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FACULTY OF CHEMISTRY AND PHARMACY

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INVESTIGATING THE POSSIBILITIES OF STEREOSELECTIVE CLICK RADIOLABELING WITH ^{18}F-FLUORODEZOXY GLUCOSE

ABSTRACT

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The dissertation consists of 135 pages, contains 63 figures, 15 tables and 143 references are cited. The numbering of the figures in the abstract does not correspond to that of the dissertation.

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The materials for the defense of the dissertation work are available in the Dean's Office of the FCF at Sofia University, as well as on the website of the Faculty of Chemistry and Pharmacy.

CONTENTS

ABBREVIATIONS USED

iTCO – trans-cyclooctene used: (Z)-O-(cyclooct-4-en-1-yl)hydroxylamine

PET-CT – Positron emission tomography

BOC – tert-butyloxycarbonyl

DAD – diode array detector

[^{18}F]FDG – [^{18}F]2-fluoro-D-deoxyglucose

HPLC – High Performance Liquid Chromatography

IEDDA – [4+2] Diels-Alder cycloaddition between tetrazine and trans-cyclooctene

K1 – aniline

K2 – p-methoxyaniline

K3 – p-diaminobenzene

K4 – 3,5-diaminobenzoic acid

K222 – Cryptofix or (4,7,13,16,21,24-hexaoxa-1,10-diazobicyclo-[8,8,8]-hexacosane

Pr1 – oxime product between [^{18}F]FDG and Tz1

Pr2 – hydrazone product between [^{18}F]FDG and Tz2

Pr3 – oxime product between [^{18}F]FDG and R1

Pr4 – monosubstituted oxime product between [^{18}F]FDG and R2

Pr5 – disubstituted oxime product between [^{18}F]FDG and R2

Pr6 – monosubstituted oxime product between [^{18}F]FDG and R3

Pr7 - disubstituted oxime product between [^{18}F]FDG and R3

QC - quality control

R1 – aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester

R2 – 4-(6-(4-(-2-(aminooxy)acetoxy)-1,2,4,5-tetrazin-3-yl)phenyl-2-(aminooxy)acetate

R3 – aminooxy-acetic acid 6-(2-aminooxy-acetoxy)-[1,2,4,5]tetrazin-3-yl ester

RAD – radioactivity detector

RI – refractive index

Tz1 – O-{10-[4-(6-Phenyl-[1,2,4,5]tetrazine-3-yl)-phenoxy]-decyl}-hydroxylamine

Tz2 – {3-[4-(6-Phenyl-[1,2,4,5]tetrazin-3-yl)-phenoxy]-propyl}-hydrazine

TCO – trans-cyclooctene

TFA – trifluoroacetic acid

TLC – thin layer chromatography

UV/VIS – ultraviolet/visible light

INTRODUCTION

The oncological diseases are one of the leading causes of death in the world. Despite significant improvements in current treatment options such as surgery, radiation therapy, chemotherapy, and immunotherapy, there are also known shortcomings in the treatment of cancers. Therefore, the early and precise diagnosis of malignant tumors plays a leading role in choosing the right approach to therapy and the prognosis of cancer patients' survival. This task is solved to a significant extent with the help of nuclear medicine through in vivo studies based on the distribution of compounds labeled with radionuclides. The main imaging technique in nuclear medicine is positron emission tomography (PET-CT). It aims to non-invasively visualize, characterize and quantify biological processes at the cellular and molecular level in vivo. Its principle consists in the detection of positron emitters, as a result of which two- and three-dimensional images of the distribution of radioactivity in the patient's body are reconstructed. The main radionuclide for the preparation of PET-radiopharmaceuticals is ^{18}F. It is characterized by easy accessibility, optimal physical properties and a relatively short half-life (109 min.). For the purposes of radiopharmaceutical synthesis, it is primarily produced in a cyclotron by proton bombardment of ^{18}O-enriched water by the following nuclear reaction $^{18}O(p,n)^{18}F$.

The [^{18}F]FDG is a glucose analog and is the most commonly used PET radiopharmaceutical due to its highly efficient radiosynthesis and automation capability. It is used in oncology, cardiology and neurology. PET CT with [^{18}F]FDG is a sensitive imaging method to detect and characterize malignant tumors, to evaluate the effect of treatment, to plan therapy and also to determine the exact site for biopsy. It is mainly obtained by nucleophilic radiofluorination, and the reaction proceeds according to the S_{N2} mechanism, i.e. as a bimolecular stereospecific reaction followed by acid hydrolysis. Although [^{18}F]FDG is used as a universal, it does not fit into the modern concept of a highly specific and selective radiopharmaceutical. This necessitates the modification of the molecule. [^{18}F]FDG has been reported as a suitable prosthetic group that can be used to indirectly label sensitive macromolecules. The presence of a carbonyl group in the structure suggests the participation of the molecule in reactions to form an oxime or hydrazone bond. For this reason, the development and proposal of a methodology for the modification of one of the most commonly used radiopharmaceuticals in nuclear medicine, [^{18}F]FDG, is the main idea underlying the present dissertation.

The ability to use specific click reactions to modify ^{18}F-labeled radiopharmaceuticals will further increase their specificity and efficiency. An interesting click reaction is the cycloaddition between tetrazine and trans-cyclooctene. It is characterized by fast kinetics and irreversibility, takes place in an aqueous environment and in the absence of a catalyst. The reaction enables the preparation of radiopharmaceuticals for pre-targeted imaging. This is a strategy where the radiolabeling of the desired biomolecule is performed in vivo. First, target-specific molecules or immunoconjugates that bind to the target site are injected. Radiolabeled compounds are then added that selectively conjugate to the pretreated molecules at the target site. This method has several advantages, including better image contrast, the ability to use short-lived PET radionuclides, and reduction of radiation doses to non-target tissues.

The possibility of indirect [^{18}F]FDG radiolabeling of clickable bifunctional tetrazine and TCO derivatives is an important step towards the development of personalized medicine related to the early and accurate diagnosis of cancers and also to the refinement of therapy. We believe that the radiolabeling method based on the bioorthogonal click reaction of tetrazine with trans-cyclocene (IEDDA) may allow rapid conjugation at very low concentrations, leading to the optimal production of new PET radiopharmaceuticals with high specific activity and selectivity.

AIM AND OBJECTIVES OF THE DISSERTATION

The present work aims to investigate the feasibility of using [^{18}F]FDG as a prosthetic group for stereoselective "click" radiolabeling of macromolecules. The reaction should be simple to implement, involve a minimal number of steps, be efficient and rapid given the relatively short half-life of ^{18}F (109 min). It is important to allow it to be performed in standard clinical conditions at a moderately high temperature (up to 80°C). Development of a methodology to modify [^{18}F]FDG using a bifunctional tetrazine derivative will provide a fluorinating agent clickable to bioactive TCO structures. After carrying out the synthesis, it is necessary to characterize the final product with modern methods of separation and analysis.

The following tasks have been set for the fulfillment of the set goal:

1. Development of a methodology for oxime modification of aminooxy functionalized tetrazines.

2. Development of a methodology for hydrazone modification of hydrazine containing tetrazines.

EQUIPMENT, REAGENTS AND MATERIALS USED

The experiments presented in this dissertation, related to the production and quality control of [^{18}F]FDG, the labeling of bifunctional tetrazine derivatives and TCO with [^{18}F]FDG by formation of an oxime or hydrazone bond, were performed at the Clinic of Nuclear Medicine at St. Marina" – Varna. Further analyses and characterization of some of the obtained products by HPLC and IR-ATR, as well as investigation of the kinetics of IEDDA reactions by absorption spectrophotometer are performed at the Faculty of Chemistry and Pharmacy, Sofia University "Sv. Kliment Ohridski.

I. Apparatus used

- Cyclotron complex

For the production of [^{18}F]FDG in the Clinic of Nuclear Medicine at the University Hospital "St. Marina" - Varna a biomedical small cyclotron of ABT Molecular Imaging, model BG-75, complete with automated radiochemical synthesis module and automated QC-system is used. The automated QC system includes a pH-microelectrode, QC-pump, standard HPLC-system with UV/VIS, RI and RAD detectors. The HPLC - column used was Phenomenex, Rezex ROA-Ogranic Acid H+ (8%), 4,6x250 mm, which was maintained at 76 ^{o}C in an oven.

- Activimeter (dose calibrator)

A Dose Calibrator ISOMED 2010, SN 192148, certified according to the regulations of the local radiation control agencies, is used to measure the activity of the resulting radiopharmaceutical [^{18}F]FDG and to determine the half-life of the radionuclide.

- Multichannel analyser (gamma spectrometer)

A Digital Spetrum Analyzer DSA-1000 multichannel analyzer with a germanium detector (model 7500) is used for radionuclide purity determination.

- Instrumentation for bacterial endotoxins

An Endosafe PTS Charles river system (model ES-VAL PTS001-12, SN 5821) using licensed disposable instrument-specific cassettes is used to determine the presence of bacterial endotoxins.

- Radio-TLC

The detection of successful reactions and the production of radiolabelled [^{18}F]FDG bifunctional compounds by oxime and hydrazone bond formation is performed with a TLC radio-TLC instrument (Scan-Ram PET/SPECT radio TLC-scanner).

- HPLC-DAD system

HPLC analysis using a Varian ProStar HPLC system was applied to prove the success of the reactions and to obtain the desired products Separation was performed with a C18

chromatography column (Microsorb-MV, 100-5, C18, 150 x 4,6 mm). The chromatographic analysis parameters are given in Table 1.

Table 1. HPLC analysis parameters

Chromatographic separation parameters	Mobile phase	H_2O (A) and CH_3CN (B)
	Flow	1 $mL.min^{-1}$
	Gradient	95% A:5% B for 2 min → 0% A:100% B for 20 min
	Duration	22 min
Detector parameters	Wavelength	260 nm

- Radio - HPLC system

Radio-HPLC is used as an independent method using the SCI8100 Plus radio-HPLC system including: vacuum degasser, binary pump; UV/VIS detector with dual wavelength capability; radioactivity detector. Separation is carried out with the same C18 chromatography column (Microsorb-MV, 100-5, C18, 150 x 4,6 mm). Analyses were performed using two different gradient methods, the parameters of which are presented in Table 2.

Table 2. Radio-HPLC analysis parameters

Parameter	I method	II method
Mobile phase	0.1%TFA/H_2O (A) and 0.1%TFA/CH_3CN (B)	0.1%TFA/H_2O (A) and 0.1%TFA/CH_3CN (B)
Flow	1 $mL.min^{-1}$	1 $mL.min^{-1}$
Gradient	95% A:5% B for 2 min → 0% A:100% B for 20 min	100% A: 0% B for 4 min 40% A: 60% B for 10 min 5% A: 95% B for 6 min
Duration	22 min	20 min
Wavelength	260 nm	280 nm

- Auxiliary equipment

To prepare solutions with a given concentration and pH, an analytical balance KERN ABS-N-ABJ-NM, model 1.5 /08/2017 BG (1715) and a pH meter model Bante 920UK are used. A thermostated water bath shielded with lead protection is used to heat the reaction mixtures.

II. Reagents and materials used

1. Reagents and materials for the production of [^{18}F]FDG

For the production of the ^{18}F radionuclide, ^{18}O-enriched water (≥ 98%) ($[^{18}O]H_2O$) is used. The radiochemical process uses disposable synthesis cards and a reagent kit including acetonitrile, K222, ethanol 70%, 2M HCl, water for injections and an empty waste vial. And a vial containing lyophilized mannose triflate is additionally used. All reagents were obtained commercially and used without further purification.

Liquid chromatography water (Fisher) was used to perform the HPLC analysis by the automated QC system. LAL water (EMD Millipore Corporation) and disposable instrument-specific cartridges (Charles River) were used to determine bacterial endotoxins.

2. Reagents and materials required for chemoselective labeling with [^{18}F]FDG

2.1. Catalysts: aniline (K1); p-methoxyaniline (K2); p-diaminobenzene (K3); 3,5-diaminobenzoic acid (K4).

2.2. Buffers: sodium acetate with pH=4; phosphate PSB with pH=7.4; HEPES pH=5.5; potassium phthalate buffer with pH=4; aniline acetate buffer with different pH (aqueous solution of the catalyst – aniline or its derivative, acidified with acetic acid).

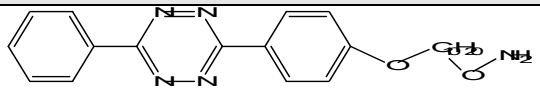
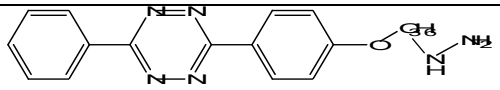
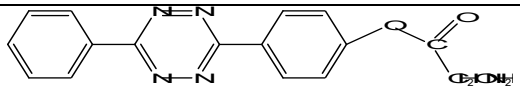
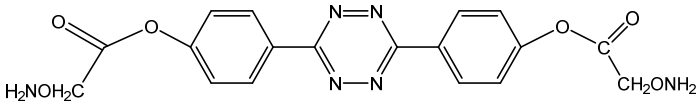
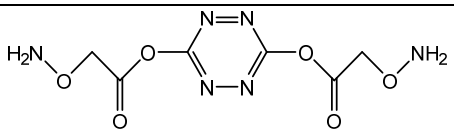
2.3. Bifunctional tetrazine derivatives:

- O-{10-[4-(6-Phenyl-[1,2,4,5]tetrazin-3-yl)-phenoxy]-decyl}-hydroxylamine (Tz1)
- {3-[4-(6-Phenyl-[1,2,4,5]tetrazin-3-yl)-phenoxy]-propyl}-hydrazine (Tz2)
- aminooxy-acetic acid 6-(2-aminooxy-acetoxy)-[1,2,4,5]tetrazin-3-yl ester (R3)
- aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester (R1)
- 4-(6-(4-(-2-(aminooxy)acetoxy)-1,2,4,5-tetrazin-3-yl)phenyl-2-(aminooxy)acetate (R2)

Appropriately functionalized tetrazine derivatives containing a free hydroxylamine or hydrazine group capable of forming an oxime or hydrazone bond were selected. In addition, a suitable spacer was selected between the tetrazine ring and the functional group. By varying the spacer, the lipophilicity and pharmacokinetic properties of the macromolecule can be influenced. The structures of the bifunctional compounds used are presented in Table 3.

