The modification of [18F]FDG for further click reactions

G. V. Simeonova[1,2]*, B. R. Todorov[2]

[1] UMHAT "St. Marina", Department of Nuclear Medicine, 1 Hristo Smirnenski Blvd., Varna, Bulgaria
[2] Sofia University St. Kliment Ohridski, Faculty of Chemistry and Pharmacy, Department of Analytical Chemistry,
1 James Bourchier Str, Sofia. Bulgaria

The synthesis of new fluorinated PET radiopharmaceuticals is facilitated by various strategies for [18F] labeling reactions. [18F]FDG, as a basic PET radiopharmaceutical (glucose analog), can be used as a building block for indirect radiofluorination under mild reaction conditions, due to the presence of a carbonyl group where chemoselective formation of an oxime bond can take place for conjugation with target moieties. The glycosylation is one of the used modification strategies that can increase the hydrophilicity of the radiotracer, and shorten the period between injection and imaging. Also, glycosylation of biomolecules such as peptides or proteins can improve *in vivo* pharmacokinetics and blood stability.

We have developed a procedure for modification of [18F]FDG with bifunctional tetrazine derivatives, by oxime bond formation and all experiments were done with standard clinical equipments. For this purpose, [18F]FDG was produced using a biomedical baby-cyclotron at the Nuclear Medicine Clinic at the University Hospital "St. Marina" – Varna. Successful modification with [18F]FDG was achieved at a temperature of 70-75ºC, in a slightly acidic environment (pH= 4-4.2), in the presence of a *p*-diaminobenzene catalyst for 25-30 minutes. TLC chromatography was used as a fast and available in clinic method to monitor the synthesis process. This is a prerequisite for the development of new PET radiopharmaceuticals and for the refinement of diagnostics and therapy in nuclear medicine.

Keywords: [18F]-FDG, bifunctional compounds, tetrazine, trans-cyclooctene, oxime formation, click reactions, personal medicine

INTRODUCTION

The early diagnosis of malignant tumors plays a leading role in the choice of treatment approach and the survival prognosis of cancer patients. This task is solved to a significant extent with the help of radionuclide diagnostics (nuclear medicine) [1-3]. The use of molecular imaging agents plays an important role in the advancement of medical procedures and is an essential part of ongoing research. At the forefront of molecular imaging is the use of positron emission tomography (PET-CT) which relies on a biomarker labeled with a short-lived positron-emitting radionuclide [4]. The molecular imaging aims to non-invasively visualize, characterize and quantify biological processes at the cellular and molecular level *in vivo* [5]. When considering the clinical applications of pre-targeted PET imaging, the ideal isotope would be fluorine-18 ([18F]) due to its general availability and optimal physical properties [6].

There are various chemical methods of introducing [18F] to the desired molecule. The main synthetic strategies can be divided into two main categories: direct fluorination, i.e. [18F] is directly attached to the molecule to be labeled, and indirect fluorination, in which [18F] is introduced in the form of an [18F]-containing prosthetic group, which usually requires multistep synthesis [7]. In recent years, various strategies for chemoselective [18F]-labeling reactions have been successfully developed, facilitating the accessibility to novel PET radiopharmaceuticals. These synthesis strategies have been improved, especially due to the development of various [18F]-labeled prosthetic groups designed mainly for chemoselective peptide labeling [8]. The use of a prosthetic group overcomes the inability to radiofluorinate certain compounds by nucleophilic [18F]-substitution of a suitable leaving group. The development of [18F] prosthetic groups makes extensive use of the concept of click chemistry [9]. The click chemical reactions should be simple, fast and modular. They are characterized by high chemical (and radiochemical) yields and production of stable products [9, 10]. Among the reactions that meet these requirements, carbonyl condensation reactions such as imine, hydrazone and oxime formation can be mentioned [11].

