A novel approach to the synthesis of [^{18}F]flumazenil, a radioligand for PET imaging of central benzodiazepine receptors*

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An express method of solid-phase extraction was proposed for the first time for isolation and purification of [^{18}F]flumazenil, a radioligand used to quantify the density of central benzodiazepine receptors by positron emission tomography (PET). This novel approach afforded the radioligand with >97% radiochemical purity and a high chemical purity (nitromazenil content <1 μg mL^{-1}) and considerably reduced the time of the synthesis (from 90 to 50 min). The nonoptimized decay-corrected radiochemical yield was 8%, and the specific radioactivity was >37 GBq μmol^{-1}. The novel synthetic procedure easily can be integrated into automatic modules for the synthesis of clinically used PET radiopharmaceuticals.

Key words: central benzodiazepine receptors, positron emission tomography, fluorine-18, radioligand, [^{18}F]flumazenil, solid-phase extraction.

Positron emission tomography (PET) studies of neurochemical processes in the central nervous system (CNS) employ labeled compounds capable of specifically binding to some types of CNS receptors. These compounds (receptor radioligands) help to obtain valuable information on the receptor mechanisms, density, and distribution in various regions of the brain.[1—4]

Any receptor radioligand must meet the following basic requirements:

— high selectivity and affinity to the receptor at the nanomolecular level;

— high specific radioactivity (>37 GBq μmol^{-1} (1 Ci μmol^{-1}); *i.e.*, the radioligand must be no carrier added since the saturation of receptors can cause unwanted pharmacological or even neurotoxic effects;

— slow (on the scale of the PET study) metabolism;

— penetration across the blood-brain barrier;

— low nonspecific binding and sufficiently high biological clearance rate.[1,2]

The GABAergic system promoting the biochemical effect of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS, is of great interest for PET studies.[2,3] Central benzodiazepine receptors (BZRs) localized on the postsynaptic membranes of the GABAergic system are part of a $GABA_A$-benzodiazepine-ionophore complex, which serves to regulate (modulate) the resistance of neurons to stimulant signals. When bound to BZRs, benzodiazepines enhance the inhibitory processes in the CNS. Because of their sedative effect, benzodiazepines have found clinical use as anxiolytics, relaxants, anticonvulsants, and hypnotics. Central BZRs play a key role in such diseases as epilepsy, anxiety disorder, dementia, alcoholism, *etc.*

To determine the density of central BZRs, labeled analogs of flumazenil (ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate, **1**) are used as radiopharmaceuticals in PET. Flumazenil (**1**) has high affinity (K_i~1 n*M*) for central BZRs containing the subunits α_1, α_2, α_3, and α_5. It is a full antagonist of central BZRs and is used to eliminate the narcotic effect of benzodiazepine-based drugs.[3] Flumazenil labeled with the ^{11}C radionuclide ($T_{1/2}$ = 20.38 min), *N*-([^{11}C]methyl)flumazenil, or [^{11}C]FMZ (**2**), is recognized as a "gold standard" for detection of epileptic foci in the case of drug-resistant epilepsy, which is the main factor for a positive effect of surgical operation.[5] In addition, [^{11}C]FMZ is used to determine the viability of brain tissues after acute ischemic stroke for assessing the efficacy of different therapeutic strategies.[6] However, the short half-life of carbon-11 restricts the use of this radioligand to cyclotron-equipped PET centers. Flumazenil analogs labeled with more long-lived ^{18}F isotope ($T_{1/2}$ = 109.77 min) (**3**—**5**) provide an alternative to [^{11}C]FMZ. The poten-