The substances used are synthesized in advance at the Faculty of Chemistry and Pharmacy at SU "St. Kliment Ohridski", as part of the implementation of a project with contract No. KP-06-H29/4 (Scientific Research Fund under the Ministry of Education and Science). The products were purified by polar phase column chromatography and characterized by 1H-NMR, then used for the purpose of our experiment.

Table 3 – Structures of bifunctional compounds

Bifunctional compound	Structure
Tz1	
Tz2	
R1	
R2	
R3	

2.5. Solvents and acids: HPLC water, acetonitrile, ethyl acetate, dichloromethane, methanol, ethanol, acetic acid, trifluoroacetic acid.

2.6. Auxiliary materials: polyethylene cuvettes of 0.5 and 1.5 mL; 10 mL glass vials with rubber stoppers; automatic pipettes from 5 μL, 100 μL and variable from 200 to 1000 μL; silica-coated aluminum plates for TLC; tray for TLC plates; 2 mL syringes and needles for them; 0.45μm filters.

PRODUCTION CYCLE OF [^{18}F]FDG

The Nuclear Medicine Clinic at UMBAL "St. Marina" – Varna has its own cyclotron, with the help of which the production of [^{18}F]FDG is carried out for routine clinical purposes. Production is standardized and fully automated, characterized by good reproducibility. The radiopharmaceutical is supplied in a syringe as a ready-to-inject dose. The duration of the production process is 95±5 minutes. While the radiochemical process takes place in the synthesis module, the radionuclide for the next dose is produced in parallel in the cyclotron. Due to the overlapping of the mentioned processes, the reception of the doses after the first one is at an interval of 50 minutes.

In the first stage of the production cycle, the production of the ^{18}F radionuclide is carried out by bombarding ^{18}O-enriched water (>95%) with protons with an energy of 7.5 MeV at an average current of 4 μA for 40-45 minutes. After entering the radionuclide (in the form of an

anion in an aqueous solution) into the reactor of the synthesis card, the radiochemical synthesis of [^{18}F]FDG is carried out for 30-32 minutes. For this purpose, nucleophilic radiofluorination with the precursor mannose triflate followed by hydrolysis with 2M HCl was applied, presented in Figure 1.

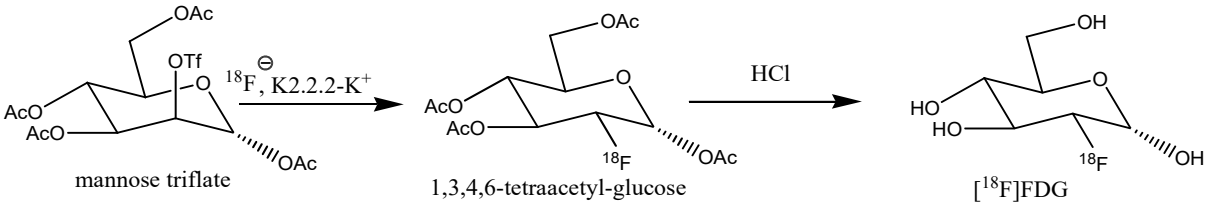


Figure 1 - Synthesis of [^{18}F]FDG

The resulting product undergoes various quality controls to ensure purity, safety and effectiveness. After successful quality control, the resulting product is released and used for clinical purposes. Non-patient administered amounts of [^{18}F]FDG were used for the purpose of our experiment to modify bifunctional tetrazine derivatives. The use of [^{18}F]FDG as a prosthetic group is due to the fact that in aqueous solution an equilibrium is observed between the cyclic and acyclic forms of the molecule, represented in Figure 2. The acyclic form is favored at high temperature and low pH.

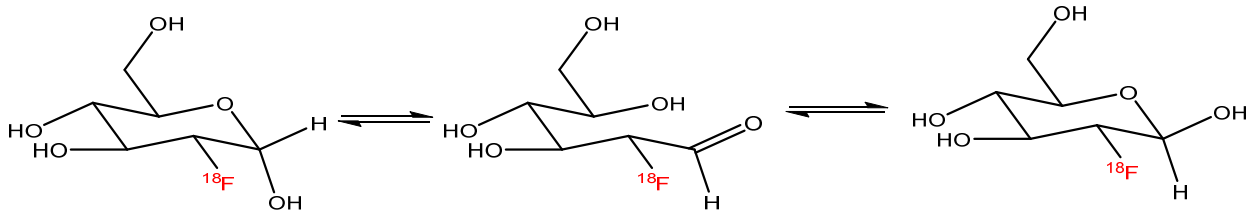


Figure 2 – Equilibrium of [^{18}F]-FDG in aqueous solution

STEREOSELECTIVE CLICK RADIOLABELING WITH [^{18}F]FDG

1. Reaction conditions of the conducted experiments

The reactions for modification with [^{18}F]FDG of the bifunctional derivatives of tetrazine are carried out entirely in clinical conditions on the territory of the Clinic of Nuclear Medicine at the "Sveta Marina" UMBAL – Varna, and not in a specialized radiochemical laboratory. From the point of view of radiation safety, the experiments are carried out at a low initial radioactivity of [^{18}F]FDG. Over two hundred syntheses are conducted. In order to find the optimal reaction conditions for the conjugation between the bifunctional tetrazines and [^{18}F]FDG, the reactions were carried out at different temperatures: room temperature, 30, 40, 50, 70, 80 and 100°C, heating was carried out in a thermostated water bath. The progress of the

reactions was monitored by radio-TLC of samples after 1, 5, 10, 20 and 30 minutes after mixing the reagents. The pH of the medium is varied between 0.5 and 8.5. Syntheses are carried out in the absence and in the presence of a catalyst (aniline or its derivatives). The type of buffer used (sodium acetate, phosphate, HEPES, aniline acetate) varies. The amounts of reagents used, the concentration of the catalyst, the ratio of tetrazine to [^{18}F]FDG and the method of mixing the reagents are varied. In some syntheses, all the reactants are mixed at the initial moment, immediately before heating. It was then found that stepwise mixing proved to be more efficient, mixing the catalyst buffer solution with [^{18}F]FDG in the first step, and adding the tetrazine pre-dissolved in acetonitrile in the next step. In some of the experiments, the carbohydrate component was used in a tenfold excess over tetrazine. To compensate for the low radioactivity of [^{18}F]FDG (corresponding to a low molar amount), a certain amount of non-radioactive glucose is added as a carrier. The administered radioactivity was between 20 and 70 μCi (0.74-2.59 MBq). Finally, the syntheses were carried out without the addition of non-radioactive glucose, and in this group of experiments a significantly higher activity was applied - on the order of 150 - 750 μCi (5.55 - 27.75 MBq).

In initial experiments, radio-TLC analysis was carried out with DCM (dichloromethane) as eluent, but proved unsuitable for the separation of the reaction components, as both [^{18}F]FDG and the resulting product were retained at the start (at the drop point). A 1:1 DCM/CH_3OH mixture was then used and slightly better separation was obtained. Subsequently, it was decided to use a more polar solvent – ethyl acetate. Until the optimal conditions are established, the syntheses are primarily carried out with one of the provided tetrazines, namely Tz1. The first experiments were carried out in very small quantities (total volume of the reaction mixture was 0.27 – 0.30 mL) and with very low [^{18}F]FDG radioactivity, and the reagents were simultaneously mixed at the initial time. Due to the small volume of the reagents, polyethylene cuvettes with a volume of 0.5 mL were initially used as reaction vessels (however, they are not suitable for heating at a higher temperature). Figure 3 shows reaction mixtures of selected initial experiments.

At a later stage of the work, the syntheses were carried out in 10 mL glass vials shown in Figure 4. The vials are heated with a ventilation needle stuck in the rubber stopper to equalize the steam with the atmospheric pressure. In this way, they are prevented from opening and possible splashing out of the radioactive reaction mixture.

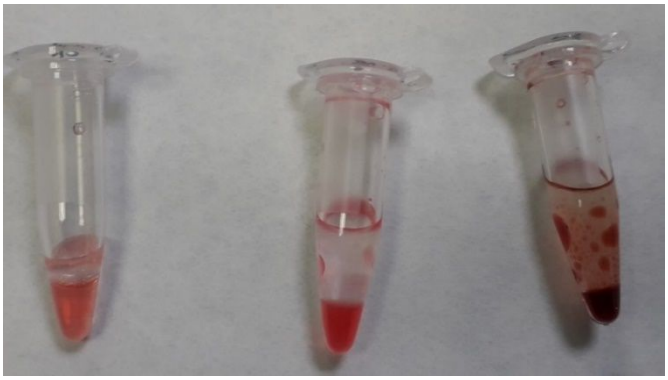


Figure 3 – Reaction mixtures in initial experiments

Figure 4 - Reaction vials

2. Description of the experimental procedures

2.1. Experiments with random ratios of reactants

In the first experiments, the reagents (100 µL sodium acetate buffer, 20 µL aniline, 100 µL [^{18}F]FDG and 50 µL tetrazine solution with a concentration of 10 µM) were mixed simultaneously and the reaction vessel was incubated at the appropriate temperature for 15 minutes. In other experiments, the addition of tetrazine is carried out at a later stage after the preheating of the remaining components of the reaction mixture. Experiments are also carried out without catalyst but in the presence of HCl. The specific conditions of the syntheses can be seen in Table 4 (rows 1-3). TLC analysis performed with DCM as eluent indicated a failed reaction under these conditions.

2.2. Experiments with the addition of non-radioactive glucose to the reaction mixture – work in excess of glucose compared to tetrazine

In this set of experiments, catalysts K1 or K2 were dissolved in the respective buffer (sodium acetate, HEPES and PBS). From the obtained solutions with a concentration of 0.2-0.8 M, transfer 500 µL to the reaction vessel. To these are added the appropriate amount of non-radioactive glucose as carrier and 100 µL [^{18}F]FDG. The reaction mixture was heated at

70-80°C for 20 minutes, after which the tetrazine solution was added. This is followed by additional heating at the same temperature for 15 minutes. After TLC analysis with DCM, colored spots were observed at the start corresponding to products obtained in some of the cases, while the starting tetrazine and catalyst were recorded as colored spots near the front. At this stage of the work, it is only qualitatively monitored whether the reaction is proceeding or not, since during the elution with DCM and the unreacted [^{18}F]FDG is retained at the start. And when scanning the plates with a radio-TLC scanner, it is not possible to tell how much of the registered radioactivity corresponds to the product obtained. Successful syntheses were observed in the presence of K2 in sodium acetate buffer and a final pH of the reaction medium of 5.4. More details on the conditions of the syntheses can be obtained from Table 4 (rows 4-7).

2.3. Experiments without adding non-radioactive glucose to the reaction mixture - working in a large excess of tetrazine

The need to obtain a labeled product with a higher specific radioactivity leads to carrying out the following syntheses without adding non-radioactive glucose, that is, in a large excess of tetrazine compared to [^{18}F]FDG, not the other way around. This suggests complete conversion of [^{18}F]FDG present in the reaction system under the optimal conditions for the reaction. Mainly following the synthesis process and product production is done by TLC, therefore it is important to be able to visualize the resulting more saturated colored spots on the plate, in which the radioactivity corresponding to the products is registered. This is the main reason for developing the procedure in excess of tetrazine over the fluorinating reagent. In order to establish the optimal reaction conditions for the formation of an oxime or hydrazone bond between [^{18}F]FDG and a bifunctional tetrazine, various methods taken from the literature are tried:

- Methodology proposed by Wuest et al (2009): A mixture of methanol and water in a ratio of 1:1 is used as the reaction medium, of which 0.5 mL is transferred to the reaction vessel. 0.2 mL of [^{18}F]FDG and 0.1 mL of the corresponding tetrazine solution are simultaneously added to it. Tz1 is used. The reaction mixture was heated at 80°C for 30 minutes.
- A methodology taken from Wuest (2008) was tested, in which a mixture of physiological saline and ethanol in a ratio of 1:5 was used as the reaction medium. From this mixture, 0.5 mL was taken and equal amounts of [^{18}F]FDG (0.2 mL) and tetrazine solution (0.1 mL) were added to it. All reagents are mixed simultaneously at the starting time. It is

heated at a temperature of 95°C for 10 minutes. In some of the syntheses, heating is 20 minutes at 100°C or 30 minutes at 80°C.

- Methodology presented by Maschauer (2014), in which 0.4% (TFA) and 16% ethanol in physiological solution are used as reaction medium. Pipette 0.5 mL of the thus prepared solution with pH=1.5. The reagents [^{18}F]FDG and R3 are added to it. The reaction mixture was heated at 100°C for 20 minutes.

The conditions of the presented methods are summarized in table 4 (rows 8-10). According to the results of the radio-TLC analysis, no colored spots with radioactivity detected in them were registered. The application of these methods in this case did not lead to successful labeling of bifunctional tetrazine by forming an oxime or hydrazone bond. For this, the methodology described in point 2.2 is again applied, where the presence of a marked product is registered. In this case, however, the experiments were performed without the addition of non-radioactive glucose and with a higher applied radioactivity of [^{18}F]FDG. The aim is to optimize the reaction conditions (temperature, pH, concentration and type of catalyst) to obtain a labeled product with high RCY and good radiochemical purity (RCP). The methodology used in the following experiments is as follows: to 0.5 mL of a solution of one of the catalysts (K1, K2, K3, K4), acidified with acetic acid to a given pH, 0.2 mL [^{18}F]FDG is added with activity in the range 0.15-0.80 mCi (5.55 – 29.6 MBq). Heat at the appropriate temperature for 10-15 minutes, then add 0.1 mL of a solution (in acetonitrile) of one of the tetrazines. Reheat for 15 minutes. With this method of mixing the reactants and with these amounts, the type of catalyst, the type of tetrazine, the pH of the medium, as well as the reaction temperature change.

