



Radiosynthesis of [^{18}F]flumazenil for imaging benzodiazepine receptors and its evaluation in human volunteers using simultaneous PET-MRI

Pardeep Kumar¹ · Chandana Nagaraj¹ · Raman Joshi¹ · N. Sridhar Goud¹ · Dinesh Kumar¹ · Vittal Korann² · Sandhya Mangalore¹ · Naren P. Rao²

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Abstract

Fluorine-18 (^{18}F) flumazenil (FMZ) was synthesized and purified using a single solid-phase cartridge and the formulation was tested in animals for biodistribution at various time points. A 90 min dynamic scan was acquired in human volunteers on simultaneous PET-MRI. The radiochemical purity was amounted to $94 \pm 2\%$. The animal biodistribution showed high uptake in the brain specifically in the cortex region. The human images showed binding in benzodiazepines (BZ) receptor-rich regions, such as the neocortex, and the lowest uptake in the pons. [^{18}F]FMZ was synthesized and purified using a sep-pak cartridge. This method allowed to apply this tracer in human studies showing the typical accumulation pattern in regions with high benzodiazepine receptors.

Keywords Radiochemistry · Fluorine-18 · [^{18}F]flumazenil · PET · Benzodiazepine receptors · Receptor imaging

Introduction

Gamma-aminobutyric acid (GABA) is a naturally occurring amino acid that works as a neurotransmitter in the brain. It is widely distributed throughout the central nervous system (CNS) and the major inhibitory neurotransmitter which involved in the pathophysiology of the various neuro-disorders such as schizophrenia, epilepsy, anxiety and sleep [1]. Benzodiazepines are a class of psychoactive drugs that are among the most prescribed drugs. flumazenil (ethyl 8-fluoro-5,6-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 ($K_i \sim 1$ nM) in comparison with other benzodiazepines and competitively inhibits the effects of benzodiazepines [2, 3]. Their affinity for GABA receptors has led to the development of

carbon-11 (^{11}C) and ^{18}F labeled FMZ for PET imaging of central benzodiazepine receptors (CBRs). [^{11}C]FMZ was the first radioligand developed for the imaging of the GABA_A receptors using PET [4, 5]. [^{18}F]FMZ was developed due to the advantage of longer half-life, longer data acquisition, and the possibility to transport nearby centers. Odano et al. (2009) have reported that there was no much difference in the kinetic and affinity of [^{11}C]FMZ and [^{18}F]FMZ for benzodiazepine receptors. Therefore, [^{18}F]FMZ proved to be more advantageous due to the longer half-life [6]. It has been used for imaging various disorders and showed its utilization in detecting epileptic foci, cortical damage after stroke or trauma, Alzheimer's disease, neurodegenerative disorders of the basal ganglia, and also in drug development [7–9].

Various groups have reported the synthesis of [^{18}F]FMZ via using various types of purification methods which involved solid phase and high-performance liquid chromatography (HPLC) purification. There are various types of precursors that have been used like diaryliodonium tosylate, aryl boronic ester precursors, and nitromazenil [10–12]. There were synthesis methods that involved isotopic exchange with the precursor (flumazenil) but yielded low specific activity due to the presence of precursor (as a carrier). The most common way is the nucleophilic substitution of nitromazenil precursor, where the nitro group

✉ Pardeep Kumar
pardeep1182@gmail.com; pardeepk@nimhans.ac.in

¹ Neuroimaging and Interventional Radiology (NIIR), National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, Karnataka, India

² Psychiatry, National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, Karnataka, India

is substituted by radioactive fluorine. The purification was done using a solid-phase cartridge and HPLC. This method yielded higher specific activity, which is of utmost importance in brain receptor imaging studies [13, 14].

In this study, we have used a simple method for radio-synthesis of the [^{18}F] FMZ and purified using a single solid-phase cartridge. The radiochemical purity and all other quality control parameters were performed. The preparation was tested in animals and five human volunteers for safety and biodistribution studies. A dynamic scan of [^{18}F]FMZ PET-MRI was performed, and the binding potential of the radiotracer in the human brain was calculated using PMOD software.