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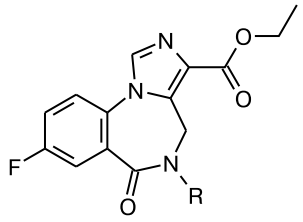
tial radioligand *N*-(2′-[^{18}F]fluoroethyl)flumazenil[7] ([^{18}F]FEFMZ, **3**) specifically binds with central BZRs but is distributed and excreted from the organism more rapidly than [^{11}C]FMZ.[8] Preclinical studies of another radioligand ([^{18}F]FFMZ[9] (**4**)) have revealed its quick metabolism accompanied by loss of the radioactive label (by 92% over 60 min). Its lipophilic metabolite penetrates across the blood-brain barrier into the brain and is the cause of the low quality of PET images,[10] the affinity of the ligand being comparable with that of flumazenil.[11] Note that radioligand **4** in the human body metabolizes much more slowly[10] and is currently employed in some PET centers.[12] [^{18}F]Flumazenil labeled at the native fluorine position ([^{18}F]FMZ, **5**) has been obtained by a ^{18}F—^{19}F isotope exchange reaction under standard conditions for nucleophilic radiofluorination with cryptand (Kryptofix 2.2.2) as a phase-transfer catalyst.[13] However, this compound contains [^{19}F]flumazenil (**1**) as a carrier and hence cannot be used in receptor studies. As demonstrated earlier,[14] complete saturation of central BZRs is achieved with as much as 15 μg of flumazenil per kilogram of body weight.



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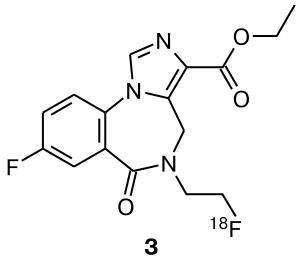

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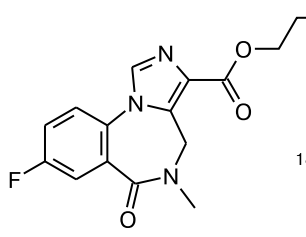
R = Me (**1**), $^{11}CH_3$ (**2**)



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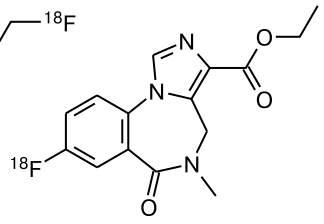

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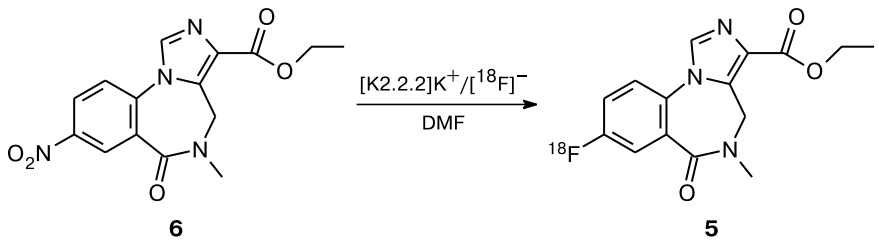
The method developed in 2005 for the synthesis of [^{18}F]FMZ involves replacement of the nitro group in nitromazenil (ethyl 5-methyl-8-nitro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,[15] **6**) by [^{18}F]fluoride (Scheme 1). The efficiency of radiofluorination of precursor **6** (Ro 15-2344, Roche Group) was 55—60% under the following reaction conditions: 160 °C, 30 min, DMF, and Kryptofix 2.2.2 as a phase-transfer catalyst. Unfortunately, such a high yield has nevermore been achieved later[16—19], probably because of the quality of nitromazenil **6** supplied by other firms. [^{18}F]FMZ was isolated from the reaction mixture by semipreparative HPLC. Cynomolgus monkey brain has been examined by PET[15] to study the [^{18}F]FMZ uptake in regions with an increased density of central BZRs; its metabolites in blood have been analyzed. Those preclinical studies and following clinical trials demonstrated identical kinetic behavior of [^{18}F]FMZ and [^{11}C]FMZ in the organism.[20]