The molecule of 2-fluoro-deoxyglucose ([18F]FDG) is an analogue of glucose, in which the hydroxyl group at the second carbon atom is replaced by [18F], and finds application in nuclear medicine. [18F]FDG allows the evaluation of glycolytic activity, which is more enhanced in tumor cells compared to normal cells. It also finds applica-

* To whom all correspondence should be sent:
E-mail: *gerimm@abv.bg*

tion in the evaluation of cardiac and neurological diseases [12]. In addition to being a basic PET radiopharmaceutical, the molecule can also be used as a building block for indirect radiofluorination under mild reaction conditions. The [^{18}F]FDG molecule has been reported as a suitable prosthetic group for indirect labeling of various aminooxy-functionalized peptides by chemoselective oxime formation [13]. The glycosylation of biomolecules such as peptides or proteins can improve *in vivo* pharmacokinetics and stability in blood [1]. The glycosylation is one of the most commonly used modification strategies that can increase the hydrophilicity of the radiomarker, increase the proportion of renal elimination, decrease the physiological uptake of the radiopharmaceutical into the digestive system, and shorten the time interval from injection of the tracer to imaging. It can also improve the contrast and usefulness of PET imaging [2]. Of particular interest are studies in which [^{18}F]FDG has been used as a building block for other, more complex compounds such as peptides that cannot be directly labeled with ^{18}F due to the harsher conditions required for their radiofluorination (high temperature, highly alkaline conditions) [3]. The glycosylation strategies for anticancer drugs have attracted considerable attention in the scientific community. This strategy improves the pharmacokinetic properties, selectivity of cytotoxic aglycones and is very useful for targeted drug delivery [14]. By a simple one-step method, [^{18}F]FDG has been applied to fluorinate aminooxy- or hydrazine-functionalized peptides by formation of oxime or hydrazone [15, 16]. The main advantages of oxime formation by a click reaction between aminooxy- and carbonyl-functional groups used for ^{18}F-fluoroglycosylation are: its high chemo-selectivity, the application of unprotected aminooxy precursors and the fact that binding to the carbonyl component can take place in aqueous media (pH 4-7) [1]. The acidic environment facilitates the unblocking of the acetal functional group, and promotes the formation of an oxime bond between the aldehyde and the aminooxy group [17]. The radiochemistry of oxime formation and the relatively high stability, both chemical and *in vivo*, allow the potential use of this approach to rapidly generate the desired [^{18}F]-labeled macromolecules for application in PET-CT imaging [18]. Another possibility for the reaction with aldehydes is the formation of hydrazones with hydrazine derivatives, which is similar to the formation of oximes - selective towards the formation of Schiff bases, but proceeds relatively slower than the reaction with aminooxy functionals [19]. The hydrazines react

more slowly with aldehydes than aminooxy derivatives, making them less common click partners in ^{18}F-labeling of peptides [9]. The reaction efficiency in oxime and hydrazone bond formation can be dramatically increased by using a range of catalysts [20]. Bifunctional catalysts are being developed that use intramolecular proton transfer from acid-base groups near a nucleophilic amino group to facilitate rapid hydrazone and oxime formation [21]. The reactions of this type are becoming an essential tool for the preparation of chemically engineered bioconjugates for applications in chemical biology. Biocompatible click reactions have the potential to be used to perform *in situ* ligation in living organisms [11].

The oxime or hydrazone formation reactions in combination with other bioorthogonal click reactions, such as the cycloaddition between bifunctional tetrazine and trans-cyclooctene (TTCO), may play an important role in the development of new radiopharmaceuticals with high specific activity and selectivity. This, in turn, will make a step towards the development of personalized medicine related to early and accurate diagnosis and refinement of therapy. The highly efficient reaction between 1,2,4,5-tetrazines and strained dienophiles is a fast and clean bioorthogonal conjugation method. Moreover, the reaction proceeds without catalyst, which is beneficial in easier purification of the final radioactive tracer [9]. The reaction proceeds rapidly at room temperature and is practically irreversible. It produces N_2 as the only by-product [22]. This type of cycloaddition of heterocyclic azadienes provides a powerful methodology for the synthesis of highly substituted and functionalized heterocycles widely used in organic synthesis and pharmaceutical industries [23]. The ligation of tetrazine with trans cyclooctene can be applied to radiolabel large biomolecules such as ^{18}F-labeled proteins and the resulting PET biomarker retains good binding ability to the desired target [24, 25]. Currently, the most promising reaction for pretargeted imaging is the ligation between tetrazine (Tz) and trans-cyclooctene (TCO). From a clinical point of view, ^{18}F-labeled Tz would be ideal for positron emission tomography (PET) applications, as ^{18}F possesses nearly perfect physical characteristics for molecular imaging [26]. The radionuclide ^{18}F can be pre-introduced into one of the two molecules involved in the click reaction, tetrazine or dienophile. A difficulty, however, is the delicate stability of tetrazines in general, which can pose a problem for the conjugation of tetrazines to biomolecules [9]. The synthesis of a fluorinated tetrazine starting from an ^{18}F-containing prosthetic