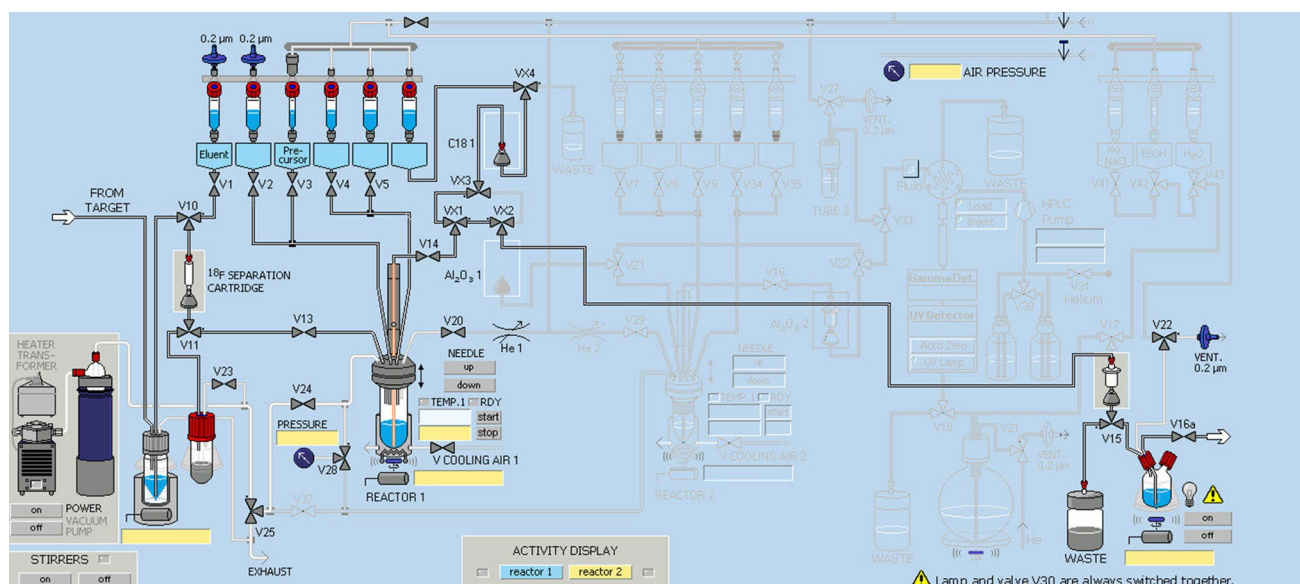
Materials and Methods

All the chemicals and reagents were procured from Sigma Aldrich (USA) and used without any further purification. The precursor nitromazenil was procured from Syncom BV (Netherlands) and ABX (Germany). The cold standard of flumazenil was procured from Sigma-Aldrich, USA. The ^{18}F radioisotope was produced in our in-house 16.5 MeV Cyclotron (PETtrace 860, GE Healthcare, Chicago, USA) by the proton bombardment on the enriched ^{18}O water using standard [$^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$] 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 and delivered to the synthesizer module (Tracerlab FX2N, GE Healthcare, Chicago, USA) using the Helium (UHP-5.5) as a carrier gas. All Sep-Pak cartridges like C18 plus (360 mg), tC18 plus (360 mg), the HLB (360 mg) was procured from Waters-India. A stock solution of kryptofix 2.2.2 (50 mg) and K_2CO_3 (10 mg) were dissolved in acetonitrile (10 mL) including sterile deionized water (0.5 mL). A solution of 0.5 M potassium dihydrogen phosphate (KH_2PO_4) buffer was prepared, and 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 pH meter (Mettler Toledo, Ohio, USA). The radiochemical purity was measured using HPLC and thin-layer chromatography (TLC) methods. HPLC system (Dionex, California, USA) equipped with UV–Vis coupled and radioactivity detector was used. The quantitative analysis was done on a C18 column (Sum 4.6 \times 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). TLC was done on an EZ-SCAN scanner (California, USA) with a multimode radiation detector (OMNI-RAD, California, USA) using 80% Ethyl acetate (in ethanol,

v/v) as a solvent on the stationary phase of silica gel coated F_{254} chromatography sheets (Merck, New Jersey, USA). The radionuclide purity was evaluated by half-life method and energy (511 keV) peak determination on CAPRAC-t well counter (S.No.10395; New jersey, USA). The levels of kryptofix were evaluated by using TLC using two different solvents. One was developing TLC in chamber saturated in the vapors of the methanol/ammonium hydroxide (9:1) and second was treating the TLC plates with potassium permanganate solution. The kryptofix standards used for testing were 500, 50, and 10 $\mu\text{g}/\text{ml}$. The residual solvents like DMF, ethanol was measured using Gas chromatography (GC) (Scion 436 GC, Netherlands) with a flame ionization detector (FID). The column was operated initially at 40 $^\circ\text{C}$ for the first 3 min and then rise 50 $^\circ\text{C}/\text{min}$ up to 8 min, and the final temperature was set at 240 $^\circ\text{C}$, and the column was BR-200 ms, 0.32 mm ID. The makeup gas consists of Helium (28 mL/min), zero air (300 mL/min) and hydrogen gas (30 mL/min) flow at the rate of 2 mL/min. The endotoxin test was carried out using Endosafe PTS cartridges on NexGen Endosafe PTS from Charles River (Massachusetts, USA). The dilution factor of 100 was made for each preparation and 25 μl of the sample was added to each well. The dose was measured on a dose calibrator (Capintec CRC-25PET, New Jersey, USA). The PET-MR imaging was performed on biograph mMR (Siemens, Munich, Germany) and the raw images were reconstructed with the syngo-via software solution. Further post-processed to derive the kinetic analysis and binding potential using Simplified Reference Tissue Model (SRTM) on PMOD (version-4.410).