The solution of the buffered catalyst is also a medium for carrying out the reaction, which is why it is used in a huge excess compared to the other reaction components. This is a condition for successful radiolabeling with [^{18}F]FDG by forming an oxime or hydrazone bond. When carrying out the syntheses according to the second strategy without the addition of non-radioactive glucose, the concentration of the starting solution of tetrazine is between 15 and 25 μM. It is run in an excess of tetrazine over [^{18}F]FDG of the order of 10^3-10^4 times.

It was established that the optimal conditions for the synthesis are the following: presence of catalyst K2 or K3 in a concentration of 0.3-0.5 M; pH 4-4.3; T 70-80ºC; stepwise mixing of reagents and excess of tetrazine over [^{18}F]FDG. Ethyl acetate was used as the eluent for TLC in these experiments. Radiolabeling of bifunctional compounds and their confirmation includes the following steps: activation of [^{18}F]FDG and opening of the glucopyranose ring (1); addition of the tetrazine solution and its modification (2); at the end of the synthesis, taking a sample for TLC (3), subsequent elution with ethyl acetate (4); scanning the plate, obtaining

a radio-TLC chromatogram (5) and processing the result (6); upon successful synthesis, additional HPLC or radio-HPLC is sometimes performed, including sample preparation (7) and analysis (8). In the cases of conducting radio-HPLC immediately after conducting radio-TLC, the resulting HPLC chromatogram appears at the 80th minute after mixing the buffered solution of the catalyst with [^{18}F]FDG. The relatively short half-life of ^{18}F and the use of low radioactivity mean that the successful progress of the reaction to form an oxime or hydrazone is primarily monitored by radio-TLC. The time steps of the radiolabeling are presented in Figure 5. Under these reaction conditions and this methodology, in addition to Tz1, the other bifunctional tetrazines (Tz2, R1, R2 and R3) are also modified.

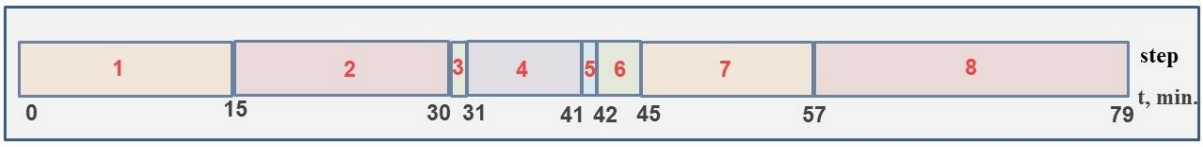


Figure 5 – Time steps of radiolabeling and product confirmation

When labeling R1 and R2, BOC-protected tetrazines are used, requiring an additional deprotection step. The removal of BOC protection is realized by carbamate hydrolysis under acidic conditions. The starting material is dissolved in water or an organic solvent such as toluene, dichloromethane or ethyl acetate. HCl or TFA can be used to acidify the medium. In this case, the following solutions were prepared: 25% TFA/DCM and 25% TFA/ethyl acetate. A small amount of the BOC-protected tetrazines was soaked in 0.60 mL of 25% TFA/ethyl acetate. Stir at room temperature for 10-12 hours. Evaporation of the solvent followed, after which the thus obtained tetrazines were dissolved in acetonitrile and modified according to the established procedure.

When labeling either of the symmetrical tetrazines (R2 and R3), it is possible to obtain two products (mono- and disubstituted). The possibility of double labeling results in a product with a higher specific activity. After column purification, the disubstituted product is separated from the unreacted tetrazine and the monosubstituted product.

Table 4. Summary of reaction conditions

No	Tz	T, °C	t, min	pH	A, mCi	Reaction medium	Presence of catalyst	Mixing method	Excess glucose	Excess Tz	TLC eluent	Result	Reaction in progress
1	Tz1	40	15	4.8	0.05-0.10	sodium acetate buffer	yes – K1	Simultaneous mixing	No	Yes	DCM	colored spots without activity	No
2	Tz1	60 40	10 20	4.8	0.10	sodium acetate buffer	yes – K1	Mix in stages	No	Yes	DCM	colored spots without activity	No
3	Tz1	70	15 20	0.6	0.35	aquatic environment	no	Mix in stages	No	Yes	DCM	colored spots without activity	No
4	Tz1	80	20 15	4.8	0.20	sodium acetate buffer	yes – K1	Mix in stages	yes (+glucose)	No	DCM	colored spots without activity	No
5	Tz1	80	20 15	6.2	0.20	HEPES buffer	yes – K2	Mix in stages	yes (+glucose)	No	DCM	colored spots without activity	No
6	Tz1	75	10 15	5.4	0.25	sodium acetate buffer	yes – K2	Mix in stages	yes (+glucose)	No	DCM	colored spots on the start with activity	Yes
7	Tz1	75	10 15	7.7	0.25	PBS	yes – K2	Mix in stages	yes (+glucose)	No	DCM	colored spots without activity	No
8	Tz1	80	30		0.12	CH_3OH/H_2O 1:1	no	Simultaneous mixing	No	Yes	DCM	colored spots without activity	No
9	Tz1	95	20		0.10	saline /C_2H_5OH 1:5	no	Simultaneous mixing	No	Yes	Ethyl acetate	colored spots without activity	No
10	R3	100	20	1.5	0.2	0,4% TFA и 16% C_2H_5OH in saline	no	Simultaneous mixing	No	Yes	DCM/ CH_3OH 1:1	colored spots without activity	No

11	Tz1	80	15 15	5.4	0.2	sodium acetate buffer	yes – K2	Mix in stages	No	Yes	DCM	colored spots on the start with activity	Yes
12	R3	75	25	4.3	0.2	catalyst solution acidified with TFA	yes – K2	Mix in stages	No	Yes	DCM/ CH_3OH 1:1	shifted color spots with activity	Yes
13	It varies	75	15 15	4- 4.3	0.15- 0.80	catalyst solution acidified with CH_3COOH	yes – It varies	Mix in stages	No	Yes	Ethyl acetate	shifted color spots with activity	Yes

RESULTS AND DISCUSSION

I. Modification of bifunctional molecules with [^{18}F]FDG

1. Modification of Tz1

Until the optimal reaction conditions are established, the experiments are carried out with Tz1. In the initial stages of the work, the syntheses are carried out in an excess of glucose, which is added together with [^{18}F]FDG to the reaction system. At a later stage, the opposite strategy is preferred, namely to use a large excess of tetrazine over [^{18}F]FDG at a molar ratio of approximately 10^4:1. The production of radiolabeled product was established by radio-TLC and confirmed by HPLC analysis. Successful modification of Tz1 was observed under the following reaction conditions: pH between 4 and 4.5; temperature between 40 and 80°C; in the presence of a catalyst (aniline or its derivative); stepwise mixing of the reagents; reaction time between 20 and 30 minutes. Tz1 was modified in the presence of one of the four catalysts used (K1, K2, K3, K4). Figure 6 shows the reaction for the preparation of Pr1 in the presence of K2, with ShB2 being obtained in the first step. Upon addition of tetrazine Tz1, the resulting Schiff base is converted to the oxime product (Pr1). Analogously, the reaction proceeds in the presence of the other catalysts (K1, K3 and K4).

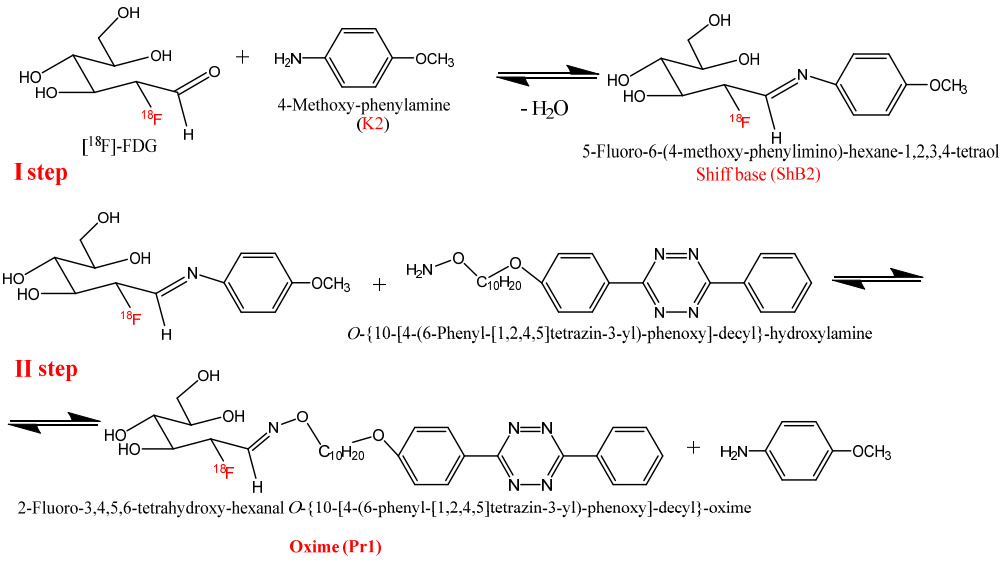


Figure 6 – Reaction to obtain Pr1 in the presence of K2

The successful progress of the synthesis is determined by radio-TLC, which is a rapid, simple and inexpensive method of analysis available in the clinical laboratory. Samples of starting tetrazine Tz1 and reaction mixture in preparation of Pr1 were dripped onto the plate in parallel. Two pink spots near the front, corresponding to baseline and unreacted Tz1, are expected in cases where a large excess of tetrazine over [^{18}F]FDG is run. But since the catalyst also moves to the front of the plate (close Rf values to the Rf of the starting tetrazines) and is

used in a significantly higher concentration than the other reaction components, it is possible to overlap the spots and only orange- the brown color due to the catalyst. Elution was performed with $CH_3CO_2C_2H_5$. Figure 7 shows the TLC chromatogram of the reaction components after Tz1 modification, with s and f denoting the start and front of elution, respectively. The distribution of the spots is as follows: 1 is starting tetrazine, and 5 – the appearance of unreacted tetrazine from the reaction mixture is expected; 2 is the unmodified [^{18}F]FDG corresponding to a radioactive peak starting at the beginning of the radiochromatogram (Figures 8 and 9); 3 (pale pink spot in which radioactivity was detected) is the product obtained, to which corresponds a new radioactive peak shifted relative to the start (Figure 8); 4 – the used catalyst.

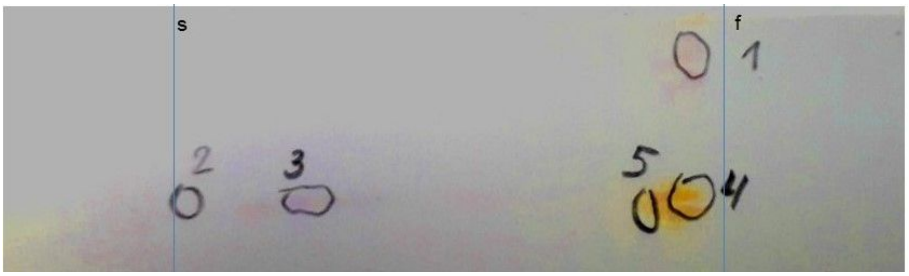


Figure 7 - TLC analysis of modified Tz1

After elution, the plates were scanned using a radio-TLC scanner and based on the obtained radio-TLC chromatogram, the approximate RCY 90±5% was calculated and the Rf of the modified bifunctional tetrazine Tz1 was determined (Rf=0.28). Figure 8 shows a radiochromatogram of a product obtained under optimal labeling conditions where the [^{18}F]FDG present in the system undergoes almost complete conversion.

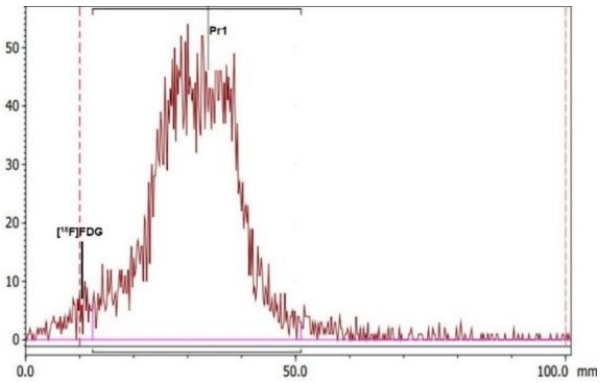


Figure 8 – Radio-TLC chromatogram of modified Tz1: Rf=0.28;RCY=92.4%

A radio-TLC chromatogram of the starting unmodified [^{18}F]FDG with Rf = 0.06 is also presented for comparison in Figure 9.

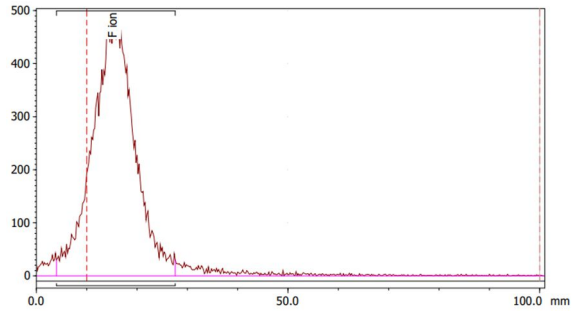


Figure 9 - Radio-TLC chromatography of unlabeled [^{18}F]FDG; Rf=0.06

HPLC analysis was performed to confirm the results obtained from the TLC chromatograms. A standard HPLC system was used in gradient mode (C18, eluent water/acetonitrile 95:5→0:100; 1 mL min^{-1}, 20 min). The chromatogram presented in Figure 10 was obtained after a synthesis carried out in a large excess of glucose over tetrazine. This allows the resulting labeled product to be detected as a new peak by the DAD detector. Peaks of unreacted tetrazine and catalyst in the reaction mixture were established by preliminary analysis of one-component solutions of the reagents used.