Currently, [^{18}F]FMZ is used for clinical and research purposes at many PET centers,[16,17,20—25] and the synthesis of this radioligand has been adapted to automated synthetic modules,[16,17,19] microreactors,[18] and microwave ovens.[19] Here the major problem is the isolation and purification of the radioligand by semipreparative radio-HPLC using a reversed-phase C18 column. This prolonged (30—40 min) and laborious procedure may be accompanied by considerable losses of the radioactive product; special care should be taken to avoid the presence of a chemical impurity of compound **6** in the final product. Purification of [^{18}F]FMZ by HPLC has been described in detail earlier.[16] Ory *et al.* have studied[26] a possible cause of the loss of radioactivity in a C18 column and the decrease in the radiochemical purity (RCP) of the product: retention of [^{18}F]fluoride in the column when an HPLC eluent has pH < 5 (an eluent with this pH is normally used in the purification of [^{18}F]FMZ). Note only that, according to the literature data, the decay-corrected radiochemical yield (RCY) of [^{18}F]FMZ obtained as described earlier[15] and isolated using HPLC varied from 10—12%[20] to 0.4—1.1%.[17] Facing this problem in the development of an automated method for the synthesis of [^{18}F]FMZ, we proposed solid-phase extraction (SPE) as an alternative method.

Results and Discussion

In the present work, we synthesized [^{18}F]FMZ as described earlier[15] (see Scheme 1). The HPLC data for the reaction mixture are shown in Fig. 1. To obtain the radioligand [^{18}F]FMZ with a high RCP, nonreacted [^{18}F]fluo-

Scheme 1



6 **5**

ride and other hydrophilic radioactive impurities ([^{18}F]) must be removed to a residual content of ≤5% (according to Pharmacopoeia[27]).

Semipreparative HPLC is not always effective for the removal of a radioactive impurity because of a stretching "tail" of the solvent (DMF) used in the radiofluorination step.[28] To remove these impurities, semipreparative HPLC is usually preceded by SPE purification on cartridges with a reversed-phase sorbent (C18), which has been proposed for the purification of [^{18}F]FMZ as well.[16,17,19] In particular, preliminary purification on C18 and optimization of the HPLC procedure (the use of the eluent with another composition) increased the RCY from 5 (as in the method proposed earlier[15]) to 15—20%,[16] the reaction time being 80 min. It was also demonstrated that additional purification of the reaction mixture prior to chromatography extends the lifetime of HPLC columns several times.[16,17] The HPLC + SPE combination provides a high RCP of the radioligand with some extension of the purification time and, accordingly, with some decrease in the yield of the radioligand because of the radionuclide decay. Korean researchers have made considerable effort to develop a preliminary purification procedure for the synthesis of high-quality [^{18}F]FMZ.[17,24] They proposed a new synthetic route to [^{18}F]FMZ starting from tosyldiaryliodonium precursors. With the most efficient precursor (4-methylphenylmazeniliodonium tosylate), the radiofluorination yield was 67%. Preliminary SPE purification of the reaction mixture on C18 cartridges prevents the penetration into the product of chemical impurities which have floating retention times in the HPLC column and a large amount of radioactive impurities resulting from the decomposition of diaryliodonium salts in a high-temperature reaction. The yield of [^{18}F]FMZ obtained according to the above method is 53.4±9.0% (~60 min, n = 94).[24] Nowadays, this radioligand is successfully employed in Korean clinical practice.

After the HPLC procedure, the target product is present in the HPLC eluate. The latter contains an organic solvent (MeCN or THF), which makes it unsuitable for intravenous administration. The organic solvent can be removed from the [^{18}F]FMZ-containing fraction either by vacuum distillation[15,19,20,23] or by sorption of [^{18}F]FMZ on the C18 cartridge followed by elution with ethanol and by dilution with an injection buffer;[17] the latter method provides smaller losses of the product.

