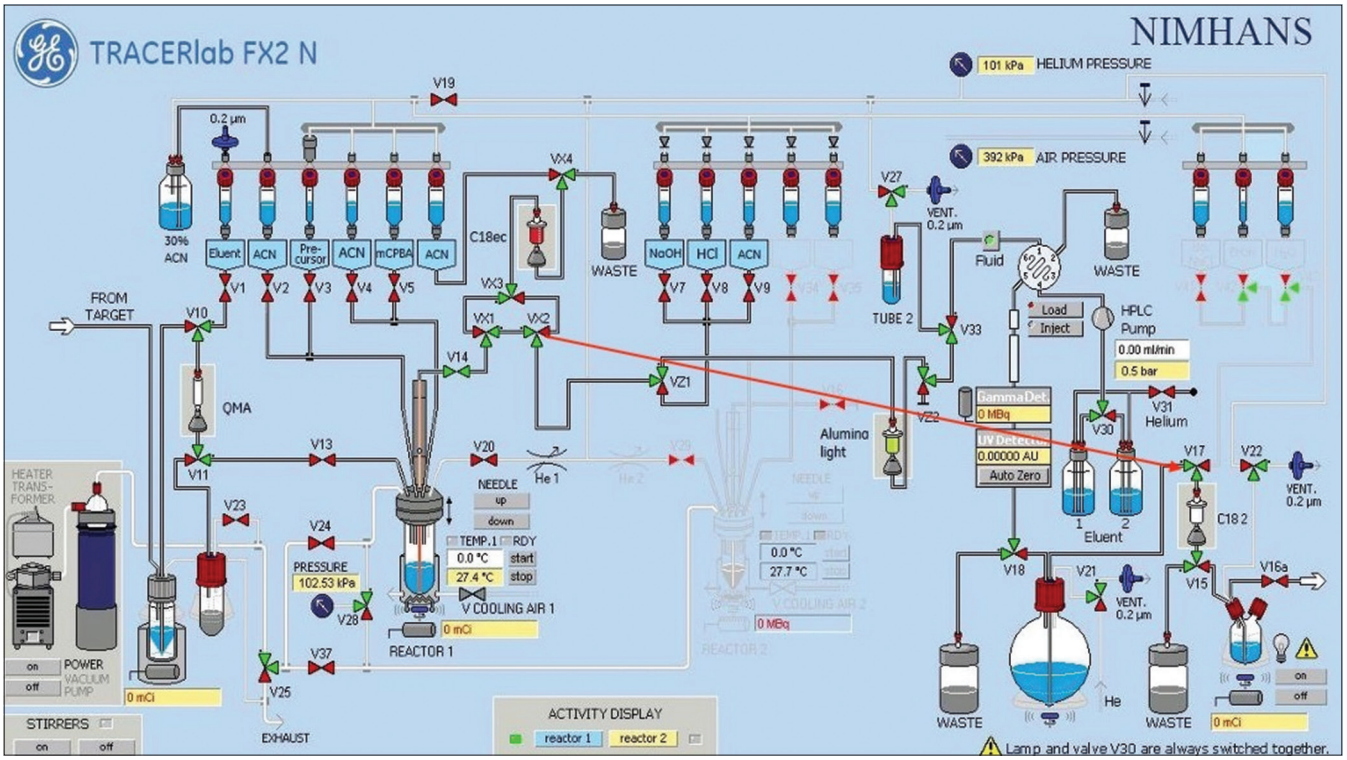

Figure 1: The schematic flow of the module. The red line showed the direct connection of VX2L with V17 for cartridge-based purification. The green valves from V14 to V33 showed the high-performance liquid chromatography-based purification pathway

Table 2: Representative of the batch report for each module

Chemistry module	Cartridge based method	HPLC based method
Purification cartridges	C18 plus	C18 prep column + C18 plus
Activity from the cyclotron (mCi)	500	475
Total activity (in reactor after elution from QMA) of ^{18}F at EOB (mCi)	505	476
Batch activity at the EOB (decay corrected) (mCi)	98	44.5
Total synthesis time (min)	40	60
Radiochemical yield (decay corrected) (%)	19.6	9.3

HPLC: High-performance liquid chromatography, QMA: Quaternary methyl ammonium, EOB: End of bombardment

