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Research Article

Radiosynthesis of 3-(2′-[^{18}F]fluoro)-flumazenil ([^{18}F]FFMZ)

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Summary

Recently, two fluorine-18 labelled derivatives of flumazenil were described: 5-(2′-[^{18}F]fluoroethyl)-5-desmethylflumazenil (ethyl 8-fluoro-5-[^{18}F]fluoroethyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylate; [^{18}F]FEFMZ) and 3-(2′-[^{18}F]fluoro)-flumazenil (2′-[^{18}F]fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*]-[1,4]diazepine-3-carboxylate; [^{18}F]FFMZ). Since the biodistribution data of the latter were superior to those of the former we developed a synthetic approach for [^{18}F]FFMZ starting from a commercially available precursor, thereby obviating the need to prepare a precursor by ourselves. The following two-step procedure was developed:

First, [^{18}F]fluoride was reacted with 2-bromoethyl triflate using the kryptofix/acetonitrile method to yield 2-bromo-[^{18}F]fluoroethane ([^{18}F]BFE). In the second step, distilled [^{18}F]BFE was reacted with the tetrabutylammonium salt of 3-desethylflumazenil (8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylic acid) to yield [^{18}F]FFMZ. The synthesis of [^{18}F]FFMZ allows for the production of up to 7 GBq of this PET-tracer, enough to serve several patients. [^{18}F]FFMZ synthesis was

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completed in less than 80 min and the radiochemical purity exceeded 98%. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: [^{18}F]FFMZ; flumazenil; fluoroflumazenil; PET; fluoroalkylation

Introduction

Benzodiazepines are used as sedative, anxiolytic, hypnotic, anticonvulsant and muscle-relaxant drugs. These drugs act via a special binding pocket on the central gaba receptor (CBR) and the binding potency correlates with the affinity of the CBRs.[1] Various diseases, such as epilepsy, Huntington's disease, Alzheimer's disease or schizophrenia can be caused by alterating these CBRs.[2] Imaging and quantification of these CBRs with positron emission tomography (PET) is helpful for the diagnosis of these diseases. An accurate localization of the epileptogenic region is especially important for the success of surgical interventions. 2-[^{18}F]Fluoro-2-deoxyglucose ([^{18}F]FDG), displaying the altered glucose metabolism of the epileptogenic region, cannot meet all the demands for detailed functional brain imaging.[3–5] Therefore, the need for the development of more selective radiopharmaceuticals is self-evident. Flumazenil (FMZ, Ro 15-1788, ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylate, **1**), a highly selective benzodiazepine antagonist has been successfully labelled with carbon-11 and is the most extensively used CBR imaging agent so far developed for PET.[6,7] However, there are some disadvantages resulting from its short half-life (20.3 min). A fluorine-18 labelled radiotracer could overcome these disadvantages and allow the examination of more patients per tracer production and the possibility of longer acquisition protocols. Furthermore, a fluorinated tracer could be distributed to PET-centers without on-site cyclotron (satellite principle). The first [^{18}F]-labelled flumazenil derivative, 5-(2′-[^{18}F]Fluoroethyl)-5-desmethylflumazenil (ethyl 8-fluoro-5-[^{18}F]fluoroethyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylate [^{18}F]FEFMZ, **2**), has been evaluated recently showing high binding values but rapid de-fluoroalkylation.[2,8] Since the [^{18}F]fluoroethyl label was introduced at the 5-position of the molecule (the nitrogen function) rapid *N*-dealkylation was observed. Therefore, a different approach was developed, labelling flumazenil at the 3-position (the carboxylic function). Recently, data were presented supporting the hypothesis

that substitution of a methyl or ethyl ester by an [^{18}F]fluoroethyl ester leads to a stabilization of the compound.[9,10] A possible synthesis of 3-(2′-[^{18}F]fluoro)-flumazenil (2′-[^{18}F]fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*]-[1,4]diazepine-3-carboxylate, [^{18}F]FFMZ, **3**) has been proposed using direct nucleophilc fluorination on previously prepared mesylated or tosylated precursor molecules.[11] Since in our department no sophisticated precursor chemistry can be conducted, we herewith present a different synthetic approach starting from a commercially available precursor (8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylic acid; 3-desethylflumazenil, **4**). The well-established two-step procedure involves the formation of 2-bromo-[^{18}F]fluoroethane ([^{18}F]BFE), its purification via distillation, conversion to [^{18}F]FFMZ and the final purification by reversed-phase high performance liquid chromatography (RP-HPLC).