Radiochemistry

Briefly, 900 ± 50 mCi of ^{18}F was transferred to the FX2N module, which was trapped using preconditioned QMA sep-pak cartridge (ABX, Germany). The ^{18}F was eluted with 1.5 mL of the QMA eluent solution (from stock solution), filled in the vial-1 of the module. The eluted ^{18}F - K_{222} complex was dried under vacuum at 95 $^\circ\text{C}$ for 6 min. The precursor nitromazenil (5 mg, 12 mmol) was dissolved in 1.5 ml of the anhydrous dimethylformamide (DMF) and filled in vial 3. The variation in the molar ratio of kryptofix to nitromazenil was varied to get a better yield. The kryptofix concentration was varied with 7.5, and 15 mg and the concentration of the precursor was varied between 5–15 mg (in DMF). The dissolved precursor was added to the reactor vial, and radiofluorination was carried out at 160 $^\circ\text{C}$ for 30 min. Afterward, the reaction mixture was cooled to 50 $^\circ\text{C}$ and diluted with deionized water (7 mL), filled in the vial 5. The reaction mixture was transferred on to the C18 sep-pak cartridge through V14 to VX1 + VX2 which was connected to V17 and on to the preconditioned C18 cartridge (Scheme 1). The C18 cartridge was pre-conditioned with 4 mL of ethanol and 10 mL of water. The product along with some



Scheme 1 Schematic diagram of the FX2N module

impurities hold in the cartridge and rest was passed to the waste. The C18 column was washed with 4 mL of water (vial-12) and 2 mL of 8% ethanol (vial-13). 2 mL of 20% ethanol (in phosphate buffer) in vial-14 was used for eluting the purified product from C18 cartridge in the collecting vial (prefilled with 10 mL of physiological saline). The final preparation was passed through cathivex GV (0.22 μ) filter and collected in a sterile vial in the dispenser. The quality control parameters like radiochemical purity, stability, residual solvents, and endotoxin were performed on the final formulation. The chemical impurity of the precursor in the formulation was calculated by comparing the area under the curve with respect to standard control.

In-vitro stability

For in-vitro testing stability, around 2.0 mL of blood was collected in a heparinized vial from the human volunteers. The blood was centrifuged at 3000 rpm for 10 min at room temperature to separate the plasma. Twenty microliters of [18 F] FMZ were mixed with 180 μ l of plasma and incubated for 60 and 240 min at 37 $^{\circ}$ C in a water bath. An equal volume of separated plasma and chloroform/methanol (4:1, v/v) were mixed and centrifuged at 6000 \times g for 5 min to precipitate the proteins. The supernatant was used for HPLC analysis.

Animal studies

Animal biodistribution studies were carried out in normal swiss albino mice ($n=9$) after the institute animal ethic committee approval (AEC/69/442/NIIR). A group of mice

(15–20 weeks, 25–35 g) were injected intravenously (in tail vein) with 4–6 MBq of [18 F]FMZ diluted with saline (in a total volume of 200 μ L) and the mice were sacrificed at 15, 30 and 60 min post-injection. Mice were then euthanized by carbon dioxide inhalation, and tissues were dissected, washed free of blood, dried, and weighed. Concomitant radioactivity was counted together with an 18 F standard solution and injected dose per gram (% ID/g) was then calculated. The blood sample was also used to calculate in-vivo stability and metabolic degradation of the tracer. A blood sample (into heparinized vials) was collected and processed in the same manner as described above.

PET-MRI

Five healthy volunteers were enrolled in the study after signing the written informed consent which was approved by the Institute human ethic committee (IEC). They were considered healthy after reviewing their medical history, blood tests, no medication, no drug abuse. Simultaneous PET-MR imaging was performed on 3 T Biograph mMR. The volunteers were asked to come fasting (4–6 h) to ensure stable basal metabolic conditions. All volunteers were screened for any metallic object or allergy to radiopharmaceuticals. All studies were non-contrast PET MRI studies. The subjects were positioned on the scanner table with 8 channel head coil and secured i.v line. The scout images were acquired, followed by 170 ± 20 MBq of the [18 F]FMZ injected intravenously and flushed with 20 ml normal saline as a slow i.v bolus. The dynamic scan was acquired in the LIST mode, over 90 min with standard and advance imaging MRI sequences (like T1 MP Rage sequence). Ultrashort

time echo (UTE) sequence was used to generate attenuation maps. The attenuated corrected images were split/reframed into [(10 frame * 30 s) + (10 frame * 60 s) + (15 frame * 300 s) = 5400 s (90 min)].