In our preliminary experiments, we combined the above approaches to HPLC optimization into a three-step procedure for [^{18}F]FMZ purification: (1) SPE on C18 cartridges for removal of radioactive impurities and DMF, (2) reversed-phase HPLC for removal of nitromazenil and other organic impurities, and (3) SPE on C18 cartridges for removal of the organic solvent from the [^{18}F]FMZ-containing fraction of the eluate. Using this procedure, we obtained [^{18}F]FMZ with the RCP >99% over 90 min (the content of nitromazenil and other chemical impurities is <1.0 μg mL^{-1}; the decay-corrected yield of the product is <5%). Failing to automate this multistep process completely, we used [^{18}F]fluoride with an initial activity of <3.7 GBq (100 mCi), which is insufficient for achieving the activity of the radioligand required even for a single brain receptor study (0.2 GBq[20]). For this reason, we tried to obtain [^{18}F]FMZ meeting the pharmacopeial requirements, using the SPE approach only.[27]

The major problem was to separate the product and the precursor having similar physicochemical properties and affinity for central BZRs. Nitromazenil is more lipophilic than [^{18}F]FMZ and hence is better retained at a reversed-phase sorbent (see Fig. 1). Our new approach involved sorption on an Oasis HLB 6cc microcolumn (Waters) filled with a copolymer of *N*-vinylpyrrolidone and divinylbenzene followed by fractional elution with aqueous ethanol of increasing concentration. The extraction capacity of the sorbent HLB is known[29] to be far higher than that of organosilicon materials (C18); this sorbent is suitable for separation of lipophilic and hydrophilic compounds at high elution rates used in automatic synthetic modules.[28,30] Many literature examples show its efficiency in the extraction of benzodiazepines from biological fluids.[31] Since flumazenil is insoluble in water, we used ethanol as an eluent (it is the only solvent allowed in injection solutions[27]). In the SPE separation of [^{18}F]FMZ and nitromazenil, it was important to minimize the amount of the latter. As shown in Table 1, a decrease in its

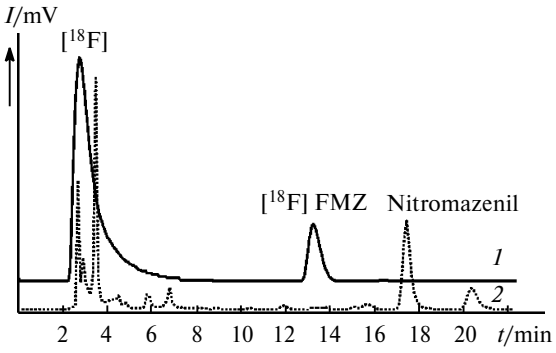


Fig.1. HPLC analysis of the reaction mixture in system 1: (*1*) radiometric detection and (*2*) UV detection.

Table 1. Parameters of the synthesis of [^{18}F]FMZ

Nitromazenil /mg	T/°C	t/min	Radiofluorination efficiency (%)*
8	150	30	18±4 (n = 4)
4	150	30	26±4 (n = 10)
1—2	150	15	30±7 (n = 9)

* The radiofluorination efficiency (a proportion of the radiotracer obtained in the reaction to the total activity of [^{18}F]fluoride) is determined in a sample of the reaction mixture using radio-TLC.

starting amount from 8 to 1—2 mg scarcely affected the efficiency of radiofluorination. Our experience demonstrated that nitromazenil should be purified before use because the impurities formed in storage can compete with nitromazenil for [^{18}F]fluoride.

Efficient sorption of [^{18}F]FMZ is achieved when a minimum amount of the organic solvent is present in a sample for SPE; for this reason, the reaction mixture is diluted with water (more than 10-fold dilution) prior to the SPE procedure. Lipophilic compounds ([^{18}F]FMZ, nitromazenil) are sorbed on an Oasis HLB microcolumn, while the major part of hydrophilic radioactive impurities ([^{18}F]) and DMF are passed through the column. In a series of preliminary experiments, we chose the composition of solutions for fractional elution. Hydrophilic radioactive (A) and chemical (B) impurities are eluted with 20% EtOH, the eluate containing no traces of [^{18}F]FMZ or nitromazenil (Fig. 2). A more lipophilic impurity is eluted with 30% EtOH; the eluate may contain [^{18}F]FMZ and traces of nitromazenil. Finally, the activity of the fraction eluted with 35% EtOH is due to [^{18}F]FMZ (>97%), while chemical impurities (*ca.* 10 mg mL^{-1} in general) are mainly represented by nitromazenil. Elution with 40—45% EtOH showed that nitromazenil is retained on the Oasis HLB; the relatively low radioactivity of the eluate is due to [^{18}F]FMZ traces.