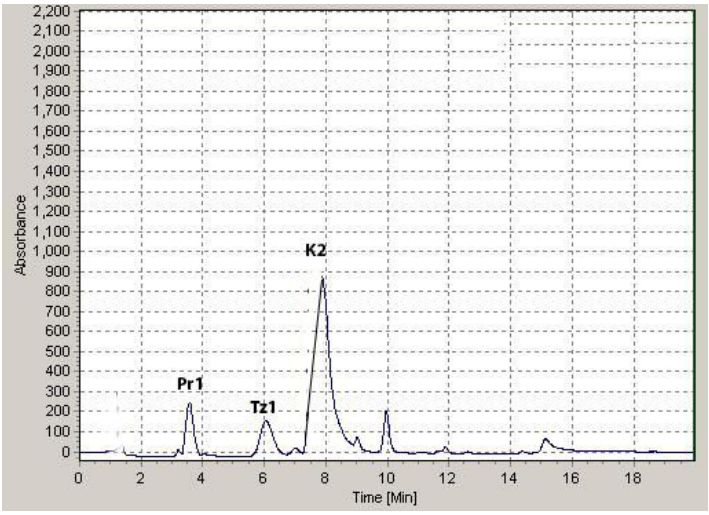


Figure 10 – HPLC chromatogram of modified Tz1 with the following tr: Pr1– 3.5 min; Tz1 – 6.0 min; K2 – 8.0 min.

The retention times of the reaction components are as follows: Tz1 – 6.0 minutes; catalyst (K2) – 8.0 minutes. The retention of the modified tetrazine (Pr1) on the column is expected to be poorer than the parent Tz1. This is explained by the fact that due to the additional hydroxyl groups of the glucopyranose residue, the Pr1 molecule is more polar and more easily passes into the polar mobile phase, with less retention time in the column. As a result of the analysis, a peak appearing with a tr of 3.5 minutes was observed. The overall sequences of the components in the reaction mixture are the same as in TLC, but in reverse order due to the reverse phase of the HPLC column. This confirms the results obtained by radio-TLC.

Table 5 gives the data from successful syntheses performed to modify Tz1 under different reaction conditions, where Ao is the applied radioactivity of [^{18}F]FDG, t is the reaction time. The third column presents the RCY obtained on the basis of the radio-TLC analysis. Under optimal reaction conditions, the RCY obtained was between 85 and 94%.

Table 5 - Data obtained from modifying Tz1

Ao, [mCi]	T, min	RCY (%), TLC
0.48	30	91.72
0.23	30	87.20
0.30	30	49.46
0.34	25	89.88
0.40	30	93.54
0.49	30	93.26
0.21	25	63.66
0.24	25	89.36
0.33	30	93.21
0.21	25	28.93
0.49	40	88.63
0.47	40	84.93
0.49	30	88.67
0.40	30	19.05
0.42	30	15.62
0.45	30	85.51

Ao, [mCi]	T, min	RCY (%), TLC
0.41	30	85.86
0.54	30	25.58
0.56	30	16.01
0.36	30	82.73
0.34	30	78.96
0.36	30	27.22
0.30	30	83.88
0.28	30	91.60
0.25	30	14.51
0.26	30	17.74
0.28	30	22.11
0.17	25	15.65
0.17	25	17.67
0.22	20	21.18
0.22	30	91.12
0.30	20	88.74
0.15	25	89.16

Table 6 presents mean, minimum and maximum RCY values. Table 6 presents mean, minimum and maximum RCY values.

Table 6 – Summary of Tz1 labeling results.

average Ao, [mCi]	0.37
average RCY % TLC	61.6
max RCY %	93.5
min RCY %	14.5

Based on the obtained results, it is proved that the reaction for modifying bifunctional tetrazines by forming an oxime product with [^{18}F]FDG is successful and efficient, takes place under relatively mild conditions and is applicable to the clinical laboratory. After establishing the optimal reaction conditions, the remaining tetrazines used are labeled in a similar way.

2. Modification of Tz2

Radiolabeling of Tz2 is carried out under the established optimal conditions, namely temperature 75°C, pH 4±0.2 and presence of any of the four catalysts used (K1, K2, K3, K4). Analogously, the synthesis is carried out in 2 stages, with the first stage passing through an intermediate product – the corresponding Schiff base (ShB1, ShB2, ShB3 or ShB4) depending on the catalyst used. After addition of tetrazine Tz2 the resulting Schiff base is converted to the hydrazone product (Pr2).

Figure 11 shows the reaction for the preparation of Pr2 in the presence of K3, with ShB3 being obtained in the first step. Analogously, the reaction proceeds in the presence of the remaining catalysts (K1, K2 and K4).

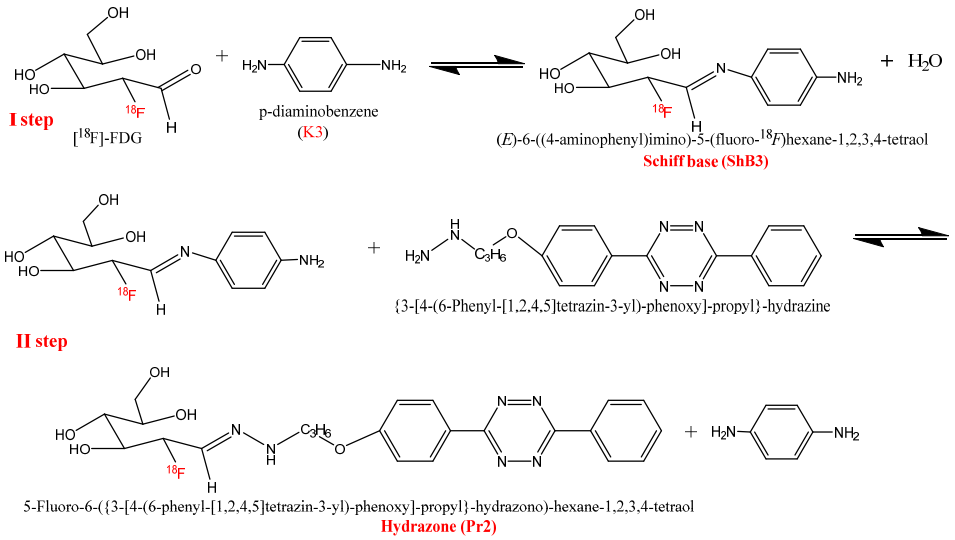


Figure 11 - Reaction to obtain Pr2 in the presence of K3

The successful progress of the synthesis was established by radio-TLC with eluent $CH_3CO_2C_2H_5$. Figure 12 shows a TLC chromatogram of the reaction components after Tz2 modification, with s and f marking the start and front of elution, respectively. The distribution of spots is as follows: 1 – [^{18}F]FDG, 2 – modified Tz2 (with reported activity in it), 3 – unmodified Tz2, 4 – catalyst.

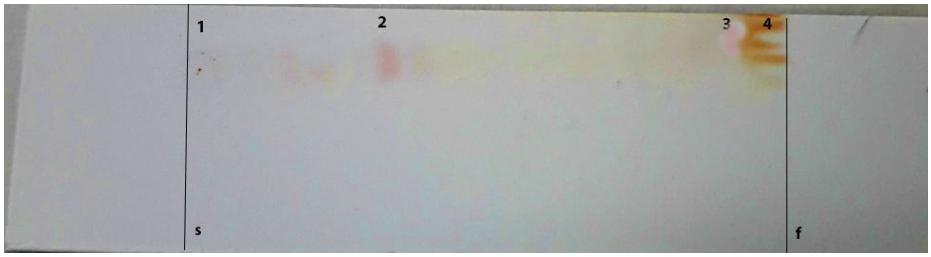


Figure 12 - TLC analysis of modified Tz2

After scanning the plate with a radio-TLC scanner, the chromatogram shown in Figure 13 was obtained. Based on it, the Rf (0.22) of the resulting product Pr2 and the approximate RCY 85% were determined.

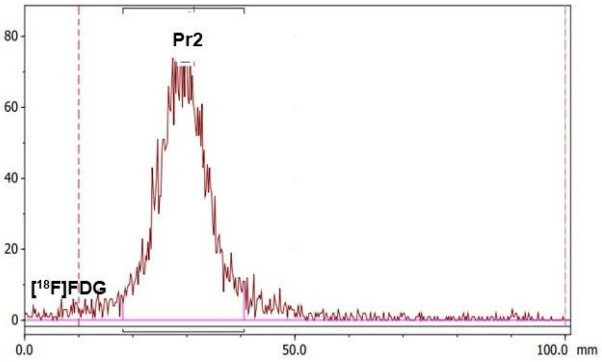


Figure 13 – Radio-TLC chromatogram of modified Tz2:Rf=0.22; RCY=85.9%

To confirm the results obtained from the TLC analysis, a radio-HPLC analysis was also carried out using a radio-HPLC system SCI8100. 0.1% TFA/H_2O (A) and 0.1% TFA/CH_3CN (B) were used as eluting phase under the conditions of the II gradient method described in Table 2. Figure 14 presents HPLC chromatograms obtained from the RAD detector of unmodified [^{18}F]FDG (chromatogram A) and reaction mixture (chromatogram B), respectively.

The RAD detector data confirms the successful reaction. The initial [^{18}F]FDG was recorded as a peak at tr=1.08 min, and upon analysis of the reaction mixture a new radioactive peak appeared at tr=2.47 min corresponding to the labeled product Pr2. A single radioactive peak (time-shifted with respect to that of the radiolabel) was recorded, since one was working in a very large excess of tetrazine relative to [^{18}F]FDG, which under optimal conditions undergoes almost complete conversion to the corresponding radiolabeled product. Moreover, due to the following facts: first, very low radioactivity is used; secondly, the time for sample preparation and analysis with radio-HPLC is longer compared to TLC analysis and thirdly – the relatively short half-life of the radionuclide, are the reason that a second radioactive peak is not registered, even if there is residual unreacted in the system [^{18}F]FDG. After the decay of ^{18}F to ^{18}O, non-radioactive glucose will be present in the reaction mixture. The results of the

radio-HPLC analysis confirmed the TLC results for the successful course of the reaction when modifying the bifunctional tetrazine Tz2 with [^{18}F]FDG.

Figure 14 – Radio-HPLC analysis with Tz2 labeling: A – RAD chromatogram of initial [^{18}F]FDG (tr=1.08 min.); B – RAD – chromatogram of labeled product Pr2 (tr=2.47 min.)

Table 7 summarizes the data from successful syntheses performed to modify Tz2 under different reaction conditions. Table 8 presents mean, minimum and maximum RCY values.

Table 7 - Data obtained from modifying Tz2

Ao, [mCi]	T, min	RCY (%), TLC
0.35	25	88.96
0.33	25	77.50
0.48	30	91.24
0.30	30	90.23
0.34	25	13.50
0.40	30	84.22
0.40	30	19.05
0.33	30	91.43
0.42	30	17.18
0.43	30	13.54

Ao, [mCi]	T, min	RCY (%), TLC
0.37	30	7.82
0.36	30	6.25
0.40	30	20.47
0.40	30	19.02
0.33	25	10.13
0.32	25	49.03
0.32	25	24.94
0.18	30	83.21
0.80	33	87.97
0.32	20	91.98

Table 8 – Summary of Tz2 labeling results

average Ao, [mCi]	0.38
average RCY %, TLC	49.4
max RCY %	91.9
min RCY %	6.3

After establishing which of the used catalysts are the most effective, the labeling of the remaining 3 tetrazines (R1, R2 and R3) is carried out precisely only in their presence (K2 and K3).

3. Modifying R1

Due to the fact that a BOC group-protected bifunctional tetrazine is used, an additional deprotection procedure described above in the text is required prior to [^{18}F]FDG labeling. The reaction scheme of this preliminary step is presented in Figure 15. The BOC-deprotected tetrazine R1 is then dissolved in CH_3CN and the established oxime modification procedure is applied at temperature 70-75°C, pH 4±0.1, presence of catalyst (K2 or K3), stepwise mixing of the reagents and duration of the synthesis 20-25 minutes. Experiments on R1 labeling are carried out only under the already established optimal conditions.

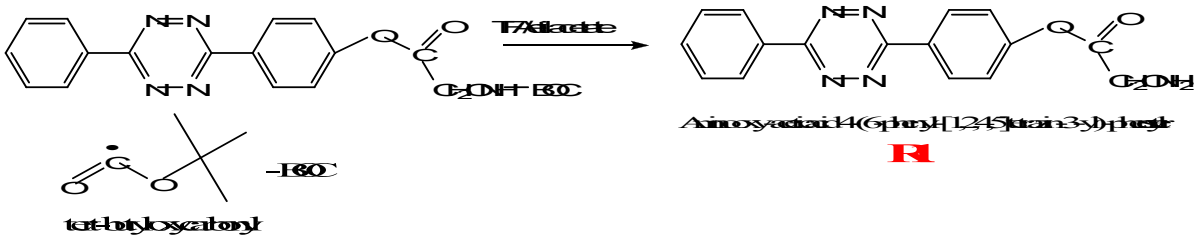


Figure 15 - Preparation of tetrazine R1

Figure 16 shows the final reaction to obtain the oxime product Pr3.

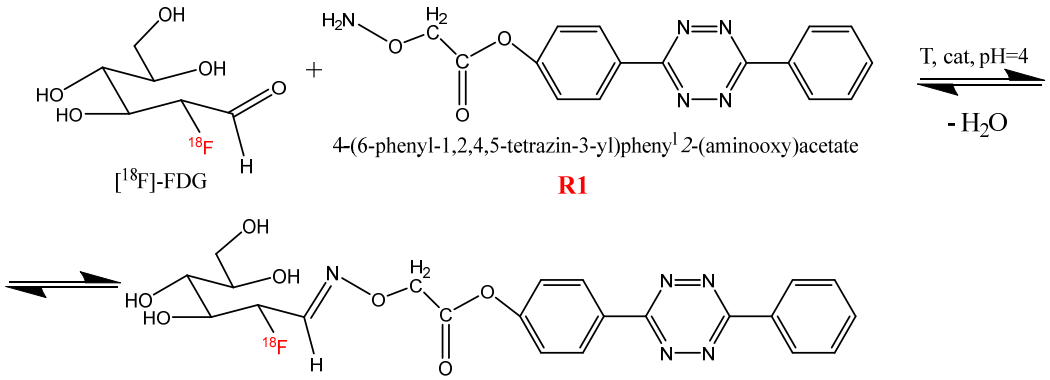


Figure 16 – Summary reaction to obtain Pr3

After TLC analysis and elution of the plate with ethyl acetate, the chromatogram presented in Figure 17 is obtained. The distribution of the spots of the reaction components after the modification of R1 is as follows: 1 is unreacted [^{18}F]FDG, if present in the system, 2 – labeled oxime product, 3 is the starting tetrazine, and 4 is the catalyst used.