Time activity curve (TAC)

The DICOM data of the reconstructed images were processed in the PMOD. Regions of interest (ROIs) were drawn on the various regions of cortex (frontal, temporal, occipital), putamen, thalamus, cerebellum, and pons. ROIs were marked bilaterally, and left and right values were averaged for analysis. The ROIs were plotted over time, the marked images were saved for kinetic analysis, and time-activity curves (TACs) were generated.

Kinetic analysis

Kinetic analysis and binding potential of the tracer in the brain were calculated by using a non-invasive approach (SRTM) using pons as reference tissue. P-MOD software was used to derive the time-activity curve and that data was used to calculate the kinetics and binding potential of the tracer in the brain. The binding potential values were obtained for each region.

Results and Discussions

Various reported studies have proved the efficacy and utilization of [^{18}F]FMZ for imaging CBRs [6, 10]. It has been used for various diseases like Alzheimer's, schizophrenia where CBRs get affected. Our study was focused on developing a simple method to get [^{18}F]FMZ with an appropriate yield which was used in clinics. The best radiolabeling was achieved with 7.5 mg of K_{222} with 3–5 mg of nitromazenil (molar ratio of nitromazenil/ K_{222} = 0.4–0.6/1). The radiochemical purity was $94 \pm 2\%$ with a $10 \pm 2\%$ yield. The overall yield was not significantly changed with increasing the concentration of precursor to 10–15 mg (Table 1). Therefore, we stick to a minimal concentration of 5 mg for clinical use. The minimum concentration of precursor leads to a decrease in the low level of the chemical impurities

in the final preparation. We have tried higher concentration of kryptofix (15 mg), which yielded higher elution of ^{18}F but did not affect the final yield of [^{18}F]FMZ. The reason may be due to the fact that a higher concentration of K_2CO_3 may cause degradation of nitromazenil and labeled product during fluorination reaction, as reported by Ryzhikov et al. [13]. 3–5 mg of precursor (in 1.5 mL of DMF) was found to be good for the reaction though in literature, around 2 mg was reported to be appropriate for labeling [13]. Therefore, a small amount of precursor is good enough for the radio-synthesis of [^{18}F]FMZ. The volume of DMF remained fixed as reported literature showed that this much volume prevents thermal degradation of nitromazenil; hence volume less than 1.5 mL was not recommended [15]. Therefore, the final preparation has 7.5 mg of K_{222} for eluting ^{18}F and radiofluorination reaction was carried out with 5 mg of the precursor (for clinical use). After trying different solid-phase cartridges, C18 plus was an ideal purification cartridge that provided us a maximum radiochemical purity and yield. The final product was eluted with 2 mL of 20% ethanolic buffer and diluted with 10 ml of physiological saline and collected into the vial after passing through 0.22 μ filter in a sterile vial. The total time of the synthesis was 50 min. Vaulina et al. (2018) has synthesized [^{18}F]-FMZ using HPLC purification method and radiochemical purity was greater than 97% with low level of chemical impurities. The authors also suggested to perform synthesis without using HPLC purification for commercial purpose as it makes convenient for the end users. Our method is just an effort in that direction. Ryzhikov et al. (2005) has standardized the labeling of nitromazenil and used various concentration of precursor from 2–8 mg with fixed concentration of kryptofix. Their results showed a molar ratio of 1/1 (substrate/kryptofix) in DMF solvent when heated at 160°C for 30 min gives maximum yield, but not significant difference was found in our experiments. They have done extensive work on finding the affinity of [^{18}F]-FMZ for benzodiazepine receptors using autoradiography and animal PET imaging. They have also analyzed radiolabeled metabolites formed during in-vivo distribution and found that most of the metabolites were polar in nature which was unlikely to pass blood–brain barrier and hence cannot affect the uptake of [^{18}F]-FMZ in the brain.