The best strategy in the SPE purification of [^{18}F]FMZ is a compromise between full removal of the precursor impurity and the minimum losses of the radioligand, as

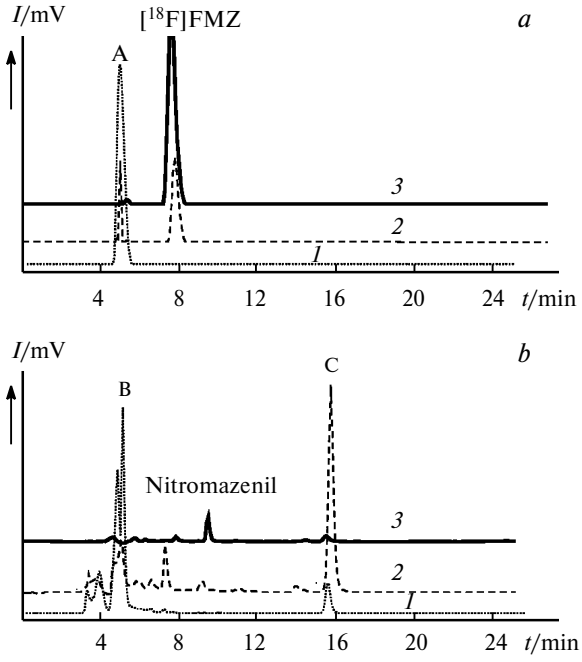


Fig. 2. HPLC analysis of the eluates obtained by purification of [^{18}F]FMZ *via* fractional SPE and containing (*1*) 20%, (*2*) 30%, (*3*) 35% EtOH. System 2 with radiometric (*a*) and UV detection (*b*).

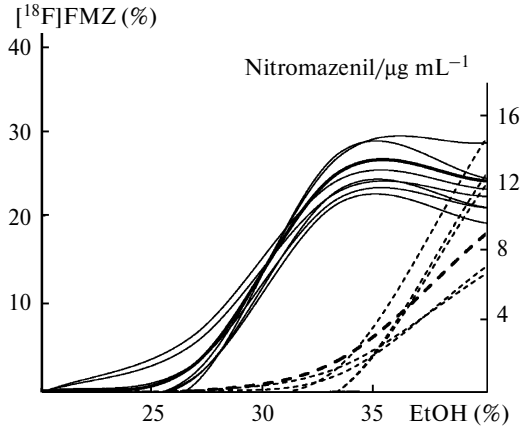


Fig. 3. Three-dimensional diagram of the elution curves showing the contents of [^{18}F]FMZ (thin solid lines) and nitromazenil (thin dotted lines) in each fraction of the eluate; the thick lines refer to their average values (n = 7).

illustrated with elution curves in a 3D diagram (Fig. 3). An increase in the concentration of EtOH results in the higher precursor content of the eluate, while the decreasing concentration of EtOH entails more considerable losses of the target product. So we took 35% EtOH as an optimal eluent extracting 27±5% (n = 12) of the [^{18}F]FMZ obtained by radiofluorination and only 3.6±0.4 μg mL^{-1} of nitromazenil (<0.2% from initial amount).