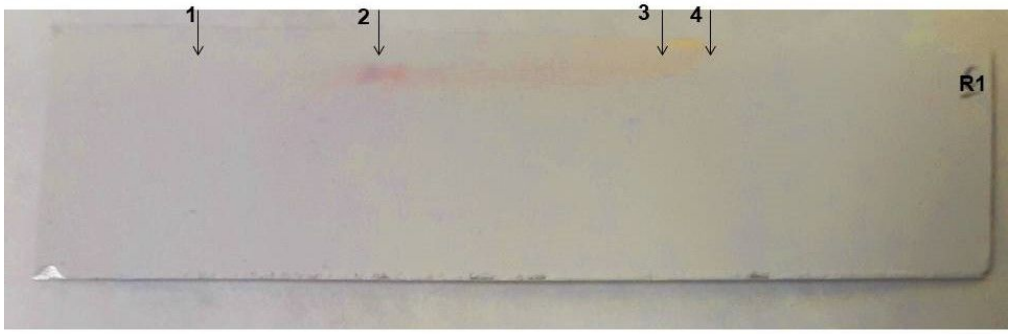


Figure 17 - TLC analysis of modified R1

After scanning the plate, the radio-TLC chromatogram shown in Figure 18 was obtained. Based on it, the Rf (0.40) of the resulting product Pr3 and an approximate RCY of 80±5% were determined.

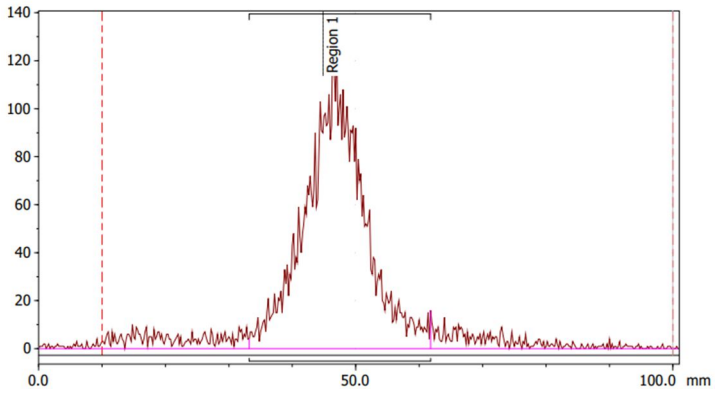


Figure 18 - Radio-TLC analysis of modified R1: Rf=0.40; RCY=85.4%

The resulting product was confirmed by radio-HPLC under the gradient conditions already described. Since the labeling of R1 (after establishing the optimal reaction conditions) is carried out in a large excess of tetrazine relative to [^{18}F]FDG, the concentration of the resulting radiolabeled product in the reaction system is negligible. Therefore, in this case, the obtained products are fully confirmed on the basis of the data from the RAD detector, which has a significantly higher sensitivity. When analyzing the reaction mixture by the RAD detector, a new radioactive peak with tr=2.24 minutes was registered, corresponding to the resulting labeled product Pr3. Figure 19 shows the corresponding RAD detector chromatograms of unmodified [^{18}F]FDG and the resulting product. Based on the results of the radio-HPLC analysis, the successful modification of R1 by forming an oxime bond with [^{18}F]FDG is confirmed.

Figure 19 - Radio-HPLC analysis modified R1: A - RAD - chromatogram of initial [^{18}F]FDG (tr=1.08 min.); B – RAD – chromatogram of labeled product Pr3 (tr=2.24 min.)

4. Modifying R2

Since a BOC-protected tetrazine group is used, an additional deprotection step described above in the text is performed to obtain R2 (represented in Figure 20). The use of symmetrical bifunctional tetrazines, such as R2 and R3, allows for dual labeling where the resulting product has a higher specific activity.

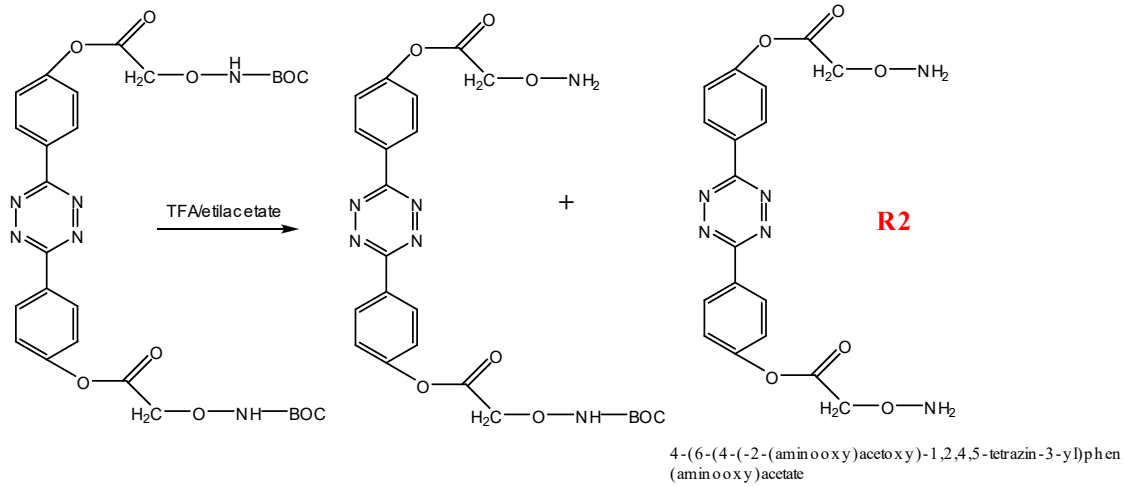


Figure 20 - Preparation of tetrazine R2

Figure 21 shows the final modification reaction of R2. It is possible to obtain two products (monosubstituted and disubstituted) depending on the amount of [^{18}F]FDG in the reaction mixture. When applying [^{18}F]FDG with a higher radioactivity corresponding to a larger molar amount, the disubstituted product Pr5 is more likely to be obtained.

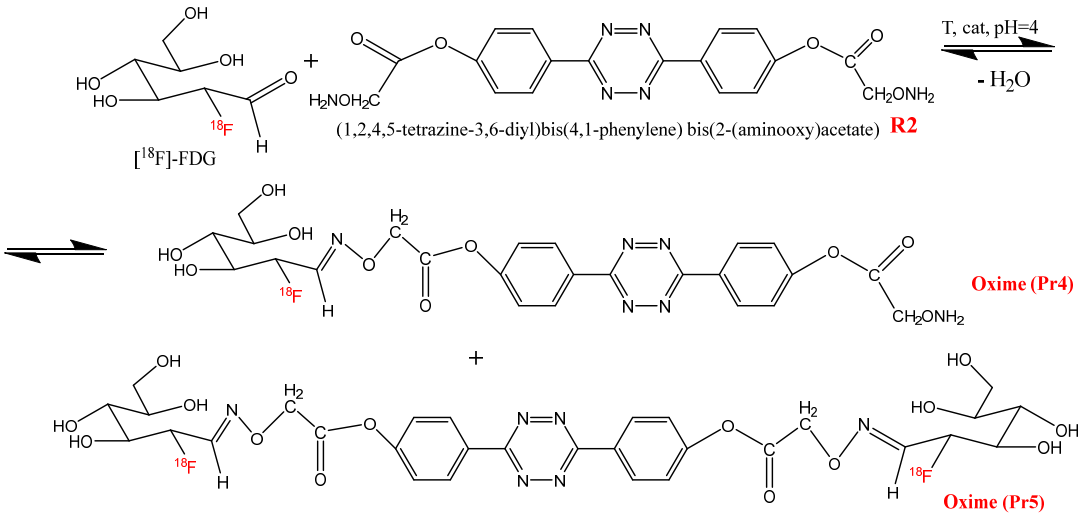


Figure 21 – Generalized reaction to obtain Pr4 and Pr5

Figure 22 shows a TLC chromatogram of the reaction components after modification of R2. The distribution of spots is as follows: 1 is the drip point where unreacted [^{18}F]FDG is retained if present in the system; 2 corresponds to the disubstituted product Pr5; 3 – monosubstituted product Pr4; 4 – unreacted tetrazine R2; 5 – used catalyst (in the case of K2). In cases where both expected products are actually obtained, the disubstituted product will have a lower Rf in comparison to the Rf of the monosubstituted product and is registered as the first pink spot after starting. This is due to the fact that the disubstituted is the carrier of more OH groups, which makes the molecule more polar and it will stick to the plate surface more strongly.

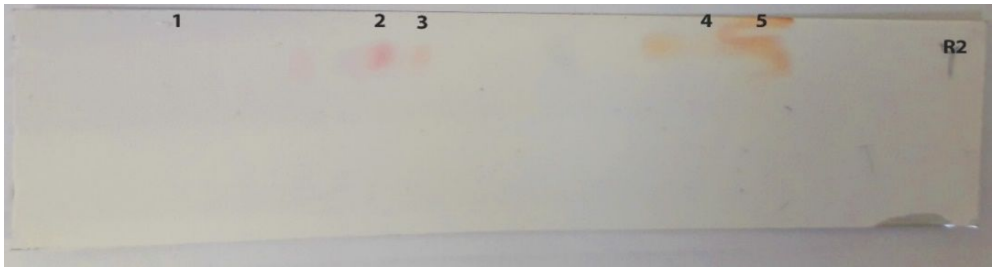


Figure 22 - TLC analysis of modified R2

After scanning the plate, the radio-TLC chromatogram presented in Figure 23 is obtained. Based on it, the Rf of the products obtained and their RCY are determined as follows: the first peak obtained with Rf=0.25 corresponds to the disubstituted product Pr5 with RCY 81.1%, and the second with Rf=0.47 – of the monosubstituted product Pr4 with RCY 18.9%.

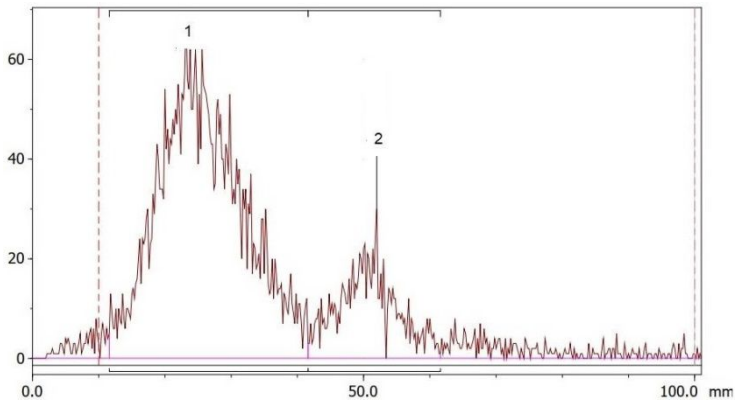


Figure 23 - Radio-TLC analysis of modified R2:Rf1=0.25, RCY=81.1%; Rf2=0.47, 18.9%

At a later stage, radio-HPLC analysis was performed to confirm the successful reaction. Figure 24 shows the obtained chromatograms from the RAD detector. The appearance of a radioactive peak (with tr=2.61 minutes) offset in time from the starting [^{18}F]FDG peak corresponding to one of the expected labeled products was observed. The second expected product was obtained at a lower concentration and due to the decay of ^{18}F to ^{18}O no second radioactive product peak was recorded in the radio-HPLC chromatogram of the reaction mixture.

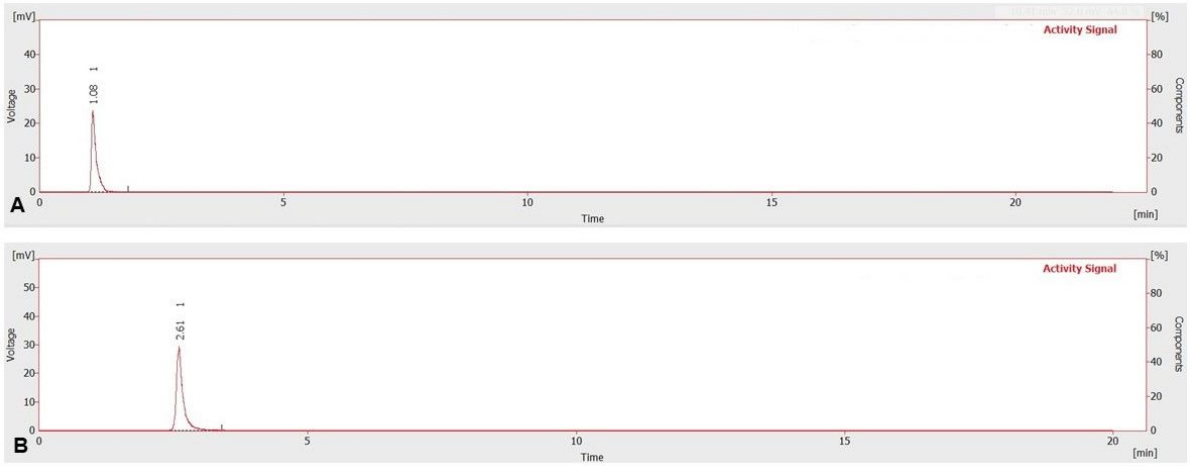


Figure 24 – Radio-HPLC analysis with R2 labeling: A – RAD – chromatogram of initial [^{18}F]FDG (tr=1.08 min.); B – RAD – chromatogram of labeled product Pr5 (tr=2.61 min.)

5. Modifying R3

R3 is modified under the conditions found to be optimal for the reaction. Since it is symmetrical, it is possible to obtain two radiolabelled products. Figure 25 shows the final modification reaction of R3, without indicating the intermediates it passes through.