group is necessary, as tetrazines are unstable under the commonly used direct radiofluorination conditions. The resulting ^{18}F-labeled tetrazine can be used for pretargeted PET imaging [16, 27]. The glycosylated ^{18}F-labeled tetrazine is an excellent candidate for *in vivo* bioorthogonal chemistry applications in pretargeted PET imaging approaches [6]. A combination of the two chemoselective (click) reactions, in which functionalized tetrazines with a free aminooxy group capable of forming an oxime bond, have been successfully applied for indirect radiofluorination of monoclonal antibodies [28]. These bifunctional, clickable compounds are a new class of conjugates with promising applications in the nuclear medicine, providing a step towards the development of the personalized medicine related to early and accurate diagnosis and precision therapy.

In the pretargeted imaging approach based on the click reaction between bifunctional tetrazine and trans cyclooctene, one of the reagents can be a carrier of a specific biomolecule and the other of the radionuclide. The biomolecule will be responsible for the accumulation of the complex in the specific organ, and the radionuclide - for the visualization of the areas of interest. The variant in which the tetrazine derivative is indirectly radiolabeled by forming an oxime bond with the [^{18}F]FDG molecule is proposed. Glycosylated ^{18}F-labeled bifunctional tetrazine will provide a reagent for indirect radiolabeling of sensitive macromolecules *via* a click reaction with trans-cyclooctene. Figure 1 shows the general reaction scheme between [^{18}F]FDG-modified tetrazine and bifunctional trans-cyclooctene. Such reactions are easy, fast, and practically irreversible. Most often, they take place at equivalent amounts of the reactants, under physiological conditions and room temperature, without the presence of a catalyst [29, 30]. 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 pharmacologically most relevant TCO derivatives and Tz are investigated. The corresponding rate constants are experimentally determined. Studying the kinetics of a cold (non-radioactive) click reaction is an important step in the development of a radiolabeling procedure using TTCO. From the obtained results, clarity is obtained about the speed of the reaction and the applicability of the procedure for the purposes of nuclear medicine. 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.

New bifunctional compounds have been synthesized within the framework of the above-mentioned project. Appropriately functionalized tetrazine derivatives containing a free hydroxylamine group capable of forming an oxime 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 compound can be influenced. The products were purified by polar phase column chromatography and characterized by ^{1}H-NMR, then provided and used for modification with [^{18}F]FDG.

The aim of the study is to develop and optimize a highly efficient and rapid method for indirect radiofluorination of bifunctional tetrazine structures with an aminooxy group by [^{18}F]FDG conjugation under standard clinical laboratory conditions. In our previous research, we mention the development of a modification procedure using another tetrazine (O-{10-[4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenoxy]-decyl}-hydroxylamine), which can be seen in more detail in reference [32]. The established procedure was applied to the successful radiolabeling of another tetrazine derivative.
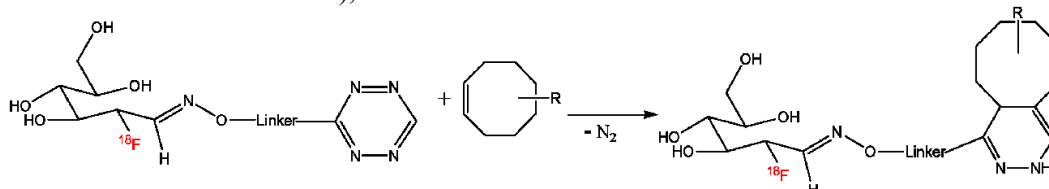