Radiosynthesis, evaluation of conversion and purification parameters as well as quality control of [^{18}F]FFMZ are discussed in the present paper.

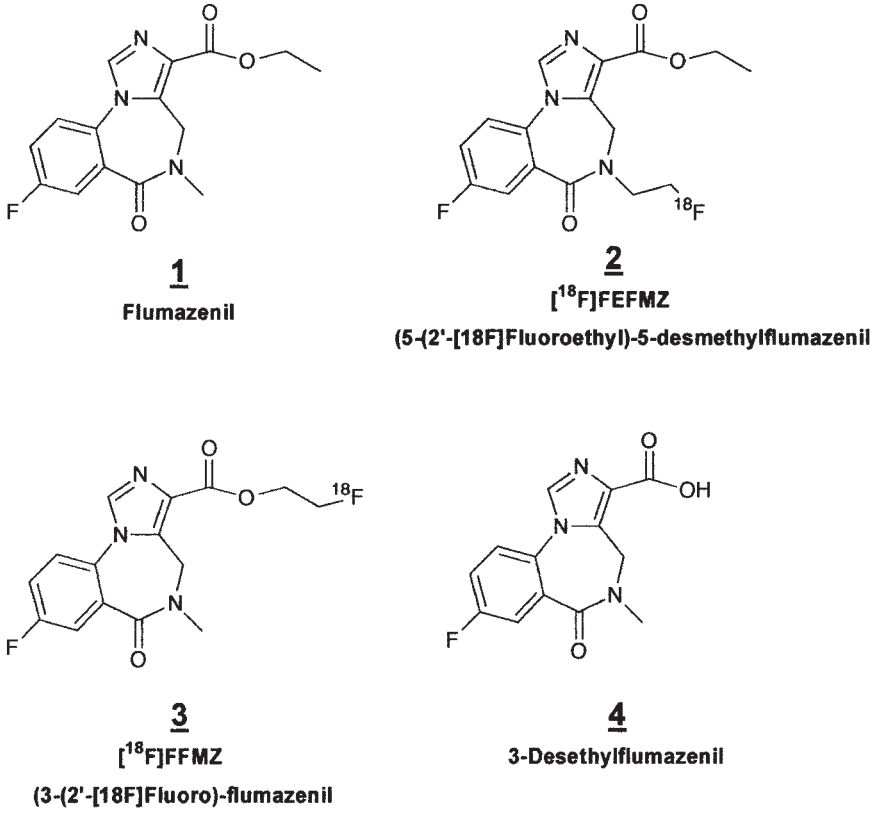
Results and discussion

Our synthesis route offers a highly reproducible and feasible method for the synthesis of [^{18}F]FFMZ in less than 80 min. So far, only 1 out of 67 (1.5%) syntheses failed (due to technical problems with the synthesizer module). Radiochemical yields at the end of synthesis (EOS) were up to 20% decay corrected (d.c.). (Table 1) Radiochemical impurities of the purified product solution as detected by radio-HPLC were well below our internal thresholds of 3%. No major chemical impurities were detected by HPLC (UV, 254 nm). Residual Kryptofix 2.2.2, as determined by thin layer chromatography (TLC), was below our detection limits.