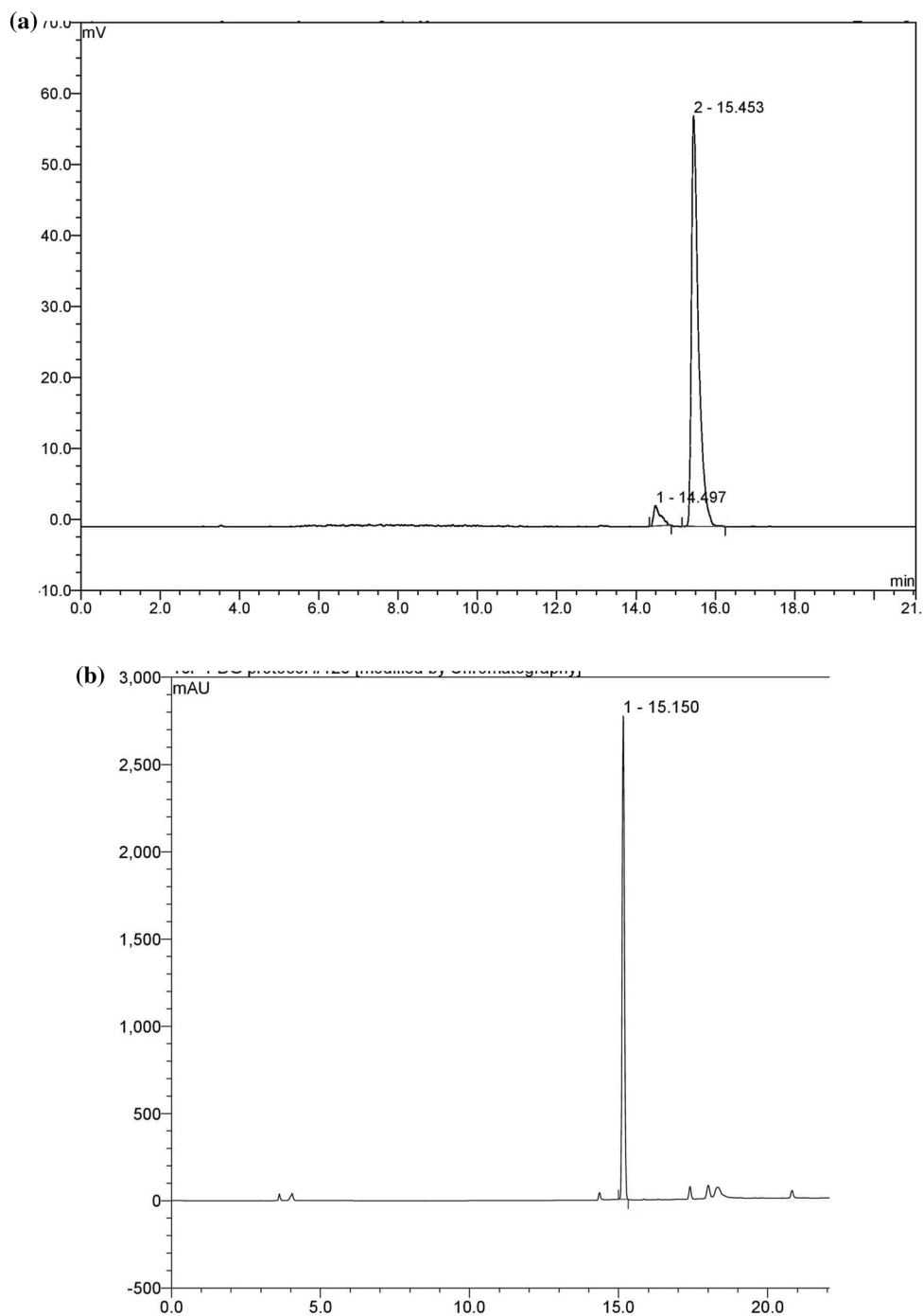
Table 1 Showing conditions for labeling nitromazenil with ^{18}F

S. No.	K_{222}	NMZ	Molar ratio NMZ/ K_{222}	Reaction Temp (°C)/Time	Purification cartridge	% yield /number of synthesis
1	15 mg (40 mmol)	5 mg (12 mmol)	0.3/1	160/30 min	C18 plus cartridge (360 mg)	1–2% (n = 3)
2	7.5 mg (20 mmol)	5 mg (12 mmol)	0.6/1	160/30 min	C18 plus cartridge (360 mg)	$10 \pm 2\%$ (n = 15)
3	7.5 mg (20 mmol)	10 mg (24 mmol)	0.8/1	160/30 min	C18 plus cartridge (360 mg)	$8 \pm 2\%$ (n = 3)
4	7.5 mg (20 mmol)	15 mg (30 mmol)	1.5/1	160/30 min	C18 plus cartridge (360 mg)	$9 \pm 2\%$ (n = 3)

The radiochemical purity of the $[^{18}\text{F}]$ -FMZ was $94 \pm 2\%$ as evaluated by HPLC with radioactive peak retention time at 15 ± 0.3 min with $4 \pm 1.5\%$ of an adduct product at 14 ± 0.3 min as compared to free ^{18}F which was $1 \pm 0.6\%$ in the final formulation with retention time of 4 ± 0.6 min ($n = 20$) (Fig. 1a). The cartridge purification of the final product has simplified the labeling of the nitromazenil, but the main limitation was presence of small amount of radiochemical (adduct product) impurity. Though this adduct

product has not impacted the distribution of the $[^{18}\text{F}]$ FMZ. The peak of $[^{18}\text{F}]$ FMZ was identified by comparing it with the UV/Vis peak of standard flumazenil solution at 254 nm under the same conditions. The retention time of the standard flumazenil solution was 15 ± 0.4 min (Fig. 1b). The confirmation of the final product peak was done by co-injection of the radioactive formulation with standard solution of flumazenil and coincidence of both

Fig. 1 **a** Radioactive peak for $[^{18}\text{F}]$ FMZ at 15.4 min as detected by radio-detector of HPLC. **b** UV–Vis peak of flumazenil standard at 254 nm at 15.1 min as detected by UV detector of HPLC



the peaks around 15 min confirmed that the [^{18}F]FMZ was eluted at 15 min with 94% purity.