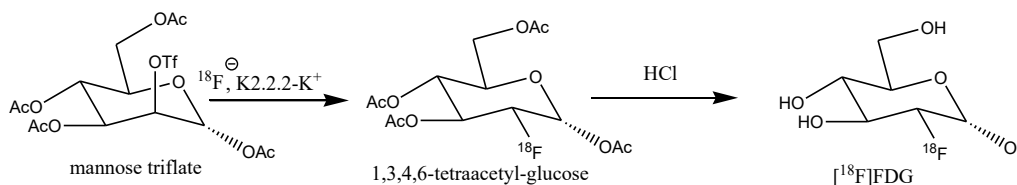**Figure 1.** Scheme of the click reaction between modified tetrazine and trans-cyclooctene

Figure 2. Synthesis of [^{18}F]FDG

EXPERIMENTAL

A procedure is proposed to modify the most common and widely used radiopharmaceutical [^{18}F]FDG by oxime bond formation. The procedure is performed at a temperature between 70 and 80 °C and is fully adapted to the clinical conditions and the equipment available in the clinic. The synthesis procedures were performed entirely at the Clinic of Nuclear Medicine at St. Marina University Hospital, Varna, Bulgaria. For this purpose, [^{18}F]FDG was synthesized using a small biomedical cyclotron (ABT Molecular Imaging biomedical, model ABT BG-75) equipped with an automated radiochemical synthesis module and a quality control system. In the first stage, the radionuclide ^{18}F is produced by proton bombardment of ^{18}O-enriched water, and the following ^{18}O(p,n)^{18}F nuclear reaction is performed. The production of [^{18}F]FDG was based on a nucleophilic radiofluorination method with mannose triflate as precursor followed by acid hydrolysis with 2M HCl [31]. All reagents required for the production of radiopharmaceuticals were commercially obtained and used without further purification. Figure 2 shows the reaction scheme of the process.

In this paper, we present the modification of the following aminooxy-functionalized tetrazine: aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester, which we will denote as R1 for brevity. Its structure is shown in Figure 3.
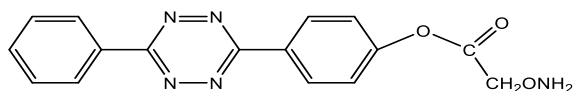
Aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester

Figure 3. Structure of used tetrazine R1

Since we are provided with a BOC (tert-butyloxycarbonyl) group-protected bifunctional compound, an additional deprotection step is required before labeling it with [^{18}F]FDG. A small amount of the protected tetrazine was mixed with 0.5 ml of 10% trifluoroacetic acid in anhydrous ethyl acetate for 12 hours at room temperature, after which the vials were placed in an air oven to evaporate the solvent. The reaction scheme of this preliminary step to obtain R1 is presented in Figure 4.

After elimination of the BOC protection, the bifunctional tetrazine was dissolved in acetonitrile. The resulting solution was used to modify [^{18}F]FDG by forming an oxime bond. We performed the syntheses in a large excess of tetrazine over radiofluorinated glucose (approximately 10^4:1), suggesting its complete conversion. The following procedure was used. From a previously prepared 0.25 M solution of the catalyst p-diaminobenzene

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acidified with acetic acid to a pH of about 4, 0.5 ml were transferred into the reaction vessel. To this was added 0.1 ml of [^{18}F]FDG of radioactivity between 5 and 25 MBq. This was followed by heating at 70-75 °C for 15 minutes to activate the molecule and open the glucopyranose ring. As an intermediate, the corresponding Schiff base was obtained with the catalyst. In the next step, 0.2 ml of the prepared R1 solution was added and further heated for another 10-15 minutes at the same temperature [32]. As an inexpensive and clinically available method of analysis, we used radio-TLC to follow the course of the reaction and confirm the production of the labeled product [^{18}F]FDG-R1. The TLC analysis was performed with silica gel-coated aluminium plates (ALUGRAM Sorbent Silica G/UV254, 40×80 mm) and ethyl acetate was used as eluent. After addition of the sample and subsequent elution, the plates were scanned using a Scan-Ram PET/SPECT radio TLC-scanner.

RESULTS AND DISCUSSION

According to literature data, the most suitable conjugation for [^{18}F]FDG is *via* oxime or hydrazone bond formation, and this is reported to be an efficient and chemoselective reaction occurring in aqueous media under mildly acidic conditions in the presence of a catalyst. The aim of the presented research was to verify the applicability of such a modification under standard conditions and equipment of a clinical laboratory. By forming an oxime bond, we were able to modify the bifunctional derivative of tetrazine.