The fraction containing 40% EtOH extracts well [^{18}F]FMZ, but the precursor (~10 μg mL^{-1}) is also eluted. To prepare the radioligand, the eluate containing 35% EtOH (2 mL) was diluted with a phosphate buffer to 15 mL. The nonoptimized decay-corrected RCY of the radioligand was 8%. According to HPLC data, the specific radioactivity of [^{18}F]FMZ in the resulting 4% ethanolic solution exceeds 37 GBq μmol^{-1} and RCP is more than 97%; the precursor and Kryptofix 2.2.2 contents are less than 1 μg mL^{-1} and 0.1 mg mL^{-1},[32] respectively). These quality parameters meet the pharmacopeial requirements for PET radiopharmaceuticals.[27] The content of unidentified chemical impurities does not exceed 1 μg mL^{-1}, in compliance with the present-day requirements.[33] The overall time of the automated synthesis (reaction + purification) is 50 min. The module for the radioligand synthesis (Fig. 4) has been designed at the N. P. Bechtereva Institute of Human Brain of the Russian Academy of Sciences (IHB RAS). This procedure proposed for the preparation of [^{18}F]FMZ can easily be integrated into currently available modules for the synthesis of PET radiopharmaceuticals.

We assume that replacement of the nitro group in the precursor molecule by trimethylammonium (they are both good nucleofuges) will afford a quantitative yield of [^{18}F]FMZ *via* SPE because the properties of this precursor (salt) and the radioligand will differ greatly.[34] This assumption calls for further investigations.