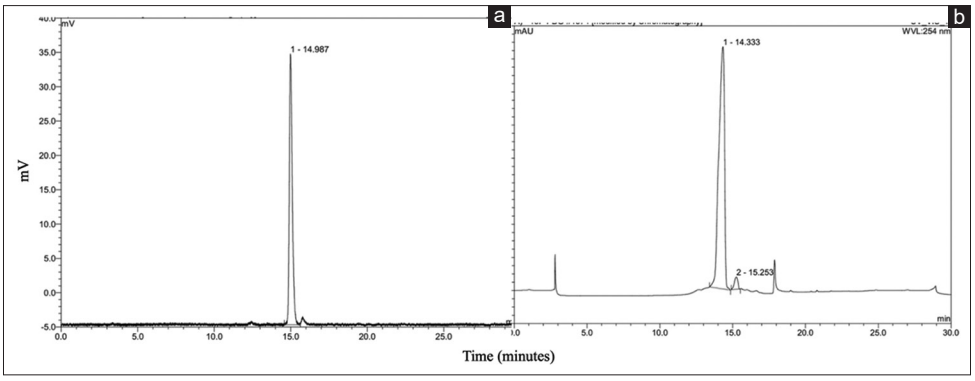

Figure 2: (a) The radioactive peak of cartridge-based purification of Fluorine-18 flumazenil (FMZ) at 14.9 min. (b) The ultraviolet/Visible peak for FMZ standard at 14.3 min

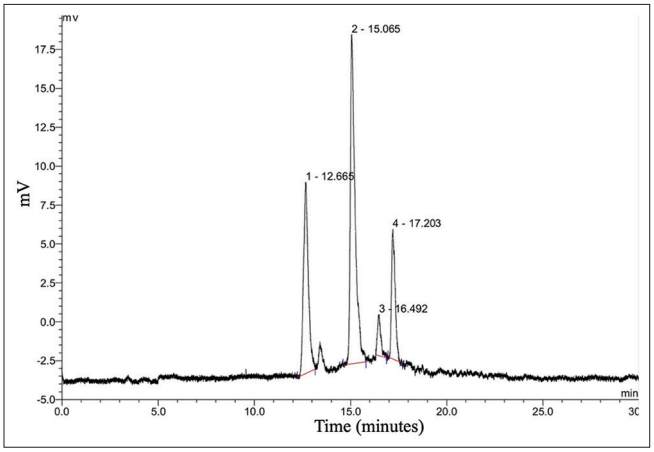

Figure 3: Multiple radioactive peaks were observed when the labeling reaction was above 110°C and purified through cartridge-based purification

reaction was carried out at temperature beyond 130°C showed multiple peaks [Figure 3]. The radiochemical yield and purity when labeling reactions were carried out at 130°C–160°C was not reproducible.

In the HPLC-based purification method, the crude mixture, after labeling, was loaded onto the preparative HPLC column, and the mobile phase was run through it. The peaks were observed at 7 ± 2 min and 12.5 ± 1.5 min [Figure 4]. It was observed that the (^{18}F) FMZ peak was at 12.5 ± 1.5 min. The product solution was loaded onto the C18 plus (preconditioned) and eluted with 20% ethanolic buffer. The yield was 10 ± 4% [Table 2] with radiochemical purity of 97.3 ± 1.4% (n = 5). The radio-HPLC showed a radioactive peak of (^{18}F) FMZ at 15.1 ± 0.6 min [Figure 5]. The HPLC purification ensured the single product irrespective of labeling reaction temperature. In preparative HPLC, we were able to separate the final product from the other byproducts. The HPLC-based purification enabled to get the purified product. However, the yield was decreased when the reaction temperature was kept above 110°C. The products formed due to high-temperature drying the labeling reactions got filtered while passing through preparative HPLC and only the identified peak of the product was separated and loaded onto the tC18 column. Therefore, in the HPLC purification method, the high temperature of labeling does not interfere with the final formulation.

The final product remained stable for up to 4 h in the water, saline, and PBS, and the stability did not fall below 90%. The stability was tested in solvents, which are possibly diluents for human injections. Our observations highlight some of the unique features of cartridge and HPLC-based purification. However, the major limitation of the method is lower molar activity, which may be due to the presence of cold FMZ.

The radionuclide purity of radionuclide (^{18}F) fluoride was more than 95%, and a half-life of 111 ± 4 min proved the radionuclide identity with a 511 KeV peak. The final solution was clear with a pH of 5.5 ± 0.6. The Kryptofix levels were <50 μg/mL. The ethanol content in the final preparation was <5% (reference value <5000 ppm) due to elution of the final product via 20% ethanolic buffer. The European Medicines Agency and United States Food and Drug Administration may accept high amounts of ethanol (Class III residual solvent) provided they are realistic in relation to manufacturing capability and good manufacturing practice (Guideline 2005). As per guidelines for radiopharmaceutical use in humans, the content of ethanol shall be below 10%.[16] A detailed report on quality control is given in Table 3.

Cartridge-based purification is convenient and is followed by most of the radiotracers used in routine clinical radiopharmaceutical synthesis. Our group has also developed a cartridge-based synthesis of (^{18}F) FMZ from FMZ using an isotopic approach. The isotopic approach to synthesize (^{18}F) FMZ from FMZ was published by Ryzhikov *et al*. (2004), they used the tC18 column (cartridge),[13] whereas we have used both preparative HPLC and cartridge-based methods for purification. In this study, we successfully developed an HPLC-based purification method for (^{18}F) FMZ. The specific activity was calculated from the standards using HPLC and found to be 120 ± 20 GBq/μmol.