The modification of R1 in the presence of p-diaminobenzene proceeds in two steps. In the first step, a Schiff base is obtained as an intermediate between the catalyst and the [^{18}F]FDG molecule, which after addition of R1 turns into the desired oxime product [^{18}F]FDG-R1. In Figure 5 the reaction scheme is presented. After the synthesis, a sample of the reaction mixture was taken and spotted onto the TLC plate. After eluting it with ethyl acetate, we obtained the TLC chromatogram presented in Figure 6. Under these elution conditions, the catalyst and unreacted tetrazine move to the front as a brown and pink spot, respectively. The unreacted [^{18}F]FDG remains at the start (at the drip point), where we also detect a radioactive peak. A new pink spot with reported activity corresponding to the labeled oxime product [^{18}F]FDG-R1 is observed close to the start. The distribution of spots corresponding to the reaction components after modification of R1 is as follows: 1 is unreacted [^{18}F]FDG if present in the system, 2 - labeled oxime product, 3 is starting tetrazine, and 4 is catalyst used.
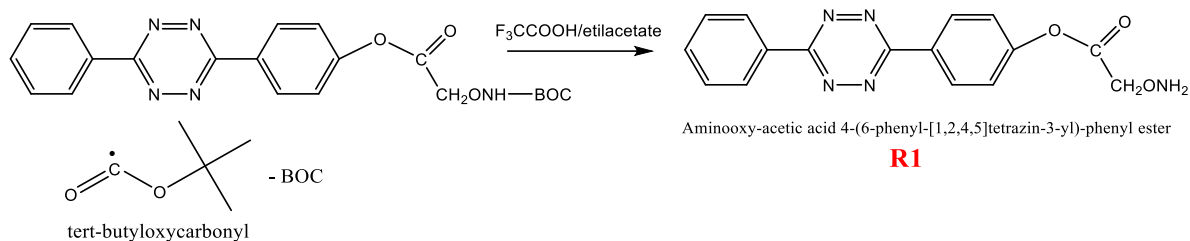
Aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester

R1
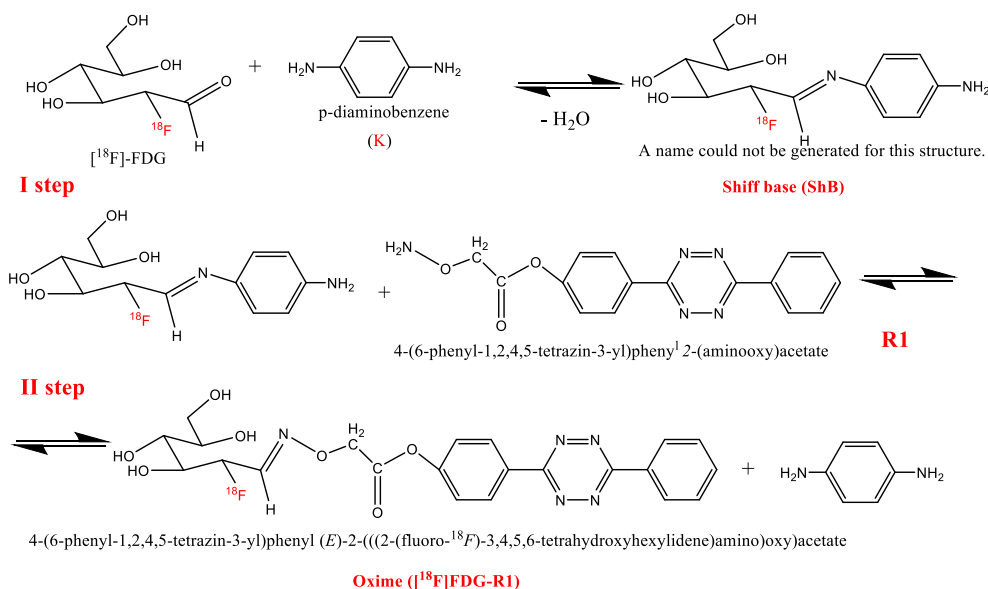
Figure 4. Preparation of tetrazine R1