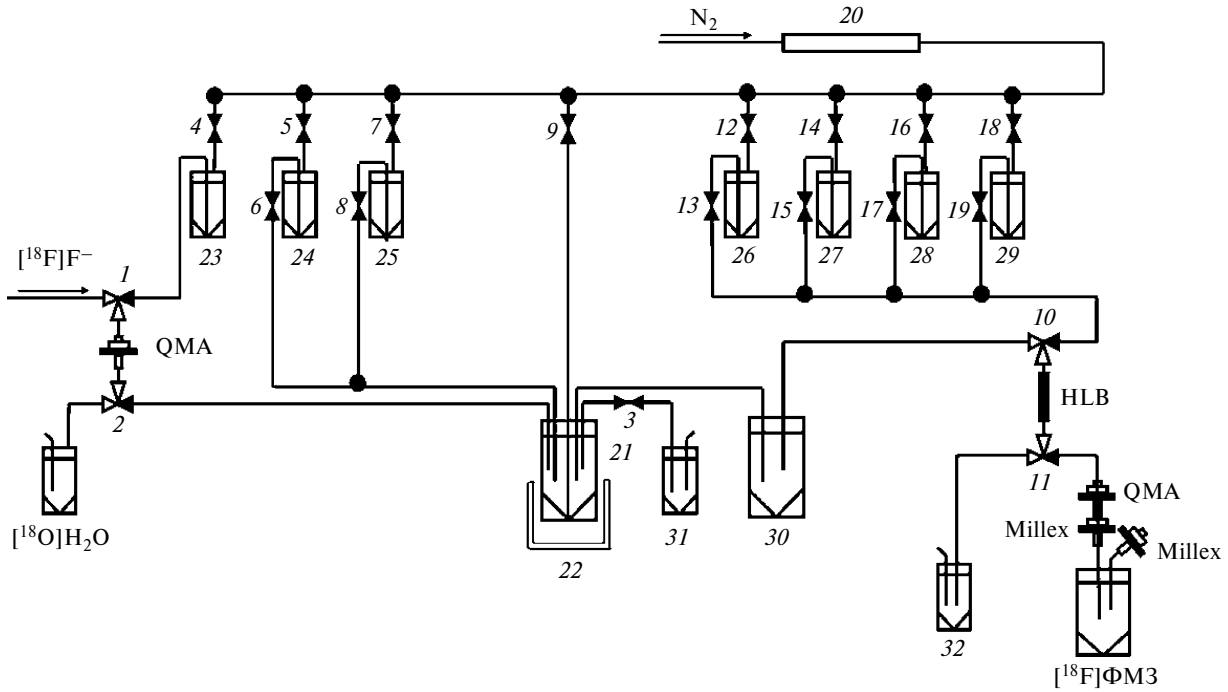


Fig. 4. Schematic diagram showing a module designed at the IHB RAS for the synthesis of [^{18}F]FMZ. The legend: (*1—19*) two- and three-way valves with electrical actuators; (*20*) flow controller; (*21*) reaction vessel; (*22*) heating unit; (*23—29*) vessels with (*23*) an eluent for QMA, (*24*) nitromazenil, (*25*) water (3 mL), (*26*) water (5 mL), (*27*) 20% EtOH (4 mL), (*28*) 30% EtOH (2 mL), (*29*) 35% EtOH (2 mL); (*30*) vessel for dilution (H_2O, 8 mL); (*31*) and (*32*) waste products; QMA is the cartridge with anion-exchange resin; HLB is the microcolumn with a reversed-phase sorbent; Millex is a sterilizing filter; [^{18}F]FMZ is a bottle for [^{18}F]FMZ.

Experimental

Materials and reactants. The following solvents were used: DMF (water content < 0.005%, Sigma-Aldrich), MeCN (water content < 0.03%, Kriokhrom, St. Petersburg, Russia), EtOH, THF (HPLC grade, Merck), and water enriched with oxygen-18, [^{18}O]H_2O (Global´nye Nauchnye Tekhnologii, St. Petersburg, Russia). Other compounds included 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (Kryptofix 2.2.2, or cryptand K2.2.2.), anhydrous K_2CO_3 (Sigma-Aldrich), flumazenil, and nitromazenil (ABX, Germany). Nitromazenil was additionally purified by flash chromatography on silica gel (Acros 35–70, 60 Å) with CH_2Cl_2 : MeOH = 95 : 5 (v/v) as an eluent. The fractions were analyzed using TLC; spots were visualized with a solution of $KMnO_4$ (1.0 g), K_2CO_3 (6.7 g), and NaOH (0.08 g) in water (100 mL). Cartridges containing tetramethylammonium anion-exchange resin (QMA light, Waters) were activated with 0.5 *M* K_2CO_3 (10 mL) and water (15 mL) immediately before use; Oasis HLB 6cc microcolumns (Waters) filled with a reversed-phase sorbent were also activated with EtOH (4 mL) and water (10 mL). A phosphate buffer (pH 6.4) was prepared by dissolving Na_2HPO_4 (2.5 g), NaH_2PO_4 (2.5 g), and NaCl (8.2 g) (all Pharm grade, Panreac, Spain) in water (1 L), with addition of 1 *M* NaOH to pH 6.4.

Radionuclide fluorine-18 was obtained by a nuclear reaction ^{18}O(p,n)^{18}F when a target filled with [^{18}O]H_2O water was bombarded with protons (17 MeV, current 40 μA) in a PETtrace cyclotron (GE Healthcare, USA). The initial radioactivity was 2—3 GBq. The synthesis of [^{18}F]FMZ was performed on a remote controlled module designed at the IHB RAS. The schematic diagram of the module is shown in Fig. 4.

Synthesis of [^{18}F]FMZ. The [^{18}F]fluoride obtained in the target was isolated from the target material by sorption on a QMA cartridge followed by elution into a reaction vessel using a QMA eluent (K_2CO_3 (2.0±0.1 mg, 12 μmol), Kryptofix 2.2.2 (9.0±0.1 mg, 25 μmol), acetonitrile—water (96 : 4, v/v; 2 mL)). The eluate solution was evaporated to dryness under nitrogen at 120 °C for 5 min. To the dry residue containing the complex [K/K2.2.2]/[^{18}F], a solution of nitromazenil (1—2 mg) in DMF (0.7 mL) was added. A radiofluorination reaction was carried out at 150 °C for 15 min; then the reaction mixture was cooled to 50—60 °C and diluted with water (3 mL).