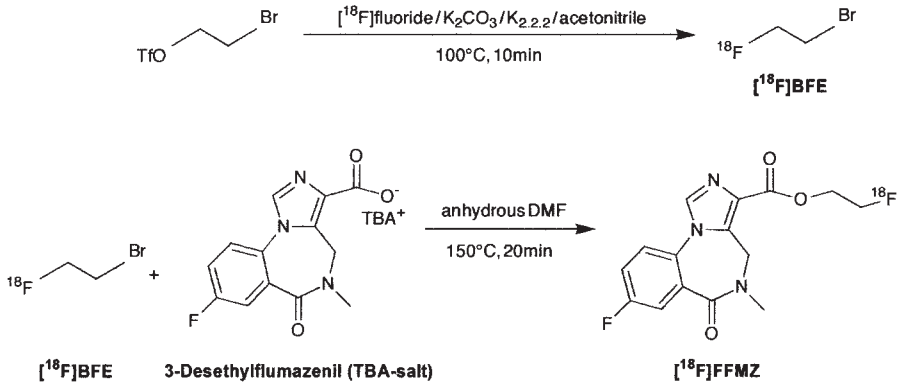
The first step of the synthesis was the formation of [^{18}F]BFE (Scheme 2), followed by its distillation into a v-vial containing anhydrous DMF through a washing flask. Although [^{18}F]BFE is a well-established synthon in the synthesis of fluorine-18 labelled radiotracers[9,12,13] we found that it was the critical and most challenging step in the whole radiosynthesis. Yields for this step varied between 13.3 and 43.7% within 10 min. Trapping yields, reactivity and quality of the distilled [^{18}F]BFE strongly depended on the nitrogen-flow as well as on the amount and ratio of DMSO/DMF used in the washing flask. Thus,

Table 1. Stepwise and overall reaction yields (decay corrected) and required times in the radiosynthesis of [^{18}F]FFMZ

Reaction step	Time (min)	Overall time (min)	Radiochemical yield (%)	Overall radiochemical yield (%)
EOB	0	0	100.0	100.0
Azeotropic drying	18	18	98.1	98.1
Formation of [^{18}F]BFE	10	28	69.7	68.4
Distillation of [^{18}F]BFE	10	38	43.7	30.5
Formation of [^{18}F]FFMZ	20	58	98.0	29.9
HPLC purification	15	73	70.0	20.9
Formulation and sterile filtration	3	76	95.0	19.9



Scheme 1. Derivatives of flumazenil



Scheme 2. Synthesis of [^{18}F]FFMZ

we determined the optimum nitrogen flow to be approximately 5 ml/min for best purification results. This value is critical, because, on the one hand, an increased nitrogen flow results in overdistillation of by-products and expulsion of [^{18}F]BFE out of the product vial and, on the other hand, a reduced nitrogen flow leads to trapping of [^{18}F]BFE in the washing flask and increased time. To complete the transfer into the product vial a total of 500 µl acetonitrile was added to the reaction vessel in 4–6 portions.

We also tried different setups for the distillation procedure using either short custom-made disposable columns filled with Ascarite and phosphorus pentoxide according to a recently published method[14] or charging the washing flask with different solvent mixtures or even using an empty washing flask. No significant improvements could be achieved be either of the described modifications.

For the second reaction step, the esterification of the tetrabutylammonium salt of 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylic acid (3-desethylflumazenil) with [^{18}F]BFE, reaction kinetics regarding temperature (Figure 1) and precursor concentration (Figure 2) were investigated. Since Zhang *et al.*[15] lately demonstrated that the addition of sodium iodide (NaI) to distilled [^{18}F]BFE improved the yields for various subsequent conversion reactions, we also compared the influence of NaI on the formation of [^{18}F]FFMZ (Figure 2). In this case the conversion exhibited lower radiochemical yields in the presence of NaI throughout all precursor concentrations and reaction times – contrary to our expectations. Furthermore, an additional by-product was observed that eluted closely to [^{18}F]FFMZ from the analytical HPLC system (retention time