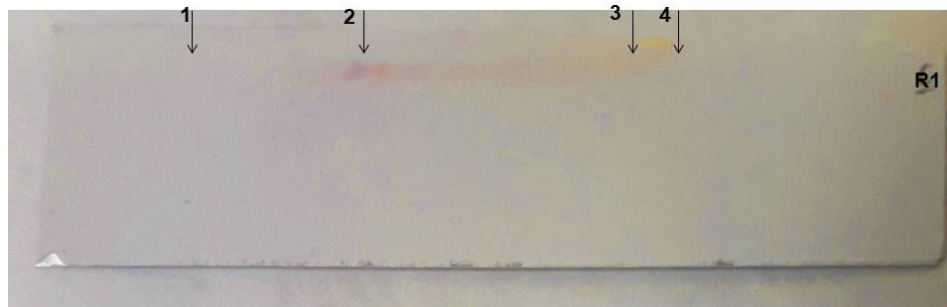**Figure 5.** Modification of R1 in the presence of p-diaminobenzene

Figure 6. TLC chromatogram showing the distribution of the reaction components after labeling R1

The plate was then scanned and the radio-TLC chromatogram shown in Figure 7 was obtained. Based on it, we determined the retention factor (Rf =0.41) of the obtained product [^{18}F]FDG-R1 and an approximate radiochemical yield (RCY = 85%), which is uncorrected for radioactive decay. A single radioactive peak was obtained because tetrazine was used in a very large excess (10^4:1) over [^{18}F]FDG, which under optimal conditions undergoes almost complete conversion to the corresponding radiolabeled product. For comparison, a radio-TLC chromatogram of the initial [^{18}F]FDG, which, after elution under the same conditions, is retained at the start (at the drop point) with Rf=0.06 is also attached. The resulting product was confirmed by radio-HPLC. Since the labeling of R1 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. As a result, the obtained products are fully confirmed on the basis of the data from the RAD detector, which has a significantly higher sensitivity. 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=2.24 min corresponding to the labeled product Pr2. Figure 8 presents the HPLC chromatograms obtained from the RAD detector of unmodified [^{18}F]FDG (chromatogram A) and reaction mixture (chromatogram B).
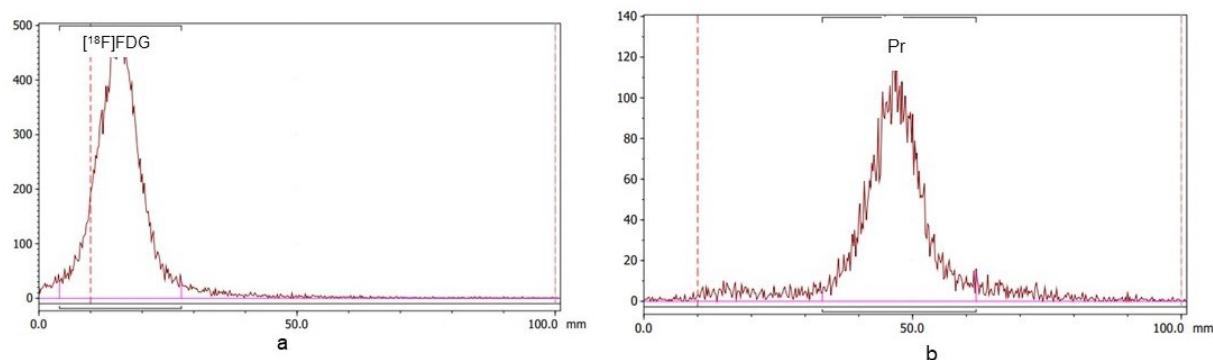
Figure 7. Radio-TLC chromatogram: a) unreacted [^{18}F]FDG; Rf=0.06; b) modified R1: Rf=0.41; RCY=85.4%

Figure 8. Radio-HPLC analysis of modified R1

CONCLUSIONS

Based on the experiments, the following conclusions can be drawn:

1) The aminooxy-functionalized tetrazine aminooxy-acetic acid 4-(6-phenyl-[1,2,4,5]tetrazin-3-yl)-phenyl ester was successfully modified under soft reaction conditions with good radiochemical yield;

2) The oxime formation with [^{18}F]-FDG is a practical method for indirect radiofluorination;

3) The developed method is fully applicable to standard clinical laboratory conditions.

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