SPE purification of [^{18}F]FMZ on an Oasis HLB microcolumn. The reaction mixture was transferred to a dilution vessel containing water (7—8 mL). The diluted mixture was passed through an Oasis HLB microcolumn to sorb [^{18}F]FMZ. The microcolumn was successively washed with water and 20% and 30% aqueous EtOH. The target product [^{18}F]FMZ was eluted with 35% EtOH (2 mL), which was passed through a QMA cartridge (for finishing purification) and a Millex sterilizing filter (0.22 μm, Merck Millipore) to a sterilized bottle containing a phosphate injection buffer (13 mL). The elution rate was 1—2 mL min^{-1}.

In preliminary experiments, [^{18}F]FMZ was isolated from the reaction mixture using semipreparative HPLC under the following conditions: μBondapakC_{18} reversed-phase column (7.8×300 mm, Waters), Gilson-305 pump, Rheodyne-7125 injector, Gilson-116 UV detector (254 nm), Beckman-170 radiometric detector, 0.05 *M* H_3PO_4—MeCN (4 : 1, v/v) as an eluent, flow rate 4 mL min^{-1}. The HPLC loop volume was 2 mL.

The retention times of [^{18}F]FMZ and nitromazenil were 18±2 and 26±2 min, respectively.

Analysis of the reaction mixtures and SPE eluates. The efficiency of radiofluorination was determined (see Table 1) and [^{18}F]FMZ was identified using radio-TLC and radio-HPLC.

Radio-TLC: Sorbfil plates with a UV indicator (LenKhrom, St. Petersburg, Russia); AcOEt—EtOH—H_2O (80 : 15 : 5, v/v) as a mobile phase. The distribution of the radiofluorination products over the plate was measured with a MiniGITA radio-TLC scanner (Raytest, Germany). The R_f value of flumazenil was determined using a UV lamp. For [^{18}F]fluoride and [^{18}F]FMZ, R_f are 0.06 and 0.50, respectively.

Radio-HPLC: Gilson chromatograph (Gilson-305 pump, Rheodyne-7125 injector), a Gilson-116 UV detector (254 nm) connected in series to a Beckman-170 flow gamma counter (radiometric detector), ACE reversed-phase column (4.6×250 mm). Two HPLC systems were used. System 1: 0.05 *M* NaOAc—THF—MeOH (80 : 10 : 10, v/v) as an eluent, flow rate 1.0 mL min^{-1}. System 2: 0.01 *M* H_3PO_4—MeCN (3 : 1, v/v) as an eluent, flow rate 1.5 mL min^{-1}. The substances were identified by comparing the retention times of [^{18}F]FMZ and flumazenil with allowance for a delay between the serially connected detectors. The retention times of [^{18}F]FMZ, flumazenil, and nitromazenil were 13.4±0.3, 13.0±0.3, and 19.2±0.3 min in system 1 and 7.5±0.5, 7.2±0.5, and 12±0.5 min in system 2, respectively.

Quality control for [^{18}F]FMZ. The RCP of [^{18}F]FMZ in the final solution was determined by radio-TLC and radio-HPLC as described above. "Cold" flumazenil in the radioligand product for specific radioactivity calculations was quantified under the conditions of HPLC system 2 (detection level 0.02 μg mL^{-1}); a calibration curve was plotted for solutions with concentrations from 5 to 0.05 μg mL^{-1}. The nitromazenil content was determined under the same conditions (detection level 0.025 μg mL^{-1}) using a calibration curve plotted for solutions with concentrations from 20 to 0.5 μg mL^{-1}. The content of cryptand K2.2.2 was determined by visualization of its spot with the iodine vapor.[32] The ethanol content was measured on a Varian-3400 gas chromatograph (flame ionization detector, Porapak Q column 4×1500 mm (Serva), helium flow rate 20 mL min^{-1}, column temperature 180 °C, injector temperature 180 °C, detector temperature 200 °C). The salt content of the radioligand product was determined by cryoscopy on an Osmomat-030 instrument.

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