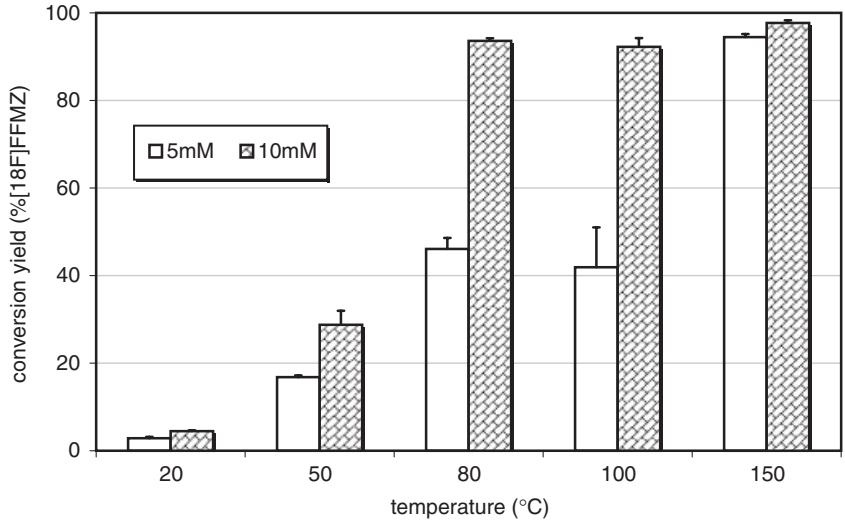


Figure 1. Temperature dependence of the conversion of [^{18}F]BFE into [^{18}F]FFMZ at two different precursor concentrations (reaction time = 20 min; n = 3–6)

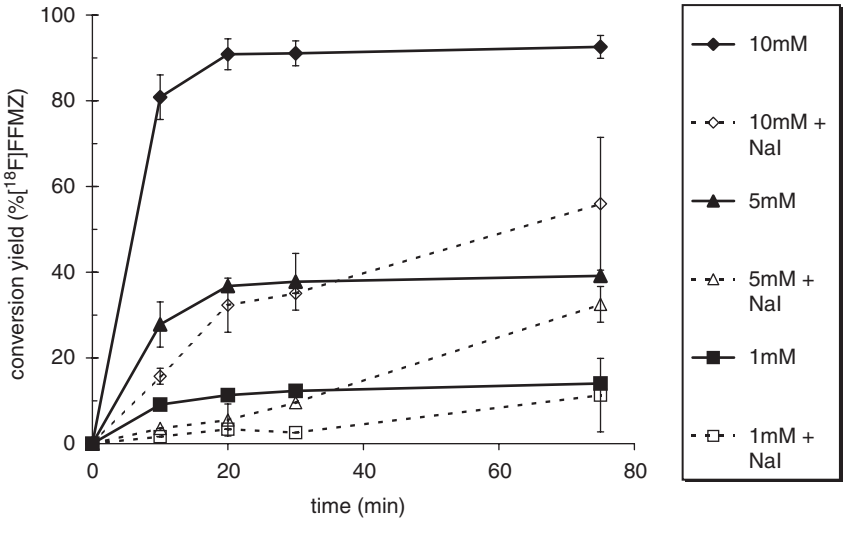


Figure 2. Kinetics of the conversion of [^{18}F]BFE into [^{18}F]FFMZ at three different precursor concentrations with and without addition of 1 mg NaI (T = 100°C; n = 3–6)

impurity: 5.5 min; retention time [^{18}F]FFMZ: 5.2 min). Since all labelling studies as performed by Zhang *et al.*[15] referred to compounds bearing an amine, phenol or amide but not a carboxylic function, we concluded that the addition of NaI to the [^{18}F]BFE/3-desethylflumazenil reaction mixture only enhanced the labelling in the 5-position by substitution of the methylgroup by the [^{18}F]fluorethyl group but not the esterification in the 3-position of the benzodiazepine molecule.

As evident from the present data a continuous increase in the esterification yields is observed throughout all precursor concentrations within the first 20 min, stagnating thereafter. Furthermore, our data explicitly indicate that a minimum reaction time of 20 min is required to achieve satisfactory conversion results ($>95\%$). This fact even applies to high precursor concentrations (e.g. 10 mM). Regarding the temperature dependance of the formation of [^{18}F]FFMZ we found that either 80°C employing 10 mM of precursor or 150°C employing 5 mM of precursor led to conversion yields higher than 95%. All these results led us to the optimum reaction conditions for the esterification of 3-desethyl-flumazenil as shown in Table 2.