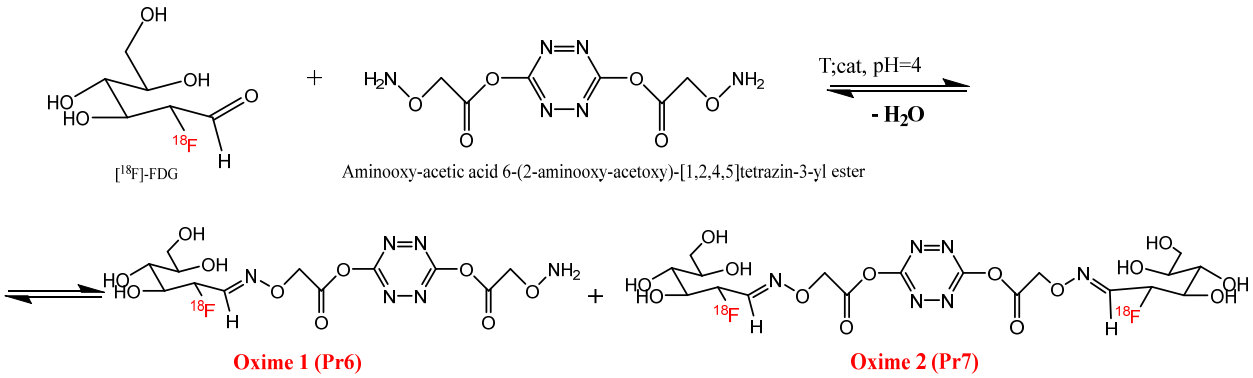


Figure 25 – General reaction to obtain Pr6 and Pr7

Figure 26 shows a chromatogram with the distribution of the reaction components after successful labeling of R3. Chromatogram a is before elution and the spot marked 0 corresponds to the starting reaction mixture. Chromatogram b is after elution with ethyl acetate and the distribution of components is as follows: 1 corresponds to unreacted [^{18}F]FDG, 2 and 3 correspond to the resulting oxime products Pr6 and Pr7; 4 – unreacted tetrazine and 5 – catalyst.

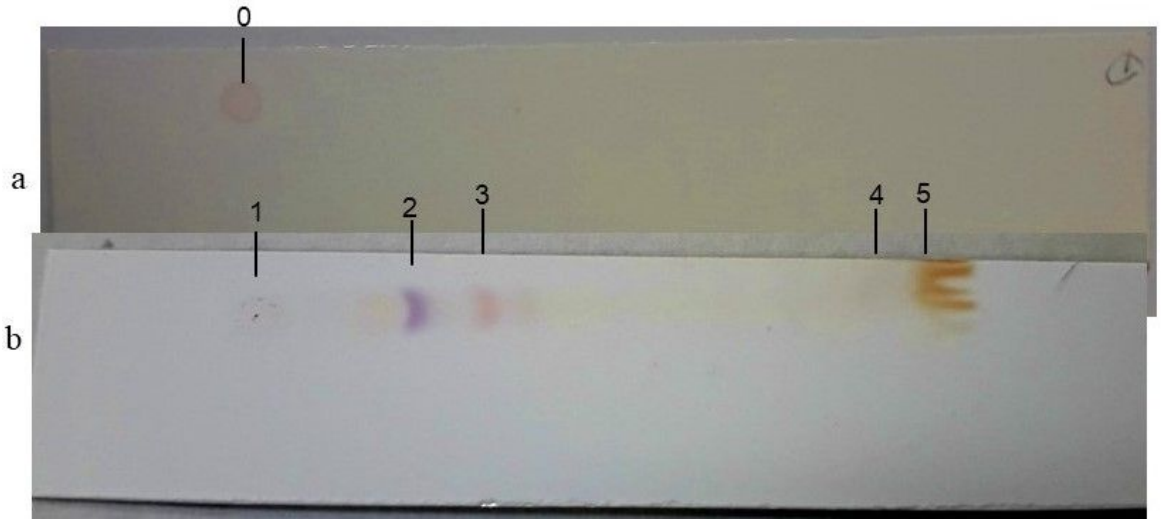


Figure 26 - TLC analysis of the preparation of Pr6 and Pr7: a) chromatogram before elution; b) chromatogram after elution with ethyl acetate

After scanning the plate using a radio-TLC scanner, the following radio-chromatogram is obtained, shown in Figure 27. Two radioactive peaks with Rf =0.30 and Rf =0.42 are obtained, corresponding to the two expected oxime products Pr6 and Pr7 with a total radiochemical yield 87.6%.

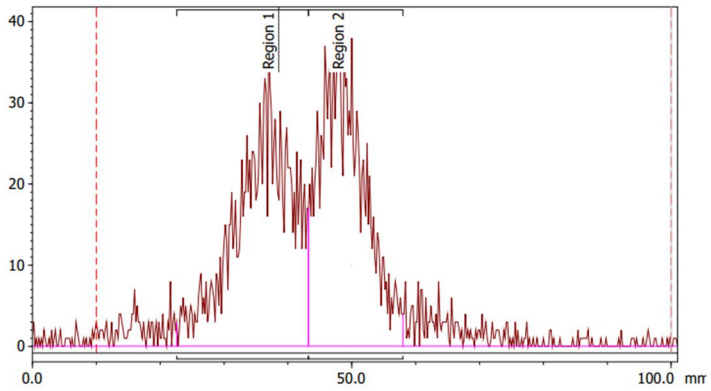


Figure 27 - Radio-TLC chromatogram of modified R3: Rf=0.30 corresponds to Pr6 with RCY=44.2%; Rf=0.42 corresponds to Pr7 with RCY=43.4%

At a later stage, the successful course of the reaction was confirmed by HPLC in gradient mode (C18, eluent water acetonitrile 95:5→0:100; 1 $mLmin^{-1}$, 20 min). The attached HPLC chromatogram (in Figure 28) is of a reaction mixture obtained in a synthesis employing the first labeling strategy, ie. working in a large excess of glucose over tetrazine. This allows detection of the resulting labeled product with a DAD detector. The analysis was carried out using an HPLC system (Varian ProStar). The resulting peaks 1 and 3 are at R3 and K3, respectively, established by preliminary analysis of one-component solutions. The appearance of a new peak (2) corresponding to one of the expected products was observed. Obtaining the disubstituted product is more likely.

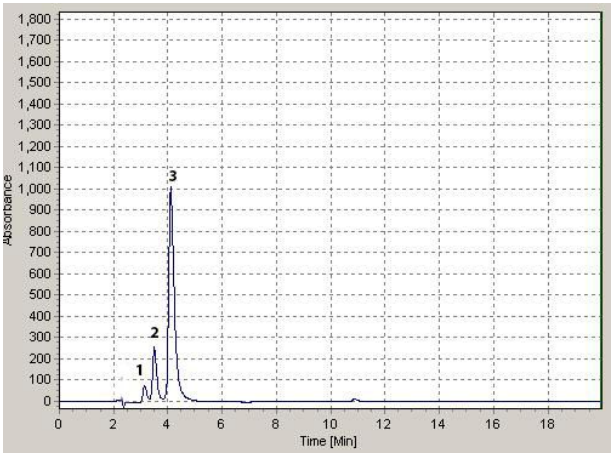


Figure 28 - HPLC analysis of modified R3. The peak distribution is as follows: –1 – R3; 2 – marked product; 3 – K3

The retention times of the components of the reaction mixture are as follows: R3 – 3.1 min; modified product – 3.5 min.; K3 – 4.1 min.

Table 9 presents data from successful R3 labeling experiments, and Table 10 presents mean, minimum, and maximum RCY values.

Table 9 - Data obtained from modifying R3

Ao, [mCi]	T, min	RCY (%), TLC
0.48	30	93.87
0.30	30	84.79
0.30	30	93.05
0.17	30	91.56
0.33	25	92.10
0.39	30	94.93
0.25	25	12.03
0.23	25	46.52
0.25	25	81.60
0.30	30	78.50
0.20	25	17.95

Ao, [mCi]	T, min	RCY (%), TLC
0.24	25	52.11
0.31	25	80.00
0.30	30	82.04
0.46	30	6.10
0.42	30	21.33
0.45	30	10.17
0.40	30	70.83
0.49	30	18.12
0.18	30	83.21
0.22	30	87.37
0.79	30	87.88

Table 10 - Summary of R3 modification results

average Ao, [mCi]	0.34
average RCY %, TLC	63.00
max RCY %	94.93
min RCY %	6.10

On the basis of the obtained results, the significance of the developed methodology can be highlighted, with the help of which bifunctional derivatives of tetrazine are successfully modified with a good radiochemical yield entirely in clinical conditions. This methodology may be useful as a future strategy to modify bioactive and organ-specific molecules by forming an oxime or hydrazone bond with [^{18}F]FDG.

6. IEDDA reactions between [^{18}F]FDG-modified tetrazines and TCO

After developing a methodology to modify bifunctional tetrazines by forming an oxime or hydrazone bond with [^{18}F]FDG, the resulting labeled products can be used for future click

IEDDA reactions with TCO. For this purpose, it is necessary to carry out the syntheses in a larger amount, which would allow isolation and purification of the labeled tetrazine before reaction with TCO. Figure 29 presents a general reaction scheme between [^{18}F]FDG-modified tetrazine and bifunctional TCO.

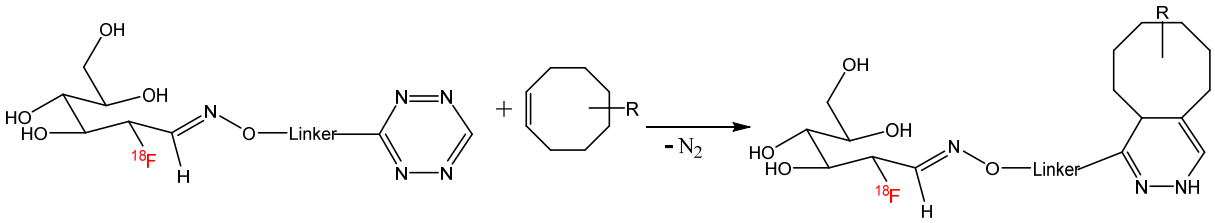


Figure 29 – General scheme of IEDDA reactions between [^{18}F]FDG modified tetrazine and TCO

The possible combinations that can be made between the trans-cyclooctene used and the modified tetrazines are presented in Figure 30.

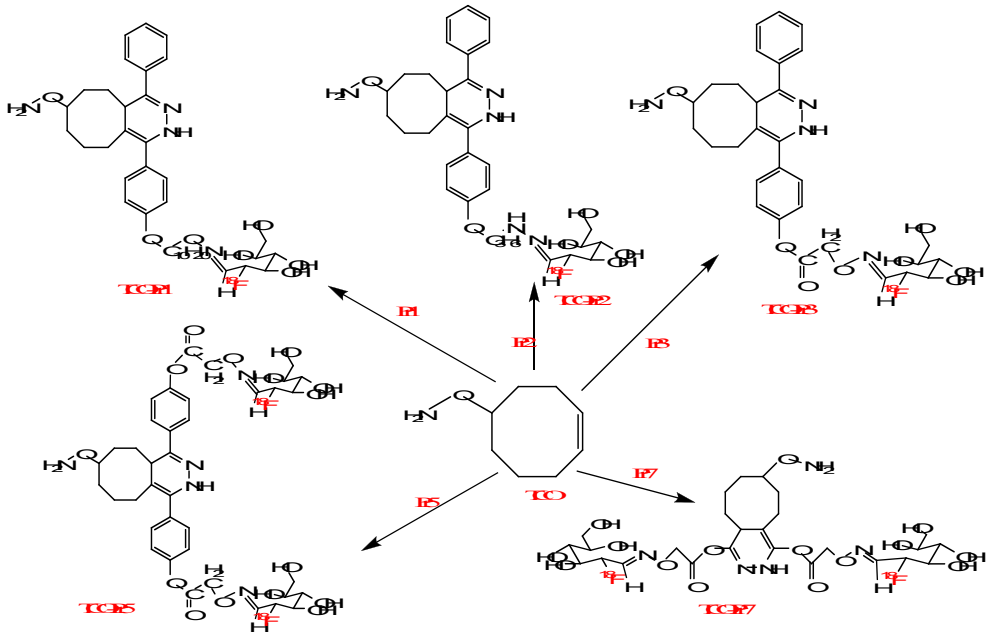


Figure 30 - Reaction scheme of possible combinations of iTCO and modified tetrazines

As known from literature data, IEDDA reactions between tetrazine and TCO are easy, fast, practically irreversible and proceed with the release of a nitrogen molecule. Most often, they take place at equivalent amounts of the reactants, under physiological conditions and room temperature, without the presence of a catalyst.

As part of the implementation of a project with contract No. KP-06-H29/4 (Scientific Research Fund of the Ministry of Education and Science), the kinetic parameters of "cold click" reactions with some of the most pharmacologically relevant TCO derivatives are investigated and Tz. The corresponding rate constants are determined experimentally. Studying the kinetics

of a cold (non-radioactive) click reaction is an important step in the development of a radiolabeling procedure using IEDDA. From the obtained results it is clear about the speed of the reaction and the applicability of the procedure for the purposes of nuclear medicine. The kinetics of the reactions were measured with a stopped-flow absorption spectrophotometer by monitoring the change of the characteristic tetrazine absorption bands at 534 nm or 270 nm as a function of time. Measurements were performed in C_2H_5OH at room temperature. The durations of the reactions are of the order of several seconds and the recording of the absorption spectra is done with a frequency of 1 kHz. The experimentally determined rate constants of the bimolecular "click" reaction in ethanol are of the same order in the range of 150-300 $M^{-1}.s^{-1}$, which ensures that the reaction takes place within seconds. This second rate is quite sufficient for the purposes of the pre-target radio tagging strategy.

II. Results obtained by varying the experimental conditions

The radio-TLC method successfully confirmed the reaction between bifunctional tetrazine and [^{18}F]FDG. This technique is used to follow the course of the reaction and to evaluate the effect of various factors (temperature, pH, type of catalyst, reaction time) on the resulting radiochemical yield. Based on the obtained results, the optimal reaction conditions are determined, under which the maximum yield is obtained.