The TLC showed a retention factor of 0.7–0.8 for [^{18}F]FMZ as compared to 0.0–0.1 for free ^{18}F . The radionuclide purity of radionuclide ^{18}F 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 5.5 ± 0.6 . The kryptofix levels were below 50 $\mu\text{g}/\text{ml}$ as evaluated by both methods. The concentration of residual solvent DMF was 40 ± 5 ppm (reference value-810 ppm). The ethanol content in the final preparation was $30,000 \pm 10,000$ ppm (reference value < 5000 ppm) due to elution of the final product via 20% ethanolic buffer (Fig. 2). The European medicines agency and USFDA 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 of radiopharmaceutical use in humans, the content of ethanol shall be below 10% [16]. The endotoxin level was less than 1.0 endotoxin units (EU)/dose as compared to the prescribed limit of 175 EU/dose. [^{18}F]FMZ was found to be stable ($\sim 90\%$) under in-vitro conditions up to 4 h.

The animal biodistribution showed early uptake in the lungs, Intestine, and kidneys at 15 min (Fig. 3). With time the activity cleared from the lungs and increased uptake were seen in intestine ($21.3 \pm 2.5\%$), liver ($11.9 \pm 1.3\%$), and kidneys ($5.1 \pm 1.5\%$). It showed that the radiotracer followed hepatorenal (dual pathway) excretion. The radioactivity was seen in the normal brain and higher uptake was observed in the cortex and a cerebral to pons uptake ratio was 3.5 ± 0.6 at 30 min. The biodistribution remained unchanged up to 1 h post-injection. The in-vivo stability was greater than 90% at

Fig. 2 GC showing the levels of ethanol and DMF in the final preparation. The retention time of ethanol and DMF was 2.2 min and 4.1 min respectively

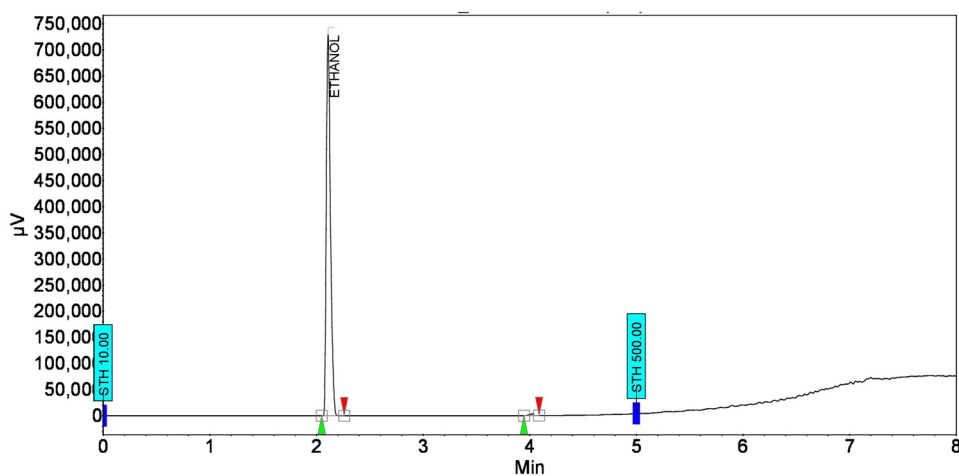
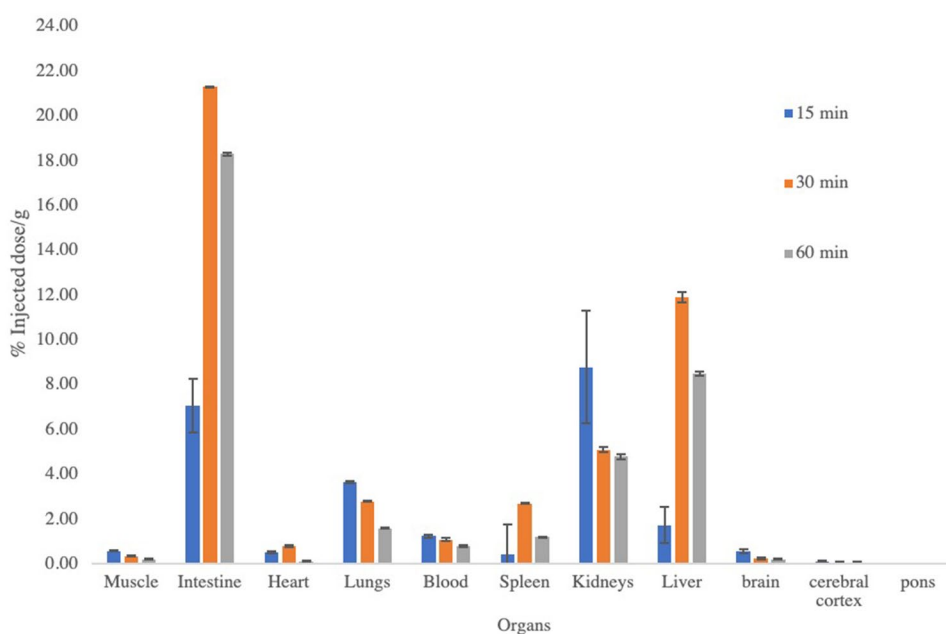