The reaction mixture was loaded on the RP-HPLC column after cooling to ambient temperature in an ice-bath. The used separation system was part of the Nuclear Interface® methylation module, remotely controlled by a laptop equipped with NINA® software. The product fraction ([^{18}F]FFMZ) showed a retention time (r.t.) of 11.1 min, whereas [^{18}F]BFE was detected at 14.9 min. All other minor radioactive impurities could also be clearly separated. Chemical impurities apparent in the crude reaction mixture such as residual solvents (r.t. 6.5 min) and precursor (e.g. 3-desethylflumazenil; r.t. 9.9 min) were completely removed by this purification method.

The collected fraction was diluted with phosphate buffered saline, transferred to a laminar air-flow cell and filtered on-line. Millex-GS filters proved to be inapplicable due to the mixed cellulose esters matrix where more than 30% of the produced radioactivity were retained. We

Table 2. Optimum reaction conditions for the conversion of [^{18}F]BFE into [^{18}F]FFMZ

Temperature	150°C
Reaction time	> 20 min
Precursor concentration	> 5.0 mmol/l

therefore used Millex-GV filters with a durapore (PVDF) matrix showing close to zero captured radioactivity.

The used quality control systems for the determination of radiochemical yields and purity of the product solution revealed excellent separation properties: HPLC retention times were 5.0–5.3 min for [^{18}F]FFMZ, 7.2–7.6 min for [^{18}F]BFE and 2.6–3.0 min for 3-desethylflumazenil, respectively. All values were verified by co-injection of inactive reference substances. In addition, the pH and the osmolality where checked and gave values of 7.0–7.4 and 250–310 mosmol/kg, respectively.

Experimental

Materials

8-Fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylic acid (3-desethylflumazenil) and FFMZ standard (2′-fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*]-[1,4]diazepine-3-carboxylate or 3-(2′-fluoro)-flumazenil or 3-(2′-fluoroethyl)-3-desethylflumazenil) were purchased from ABX – Advanced Biochemical Compounds (Dresden, Germany). 2-Bromo-1-fluoroethane was bought from ABCR (Karlsruhe, Germany). All other reagents were purchased from either Sigma-Aldrich Chemical Company (Steinheim, Germany) or Merck (Darmstadt, Germany) or Riedel-de Haën (Seelze, Germany) in the highest purity grade commercially available and used without further purification. Analytical HPLC was performed using a LiChrospher 100 RP-18 column (5 µm, 250 × 4 mm, 1 ml/min) from Merck (Darmstadt, Germany). Semi-preparative HPLC was performed using a Nucleosil 100-7 RP-18 column (7 µm, 250 × 16 mm, 6 ml/min) from Macherey-Nagel (Düringen, Germany).

[^{18}F]Fluoride was produced via the ^{18}O(p,n)^{18}F reaction in a GE PETtrace cyclotron (16.5 MeV protons). Enriched ^{18}O water (> 95%) was purchased from Chemotrade (Dresden, Germany).

Instruments

Analytical HPLC was performed with a Merck-Hitachi LaChrom L-7100 system equipped with a Merck-Hitachi LaChrom L-7400 UV detector at 254 nm and a lead-shielded NaI-radiodetector (Berthold).

Semi-preparative HPLC was performed using the original Nuclear Interface® chromatographic equipment (UV 254 nm). Osmolality (in milliosmol per kilogram, mosm/kg) of the [^{18}F]FFMZ preparations was determined using a Vapro Vapor Pressure Osmometer 5520 (Wescor Inc., Logan, USA). The pH was measured with a WTW pH 526 pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

2-*Bromoethyl triflate*

2-Bromoethyl triflate was prepared according to a literature method[16] starting from trifluoromethanesulfonic anhydride and 2-bromoethanol. Distillation (120°C, 20 mbar) gave a colorless oil in 37% yield which was stored at −18°C.