1. Effect of temperature

Syntheses were carried out according to the established procedure with Tz1, Tz2 and R3 under the following reaction conditions: pH (4.15), stepwise mixing of the reactants, in the presence of K2, a total reaction time of 30 min and varying the temperature to evaluate the the effect on RCY. Chromatograms of syntheses at different temperature are presented below and the approximate RCY is determined based on them. With Tz1, syntheses were carried out at 30, 50 and 70°C and the obtained radiochemical yields were as follows: 17.9%; 52.1%; 87.2%. Best results and almost complete conversion are obtained at a temperature of 70°C (Figure 31).

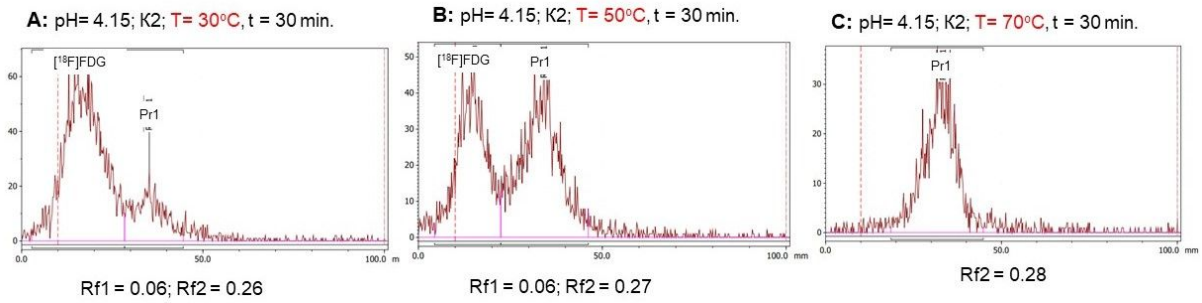


Figure 31 – Modification of Tz1 when the temperature varies: A – T=30ºC, RCY=17.9%; B – T=50ºC, RCY=52.1%; C – T=70ºC, RCY=87.2%

Figure 32 presents the chromatograms of syntheses with Tz2 at temperatures of 30, 60 and 75ºC and the corresponding yields obtained are: 13.5%; 63.6%; 86.9%. At a temperature of 60°C, a peak with Rf = 0.13 corresponding to ShB2 was observed. In this case, the free [^{18}F]FDG peak is not recorded, but the peak of the resulting Schiff base with K2, which undergoes complete conversion to the desired hydrazone product Pr2. For the modification of Tz2, the optimal temperature turns out to be 75°C.

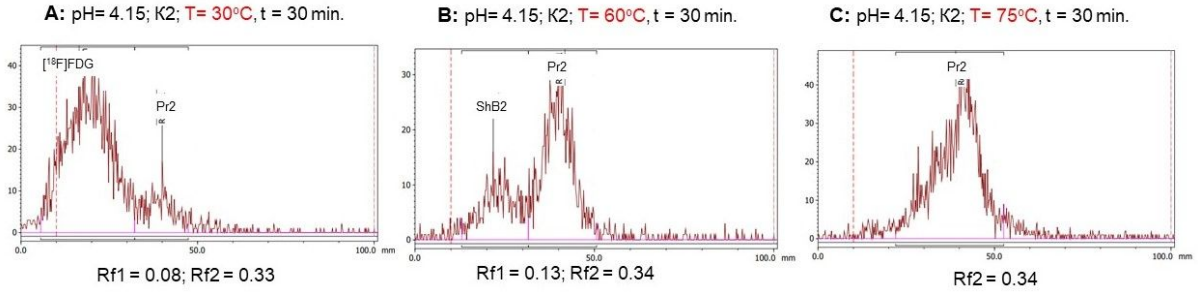


Figure 32 – Modification of Tz2 when the temperature varies: A – T=30ºC, RCY=13.5%; B – T =60ºC, RCY=63.6%; C – T=75ºC, RCY=86.9%

Figure 33 presents the chromatograms of syntheses with R3, performed at temperatures of 50 and 70°C, and the total yields obtained for the two marked products are as follows: 71% and 87.6%. At a temperature of 70°C, almost complete conversion is observed.

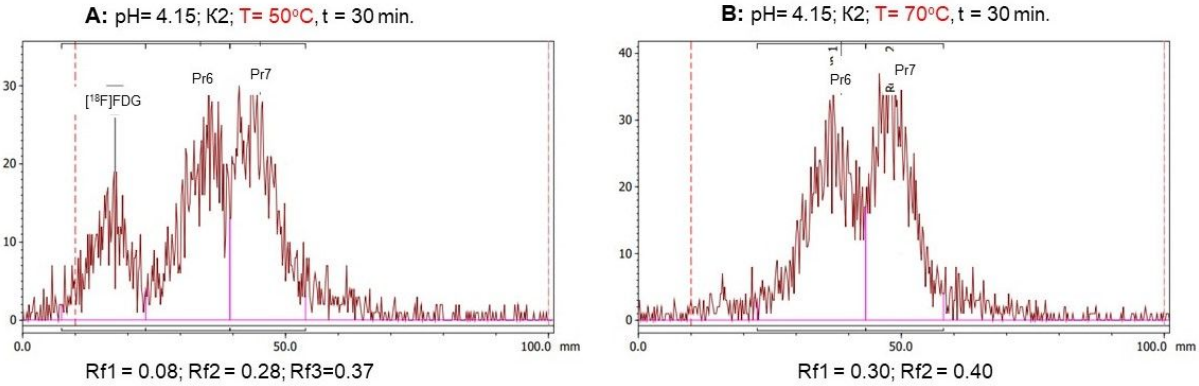


Figure 33 – Modification of R3 when the temperature varies: A – T=50ºC, RCY (Pr6)= 34.2%; RCY (Pr7)=36.8%; B – T =70ºC, RCY (Pr6)= 44.2%; RCY (Pr7)=43.4%

Table 11 summarizes the data obtained by monitoring the effect of temperature on the RCY of modified tetrazines Tz1, Tz2 and R3.

Table 11 - Dependence of radiochemical yield on temperature

T, °C	22	30	50	60	70	75	tetrazine
RCY,%	0	17.9	52.1	65.2	87.2	89.0	Tz1
	0	13.5	45.2	63.7	75.1	86.9	Tz2
	0	20.5	70.9	75.6	87.7	91.3	R3

Based on the data, it can be concluded that the optimal temperature for carrying out oxime or hydrazone formation reactions with [^{18}F]FDG is 70-75°C, at which almost complete conversion is observed.

2. Influence of the pH of the medium

Syntheses are carried out according to the established procedure with Tz1 under the following reaction conditions: temperature 70-75°C (optimal for the reaction), step-by-step mixing of the reagents, in the presence of K2, total reaction time 30 minutes and variation of pH in the range 1.5-8.5 to estimate the effect on RCY. Experiments done while varying the pH lead to the following results. At pH below 3, no second product peak was observed, only that of [^{18}F]FDG. Oxime and hydrazone formation reactions are reversible and at pH below 3, hydrolysis is favored, with the equilibrium being pulled toward the starting substances. At pH above 7.5, no labeled product was again observed due to difficulty in activating [^{18}F]FDG and opening the glucopyranose ring. At pH 3.3, a second peak was observed due to the product obtained with Rf=0.34 and RCY 11.3%. At pH 5.2, a product (Rf=0.32) and better RCY was obtained, compared to that obtained at pH 3.3 – 20%. At pH 4, a single peak corresponding to a modified tetrazine with Rf=0.23 with RCY above 85% and absence of the radiolabel peak was observed, suggesting almost complete conversion of [^{18}F]FDG present in the reaction system. Figure 34 shows the corresponding radio-TLC chromatograms.

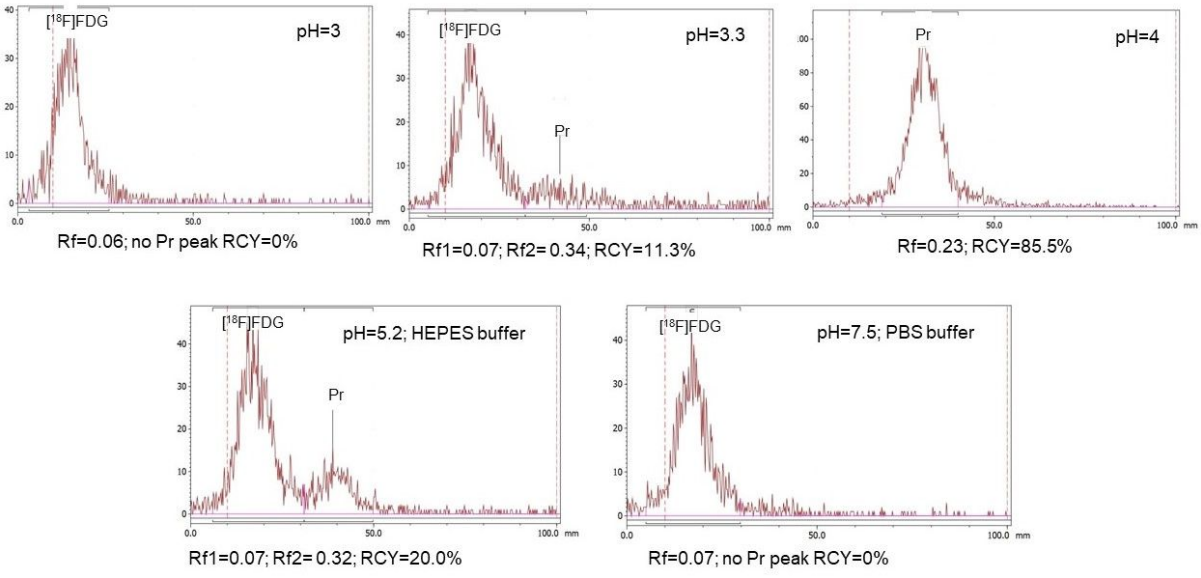


Figure 34 – Modification of Tz1 at different pH of the medium: 1 – pH 3, RCY=0%; 2 – pH 3.3; RCY=11.3%; 3 – pH 4, RCY=85.5%; 4 – pH 5.2; RCY=20%; 5 – pH 7.5; RCY=0%

Table 12 gives the results obtained by monitoring the effect of medium pH on RCY when modifying tetrazine Tz1 for the established optimum temperature of 75°C and in the presence of K2. Figure 35 graphically presents the dependence of RCY on the pH of the medium.

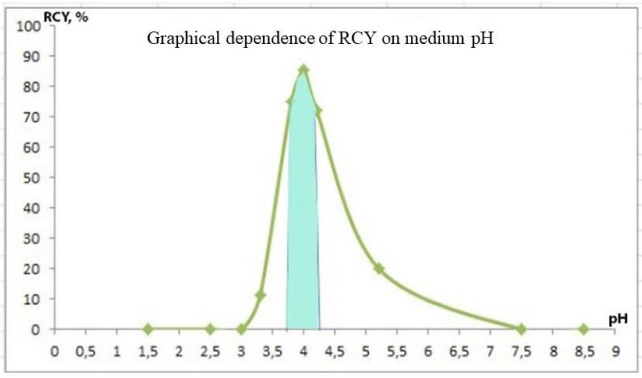


Figure 35 - Graphical dependence of RCY on medium pH

Table 12 - Dependence of the radiochemical yield on the pH of the medium

pH	1.5	2.5	3.0	3.3	3.8	4.0	4.2	5.2	7.5	8.5
RCY, %	No signal	No signal	No signal	11.3	75.0	85.5	72.4	20.0	No signal	No signal

Based on the data, it can be concluded that the optimal pH for conducting oxime or hydrazone formation reactions between bifunctional tetrazines and [^{18}F]FDG is between 3.8 and 4.2, where almost complete conversion is observed and obtaining a product with a good RCY.

3. Influence of the catalyst - its type and concentration

Syntheses were carried out according to the established procedure with Tz1 and Tz2 under the following reaction conditions: pH 4.15, temperature 70-75°C, stepwise mixing of the reactants, total reaction time 25-30 minutes and varying the catalyst and its concentration to evaluate of the effect on RCY.

Syntheses were carried out with Tz1 under the same reaction conditions and varying the type of catalyst at a similar concentration (0.30±0.03 M). For comparison, Figure 36 shows radio TLC chromatograms using K1, K2 and K3. In the three cases, the appearance of two peaks is observed, the first corresponding to the Schiff base, and the second to the final product obtained. The product obtained when using a different catalyst is with RCY as follows: 19.6% with K1; 39% with K2; 56.2% with K3. When using aniline, the RCY is below 20%, which is

an indicator of an insufficiently effective catalyst at the given concentration, therefore it is applied only in a small part of the experiments.

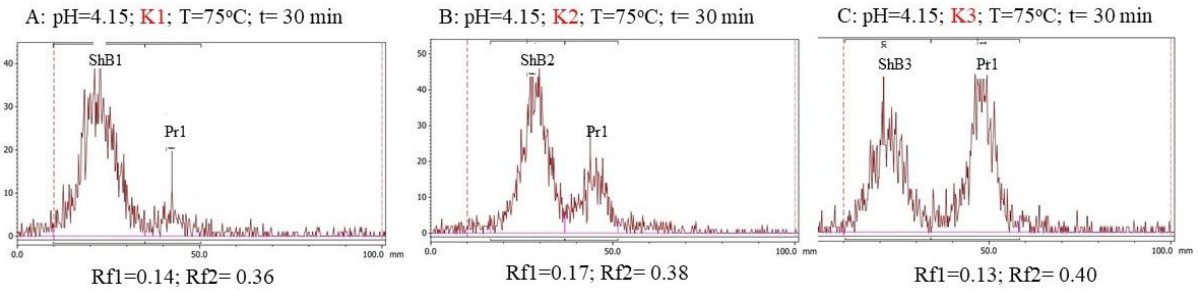


Figure 36 – Modification of Tz1 when varying the type of catalyst: A – with K1, RCY=19.6% (Pr1); B – with K2, RCY=39% (Pr1); C – with K3, RCY=56.2% (Pr1)

The use of K2 and K3 as catalysts is preferred in the development of a methodology for modifying bifunctional tetrazines. K4 is an effective catalyst, which itself has a slightly acidic pH=3.9 due to the available carboxyl group in its structure, which is why additional acidification with acetic acid is not required. In its presence, Tz1 and Tz2 are marked. But it is preferred not to be used in the labeling of the other tetrazines due to the fact that its solution is gray-black in color, which affects the color of the reaction mixture. Furthermore, in TLC analysis with ethyl acetate eluent, the resulting Schiff base (ShB4) was retained at the start, similar to unreacted [^{18}F]FDG.