Conclusions

(^{18}F) FMZ was synthesized using the isotopic exchange method using a cartridge and HPLC-based method. It may

Table 3: Quality control report of the Fluorine-18 flumazenil

Description	Method	Specification	Cartridge based	Preparative HPLC
Appearance	Visual inspection	Clear/colorless	Clear	Clear
Final pH	pH paper and Meter	4.5–7.5	6.5±0.6	6.2±0.8
Radionuclide identity	Half-life method and 511 KeV peak	110±5 min pass (must be passed)	110±2 min	110±2 min
Radiochemical purity	HPLC	≥95%	96.5±1.8%	97.3±1.4%
Chemical purity				
Ethanol	GC	Below 10% of total volume	<5%	<5%
Acetonitrile		≤500 ppm	70±8	75±8
Kryptofix (K222)	TLC plate	0.26 mg/mL	<0.1 mg/mL	<0.1 mg/mL
Radiochemical yield			16±4% (110°C)	10±4% (110°C)
				5±2% (130°C)
				6±3% (160°C)

HPLC: High-performance liquid chromatography, TLC: Thin-layer chromatography, K222: Kryptofix 2.2.2, GC: Gas chromatography

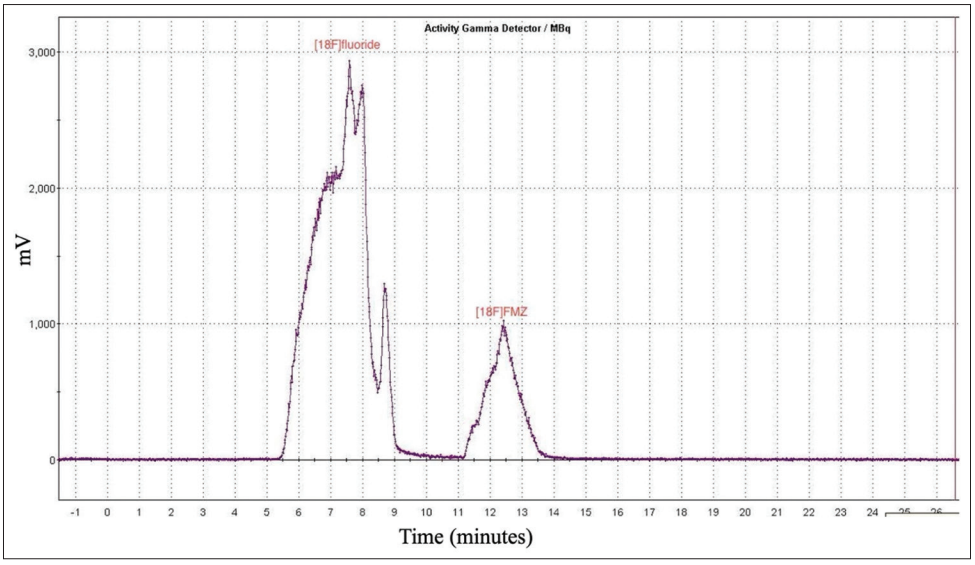

Figure 4: Preparative high-performance liquid chromatography of crude mixture of Fluorine-18 (18F)FMZ in the FX2N module. The radioactive peak at 5–9 min provided impurities while the peak between 11 and 14 min provided (18F) FMZ

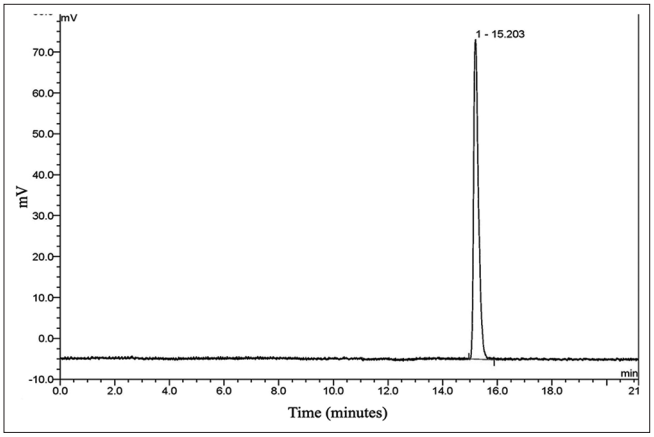

Figure 5: Analytical high-performance liquid chromatography of Fluorine-18FMZ showing radioactive peak at 15.2 min

be used as a cheaper option for producing Gaba-based PET radiotracers. Further validation is required through a clinical study.

Acknowledgments

We acknowledged the NIMHANS administration for providing us with an intramural grant (NIMH/PROJ/RD/00586 / 2019-20).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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