Fig. 3 Showing tissue distribution of [^{18}F]FMZ in swiss albino (control) mice at 15, 30 and 60 min



60 min post-injection. Though, our stability data was different from the data reported by Ryzhikov et al., which was stable up to 90% at 4 min and 52% at 90 min in monkeys. The difference in stability data may be due to difference in the metabolic rates of the species.

The clinical testing was done in five volunteers to study the kinetics, and binding potential of [^{18}F]FMZ. The dynamic images of PET-MR were acquired over a period of 90 min and processed using syngo-via software. The standard uptake value (SUV) was found to be higher in cortical areas due to the higher expression of CBRs as compare to the pons. The brain showed uptake of the tracer and major uptake was seen in cortex due to high expression of CBRs in the cortical areas as compare to brainstem region (pons). Therefore, pons was considered as a reference region. The horizontal, sagittal, and coronal views were shown with demarcated areas with regions of interest (Fig. 4). The uptake of the [^{18}F]FMZ was rapid in the brain post-injection and the peak SUV in the frontal cerebral cortex was 2.4 ± 0.4 as compared to 0.3 ± 0.2 for pons (reference tissue) at 16 min. The regional TACs showed the highest uptake in neocortical regions, intermediate

uptake in the cerebellum, thalamus, and putamen and lowest uptake seen in the pons. The time-activity curve showed clearance of the radiotracer from the cortical areas with time and peak was observed at 16.5 ± 2.5 min in the frontal cortex (Fig. 5). The binding potential values were obtained by the reference tissue models for each region (Table 2). The respective areas were chosen using automated anatomical labeling (AAL)-merge atlas. The values showed maximum binding in neocortical areas followed by the cerebellum, thalamus, and least or no uptake in the pons region. The order of uptake in various regions was as follows: frontal > temporal > occipital > parietal > parietal > cerebellum > thalamus > putamen > caudate nucleus > pons. The kinetic analysis and binding potential were calculated by a non-invasive approach using SRTM. The major advantage of this approach was that no blood samples were required, and it makes use of quantitative PET more feasible. Previous studies have shown that BP values were similar using invasive and non-invasive approaches therefore, we have used a non-invasive approach [6]. The binding potential of the [^{18}F]FMZ in the brain was concordant with benzodiazepine receptors

Fig. 4 Summation of [^{18}F]FMZ images acquired over 90 min after i.v. injection. Horizontal, sagittal and coronal sections were showing demarcated regions of interest using PMOD software