A series of solutions of the catalysts with different concentrations were prepared, and the data are presented in Table 13.

Table 13 – Concentrations of the working solutions of the catalysts

Catalyst	Molar concentration				
K2	0.47 M	0.23 M	0.12 M	0.06 M	0.03 M
K3	0.45 M	0.23 M	0.11 M	0.06 M	0.03 M
K4	0.41 M	0.20 M	0.10 M	0.05 M	0.02 M

Syntheses were carried out at medium pH 4.1 and varying the concentration of the catalysts (K2 and K3) in order to evaluate their efficiency in the formation of the oxime bond. Figure 37 shows radio-TLC chromatograms of modification of Tz1 in the presence of K3 at different concentrations. The resulting product has an RCY as follows: 13.5% at CM= 0.23 M; 8.4% at CM= 0.11 M; 7.8% at CM= 0.06 M. Experiments are carried out to modify the same tetrazine, but in the presence of K2. The data obtained for both catalysts are summarized in Table 14. The tendency of RCY to decrease significantly with decreasing catalyst concentration is observed.

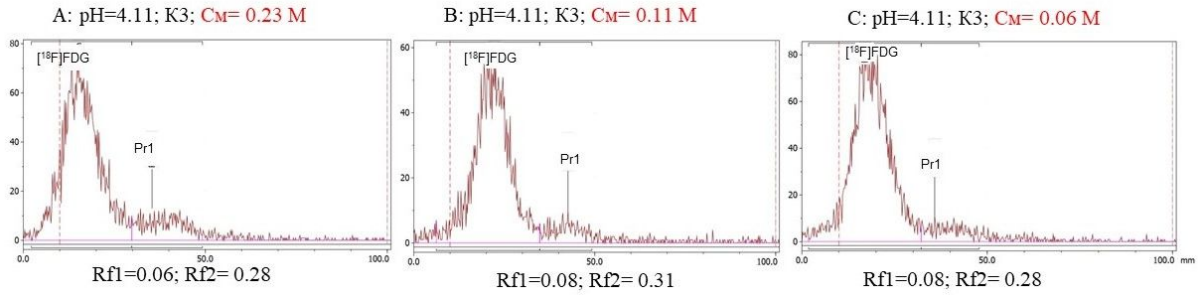


Figure 37 – Modification of Tz1 when varying the concentration of K3: A – with CM=0.23M, RCY=13.5% (Pr1); B – with CM=0.11 M, RCY=8.4% (Pr1); C – with CM=0.06 M, RCY=7.8% (Pr1)

It was found that conducting syntheses with catalyst concentrations lower than 0.3 M was not very efficient. Radio-TLC results in some of the cases indicated no product between [^{18}F]FDG and the bifunctional tetrazines. It is preferable to work with a higher concentration of the order of 0.30-0.50 M.

Table 14 - Dependence of the radiochemical yield on the concentration of the catalyst

C_M, M	0.23	0.11	0.05	0.03	catalyst
RCY,%	11.5	8.1	7.4	4.8	K2
	13.5	8.4	7.8	6.4	K3

4. Influence of the reaction time on the radiochemical yield

Syntheses are carried out according to the established procedure under the optimal conditions: temperature (70ºC), pH (4.2) and used catalyst p-methoxyaniline with a concentration of 0.50 M. The duration of the first activation stage is 10, and the second – 20 minutes. Immediately after addition of tetrazine, a sample was taken for radio-TLC analysis. This starting point is denoted as t_o. Samples are then taken at 5, 10, 15 and 20 minutes after mixing all reagents. The data are presented in Table 15 and the corresponding curves are constructed (in Figure 38) showing the dependence of the yield on the reaction time. By this procedure, the dependence of RCY on the reaction time in the labeling of Tz1 and Tz2 is followed.

Table 15 - Dependence of RCY on the duration of synthesis: A - modification of Tz1; B – modification of Tz2

	t, min	RCY,%
Tz1	0	0
	5	90.75
	10	91.55
	15	91.74
	20	93.26

A

	t, min	RCY,%
Tz2	0	0
	5	89.10
	10	91.34
	15	92.00
	20	89.33

B

As can be seen from the data, 5 min after addition of tetrazine and subsequent heating at the optimal temperature, almost complete conversion of the [^{18}F]FDG present in the system occurs. To obtain better results in the second stage of the synthesis, the heating is carried out for 10 minutes instead of 15 or 20. This also favors less radioactive decay and obtaining a product with a higher specific activity.

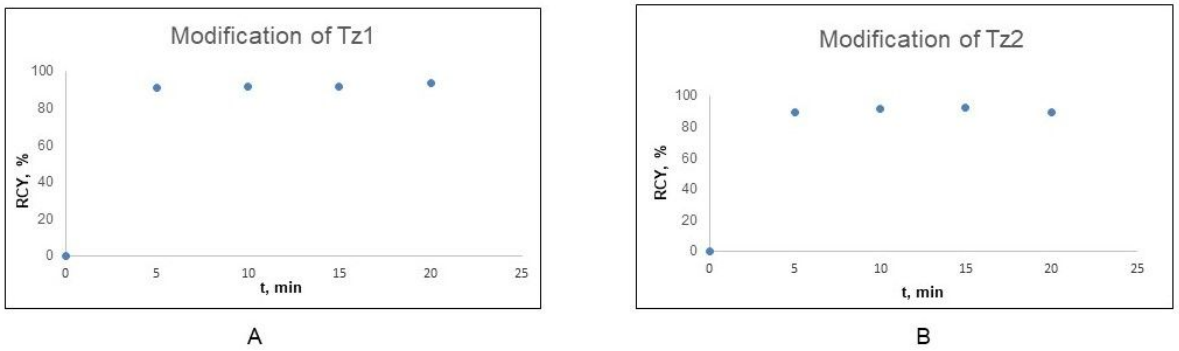


Figure 38 – Graphical dependence of RCY on the reaction time in the modification of bifunctional tetrazines: A – modification of Tz1; B – modification of Tz2

Unfortunately, the radio-TLC method only gives information about whether or not the radiochemical reaction is taking place and about the corresponding RCY obtained, but does not give an accurate and clear idea of obtaining a specific product, in case more than one substance is present in the reaction system capable of react with the [^{18}F]FDG molecule. Similar is the case with the symmetrically functionalized starting tetrazines (two functional aminooxy groups present), upon labeling of which two products are expected (monosubstituted and disubstituted), depending on the amount of [^{18}F]FDG in the reaction mixture. It also gives no information as to what fraction of the tetrazines competitively binds to the non-radioactive glucose (obtained after the decay of ^{18}F to ^{18}O) present in the reaction mixture.

CONCLUSIONS

The experiments carried out within the framework of this dissertation lead to the following more significant results:

- A methodology for modifying aminooxy functionalized tetrazine by forming an oxime bond with [^{18}F]FDG is proposed. Using this methodology, 4 tetrazines (Tz1, R1, R2 and R3) were radiolabeled.
- A methodology for modifying hydrazine functionalized tetrazine by forming a hydrazone bond with [^{18}F]FDG is proposed. Thus, 1 tetrazine (Tz2) is labeled.

- At T=70-75°C, pH=4 and in the presence of a p-methoxyaniline or p-diaminobenzene catalyst, radiolabeled products are obtained with the best RCY between 80 and 93%.
- Follow-up of syntheses and confirmation of the obtained labeled products is done with simple, cheap and clinically available methods and equipment.
- For the first time in Bulgaria, the reactions for the formation of oxime and hydrazone bonds are carried out entirely in a clinical setting, and not in a specialized radiochemical laboratory, under mild reaction conditions, in a relatively short time. The method would be fully applicable for clinical purposes.
- "Cold" syntheses (without radioactivity) between selected tetrazines and TCO derivatives are carried out and their rapid kinetics are followed, making them applicable in radiolabeling for pre-targeting.

The development and optimization of a methodology for indirect 18F radiolabeling of specific macromolecules may find a valuable application in nuclear medicine, facilitating the modification of already existing and frequently administered radiopharmaceuticals in order to increase their efficiency and selectivity. An advantage of the resulting modified products is that they can be applied in pretargeted imaging strategies that would significantly reduce patient exposure and improve image contrast.

CONTRIBUTIONS

A major contribution of the dissertation is the development of a procedure, fully adapted to clinical conditions, to modify the widely used radiopharmaceutical [^{18}F]FDG in order to increase its specificity and selectivity. A suitable radiochemical synthesis was developed, taking place under mild reaction conditions and taking into account the relatively short half-life of the PET radionuclide ^{18}F used (109 minutes). The strategy used to develop new PET imaging agents involves the chemoselective modification (by forming an oxime or hydrazone bond) of a bifunctional tetrazine capable of undergoing a subsequent click reaction with trans-cyclooctene. The reaction between a tetrazine-containing molecule (Tz) and a TCO-containing molecule proceeds rapidly under physiological conditions, yielding a stable dihydropyrazine compound. Through this approach, rapid, selective and non-toxic radiofluorination of sensitive biomolecules can be realized, which in turn will be useful in the development of new more specific and more effective radiopharmaceuticals.

Therefore, the chosen topic of the dissertation is very current and the presented approaches will be the future in nuclear medicine. The idea embedded in our work is innovative

and will initiate the creation of a new scientific direction in the field of synthesis of radiotheranostics for PET-CT studies. For the first time in Bulgaria, the use of these bioorthogonal conjugation reactions, fully adapted to standard clinical conditions, is reported.

LIST OF OWN PUBLICATIONS

1. *Modification of ^{18}F-fluororesoxy-glucose ([^{18}F]-FDG) radiopharmaceutical by oxime conjugation*, **Gergana Simeonova**, Boyan Todorov, Valentina Lyubomirova; RAP Conf. Proc., Vol. 6, 11–15; DOI: 10.37392/RapProc.2021.03 (without Q quartile)
2. *Method for indirect radiofluorination with [^{18}F]FDG by biorthogonal reaction,* **Gergana Simeonova**, Boyan Todorov, Valentina Lyubomirova; Eur. Phys. J. Spec. Top. 232, 1555–1562 (2023); DOI: 10.1140/epjs/s11734-023-00885-7 (Q2 of 2022; IF 2.8 of 2022)
3. *An approach to develop personalized radiopharmaceuticals by modifying 2-[^{18}F]-fluorodeoxyglucose (2-[^{18}F]FDG),* **Gergana Simeonova**, Boyan Todorov; Nuclear Medicine Review 2023, 26, 109–115; DOI: 10.5603/nmr.93869 (Q4 of 2022; IF 0.9 of 2022)

LIST OF PARTICIPATION IN SCIENTIFIC FORUMS AND CONFERENCES

1. XIX National Conference for students and doctoral students, organized by the Faculty of Chemistry and Pharmacy with a report on the topic: Production and modification of radiopharmaceutical [^{18}F]-fluordeoxy-glucose ([^{18}F]FDG), Gergana Simeonova, Boyan Todorov, Valentina Lubomirova; 04.06.2021
2. RAP 2021 Online Conference with a poster on the topic: Modification of [^{18}F]-fluororesoxy-glucose ([^{18}F]FDG) radiopharmaceutical by oxime conjugation, Gergana Simeonova, Boyan Todorov; 09/08/2021
3. Seventh Pharmaceutical Business Forum – Scientific and practical conference with a report on: Synthesis of ^{18}F-labeled radiopharmaceuticals and possible prospect, Gergana Simeonova, Boyan Todorov, Valentina Lyubomirova; 23.10.2021 Awarding of the first prize for the best presentation in the category of doctoral students.
4. RAP Conference 2022 with a report on the topic: Method for indirect radiofluorination with [^{18}F]FDG by biorthogonal reaction, Gergana Simeonova, Boyan Todorov; 06.06.2022
5. RAP Conference 2022 with a poster on the topic: Monitoring and evaluation of factors affecting radiolabeling with [^{18}F]FDG by oxime formation, Gergana Simeonova, Boyan Todorov; 07.06.2022

6. 11th National Chemistry Conference with report on: Use of [^{18}F]FDG as a prosthetic group for indirect radiofluorination, Gergana Simeonova, Boyan Todorov, Valentina Lyubomirova; 23-24.06.2022
7. Joint Forum SEEC-IMAB 12-th South-East European Conference of chemotherapy, infections and cancer & 32-st Annual Assembly of International Medical Association Bulgaria with a poster on the topic: An approach to develop personalized radiopharmaceuticals by modifying [^{18}F]-fluorodeoxy-glucose ([^{18}F]FDG), Gergana Simeonova, Boyan Todorov, 20-23.10.2022.
8. Eighth Pharmaceutical Business Forum - Scientific and practical conference with report on: Chemoselective radiofluorination with [^{18}F]FDG, Gergana Simeonova, Boyan Todorov, Valentina Lyubomirova; 28-29.10.2022, Awarding of the second prize for the best presentation in the category of doctoral students.
9. 12th Scientific Conference on Chemistry with a poster on the topic: Click reactions with [^{18}F]FDG and their application in the Nuclear medicine, Gergana Simeonova, Boyan Todorov; 13-15.10.2023

PARTICIPATION IN SCIENTIFIC RESEARCH PROJECTS

Participation in the implementation of a scientific project on the topic "Investigation of the possibilities of radiolabeling of bioactive molecules with [^{18}F]FDG using new bifunctional compounds" KP-06-H29/4 of 2018. to the Scientific Research Fund of the Ministry of Education and Science.

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