2-*Bromo-[^{18}F]fluoroethane ([^{18}F]BFE)*

No-carrier-added (n.c.a.) aqueous [^{18}F]fluoride was prepared by the ^{18}O(p,n)^{18}F nuclear reaction on an enriched water target. 0.4–1.6 ml of the solution was added to a 2.5 ml v-vial containing Kryptofix 2.2.2. (13.3 μmol), potassium carbonate (10.0 μmol) and acetonitrile (1.0 ml, 19.1 mmol) and heated to 100°C. Azeotropic drying was performed by subsequent addition of at least four 250 μl portions of acetonitrile. To the dried complex 2-bromoethyl triflate (20 μl, 77.8 μmol) and acetonitrile (80 μl, 1.5 mmol) were added, the vial was sealed and the contents heated at 100°C for 10 min.

Distillation

Volatiles were distilled using a smooth stream of nitrogen (5 ml/min) and 1/16″ tubing with needles connecting the reaction vessel, a washing flask and the product trap. The washing flask contained 195 μl of anhydrous DMSO and 5 μl of anhydrous DMF at ambient temperature, whereas the product trap contained 400 μl of anhydrous DMF at 0°C. A total of 500 μl of acetonitrile was added in small portions (appr. 100 μl) to the reaction vessel to achieve quantitative transfer of the intermediate ([^{18}F]BFE). The whole distillation procedure took about 10 min.

2′-[^{18}F]fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-benzo-[f]imidazo[1,5-a]-[1,4]diazepine-3-carboxylate ([^{18}F]FFMZ)

The precursor was activated by dissolving 10 mg (36.3 μmol) 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*] [1,4]diazepine-3-carboxylic acid (3-desethylflumazenil) in 300 μl dichloromethane and adding 47.9 μl tetrabutyl ammonium hydroxide (TBAH, 20%) as a phase transfer catalyst. Dichloromethane was then evaporated and the dried complex was reconstituted in 1 ml anhydrous DMF. A calculated aliquot of this solution was added to the distilled [^{18}F]BFE. The reaction was carried out for 10, 20, 30 or 75 min for the reaction kinetics and at temperatures varying between 20 and 150°C for the temperature-dependence experiments. The reaction mixture was cooled to room temperature prior to the final purification step.

Product purification

The crude reaction mixture was diluted with approximately 500 μl of water and subsequently loaded on to the semi-preparative HPLC column. The mobile phase consisted of 10 mM orthophosphoric acid/ethanol 50/50 (v/v), isocratically eluting at a flow rate of 6 ml/min. The product fraction was collected, buffered with 6 ml of 21 mM phosphate buffered saline and passed through a Millex-GV 0.22 μm sterile filter (Millipore Corporation, Bedford, USA).

Quality control

Chemical and radiochemical impurities were detected using radio-HPLC (mobile phase: 70% (water/ethanol/acetic acid 87.5/10/2.5 (v/v/v), 2.5 g/l ammonium acetate, pH 3.5), 30% acetonitrile). Residual Kryptofix 2.2.2 was analyzed by TLC according to the [^{18}F]FDG monograph in the European Pharmacopoeia (1999:1325).[17] Osmolality and pH were checked.

Conclusion

From the present investigation it is evident, that the introduced synthesis allows for the simple preparation of [^{18}F]FFMZ in a reliable manner and excellent purity in less than 80 min. The addition of small

amounts of sodium iodide to the reaction mixture decreases the achievable radiochemical yield contrary to a recently reported finding.[14]

Recent investigations regarding the *in vitro* and *in vivo* behavior of this promising compound have demonstrated its selectivity to the central gaba receptor[11,18] as well as its high stability in rats showing no increased uptake in any of the excretion organs.[18] The latter findings are different to data obtained with [^{18}F]FEFMZ.[8]

So far, the proposed synthesis allows for the production of up to 7 GBq of [^{18}F]FFMZ with high reproducibility, sufficient to serve several patients per tracer production. Hence, it may also enable PET centers without on-site cyclotron to include benzodiazepine receptor imaging into patient routine.

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