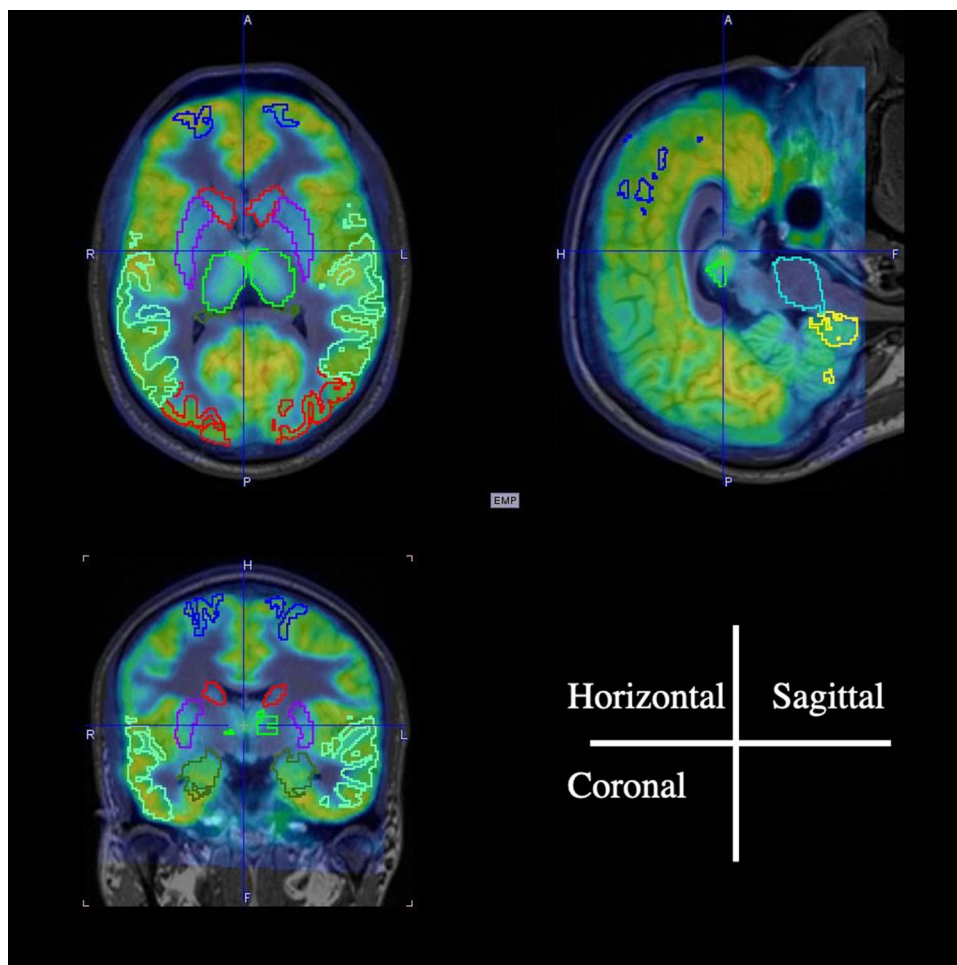


Fig. 5 Time-activity curves (TACs) showing clearance of [^{18}F]FMZ in various regions of brain

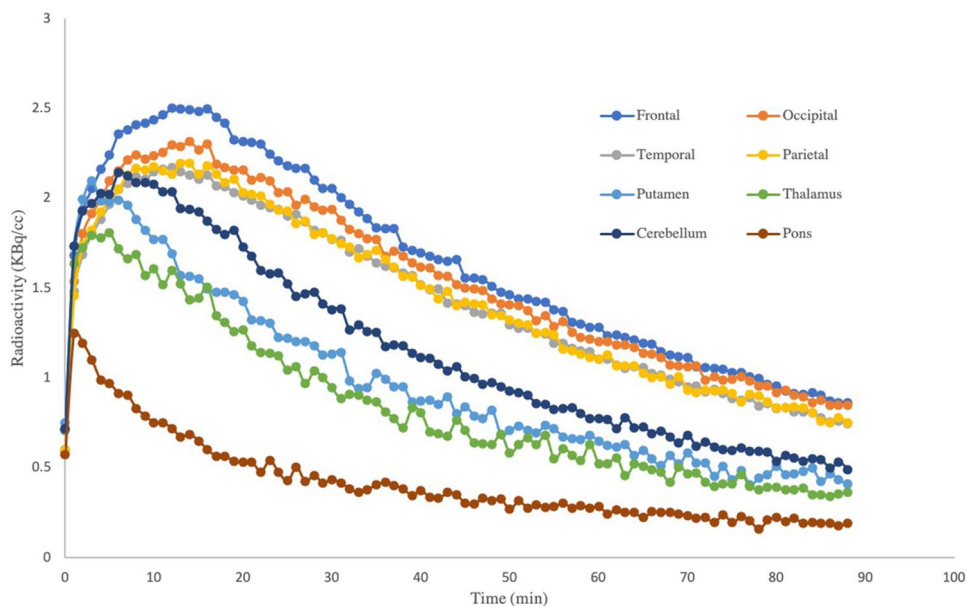


Table 2 Regional binding potential of the [^{18}F]FMZ in the brain using SRTM

Brain region	BP _{SRTM}
Frontal	2.7 ± 0.4
Temporal	2.4 ± 0.5
occipital	2.2 ± 0.6
parietal	1.7 ± 0.2
putamen	1.2 ± 0.3
Thalamus	1.0 ± 0.2
cerebellum	1.5 ± 0.4
caudate nucleus	0.8 ± 0.2
Pons	N/A

expressed in the following areas. The binding potential was higher in the frontal cortex, which has higher expression of CBRs, low in putamen and thalamus, and lowest in the pons area, which does not express any CBRs. The uptake pattern of TACs, and binding potential of [^{18}F]FMZ were similar as reported in the earlier reported studies [6, 10, 13]. Therefore, our formulation was validated through preclinical and clinical testing.

Conclusions

[^{18}F]FMZ was synthesized with high radiochemical purity and purified using a single solid-phase cartridge. The approach has been adapted to carry out the reaction in an automated module. The convenient labeling scheme and automation synthesis of [^{18}F]FMZ has encouraged us to explore its role in various neuro-disorders where CBRs get affected.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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