National University of Science and Technology
Politehnica Bucharest
Doctoral School "Chemical Engineering and Biotechnology"
Chemistry PhD

DOCTORAL THESIS

Methods of analysis for some radiopharmaceuticals

– EXTENDED ABSTRACT OF THE DOCTORAL THESIS –

Supervisor:

Prof. Dr. Eng. Alina Catrinel Ion

PhD. Student:

Chem. Maria-Roxana Cornoiu (Tudoroiu-Cornoiu)

2025 Bucharest

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CONTENTS

INTRODUCTION

Over the years, radiopharmaceuticals have been a fundamental pillar in the development of nuclear medicine, becoming an indispensable tool in the diagnosis, monitoring, and personalized treatment of complex diseases, especially oncological and cardiovascular ones.

^{64}Cu and ^{89}Zr are two of the medical radioisotopes that have stood out in oncological research due to their favourable physicochemical properties. ^{64}Cu, with a half-life of 12.7 hours, is a theranostic radioisotope, that can be used both for diagnosis (due to β^+ emissions) and for therapy (due to β^- and Auger electron emissions), allowing real-time monitoring of the patient's response to treatment and its adjustment. In contrast, ^{89}Zr, due to its half-life of 78.4 hours, is ideal for generating high-resolution images over extended periods, facilitating the long-term monitoring of biological and pathological processes. This feature makes it particularly useful in biodistribution studies and detailed monitoring of therapy.

To ensure the efficiency and safety of the administration of radiopharmaceutical compounds, the development of rigorous analytical methods is crucial. Chromatographic techniques, such as radio-high-performance liquid chromatography (radio-HPLC) and radio-thin-layer liquid chromatography (radio-TLC), are specific to the analysis of radiolabelled compounds, ensuring their separation, identification, and quantification in a precise and reliable way.

This doctoral thesis aims to develop and optimize methods for the synthesis and analysis of radiopharmaceuticals, with a particular emphasis on the use of ^{64}Cu and ^{89}Zr radioisotopes. The specific objectives of the research include: (I) investigating the fundamental characteristics and development stages of radiopharmaceuticals, (II) developing and optimizing chromatographic methods for their analysis, and (III) testing analytical methods to ensure the reproducibility and accuracy of the results. An original contribution of this work consists of labelling peptides, monoclonal antibodies, and antibody fragments with ^{64}Cu and ^{89}Zr radioisotopes, followed by evaluating optimized analytical methods to ensure their efficiency and safety in nuclear medicine applications.

The thesis is structured in two main parts: the theoretical part (two chapters) and the experimental part (three chapters), including the original contributions to the present study.

The results obtained in this thesis not only improve the understanding of radiolabelling processes and the analysis of radiolabelled compounds but also open new perspectives for the application of radiopharmaceuticals in the diagnosis and treatment of oncological diseases.

1. FUNDAMENTALS REGARDING THE IMPORTANCE OF RADIOPHARMACEUTICAL USE IN NUCLEAR MEDICINE

Radiopharmaceuticals (RFMs) represent a special class of pharmaceutical formulations in which a radioisotope attached to a pharmaceutical moiety is administered by inhalation, orally, intravenously, or interstitially for the diagnosis and/or targeted therapy of various diseases. A radiopharmaceutical is generally composed of either a free-standing radioisotope (e.g. ^{131}I) or a radioisotope bound to a biologically active molecule (inorganic or organic) responsible for its transport to specific target organs, tissues or cells [1]. The selection of the radioisotope depends on several factors, including the type of radiation emitted, its energies, the purpose for which it is used, the production methods, the half-life, the type and size of the targeted tumour, the density of the target, as well as its heterogeneity. For diagnosis, radioisotopes that directly emit γ radiation or that indirectly emit very high-energy photons, as a result of the annihilation reaction between a positron and a nearby electron, are required, while for targeted therapy, radioisotopes emitting α, β^- radiation, or Auger electrons are carefully chosen, which contribute to the gradual damage and destruction of tumour cells over time [1]. Most medical radioisotopes are produced using nuclear reactors or cyclotron-type particle accelerators, but there is also the alternative of production using generators. In general, neutron-rich radioisotopes, such as ^{99m}Tc, ^{60}Co, ^{192}Ir, ^{131}I, ^{166}Ho, and ^{177}Lu, are produced in reactors either by fission or neutron capture processes and have relatively long half-lives. On the other hand, neutron-deficient radioisotopes, such as ^{18}F, ^{201}Tl, ^{123}I, ^{67}Ga, and ^{64}Cu, are commonly produced in cyclotrons by (p,n) and (p,α) nuclear reactions and have relatively short half-lives. [2].

- *Obtaining at the cyclotron*

In this process, charged particles (protons, deuterons, and α particles) are accelerated circularly by a high-frequency oscillating electric field and steered by a perpendicular magnetic field. The ion source is positioned in the centre of the cyclotron, and as their energy grows, their movement takes on a more circular trajectory, spiralling outward. When the accelerated ions achieve the requisite energy, they are directed into an extraction window with a radius corresponding to that energy. The extracted beam is

then directed towards a specific target, where a nuclear reaction occurs to produce the desired radioisotope [3]. Positron-emitting radioisotopes of medical importance are efficiently produced by hitting stable isotopes with proton beams of up to 18-19 MeV, which can be generated in a compact cyclotron specifically built to produce positron emitters for positron emission tomography (PET). Currently, all small cyclotrons accessible for isotope synthesis are negative ion accelerators that convert H^- to protons via ion stripping [4].

Copper-64 Radioisotope

The ^{64}Cu radioisotope ($T_{1/2}$ = 12.7 hours) has decay characteristics that make it attractive as a radiotracer in PET imaging (β^+) and a therapeutic agent in targeted tumour radiotherapy (β^- and Auger electrons)[5]. Cu^{2+} produces complex combinations with different chelators that can be linked to peptides, antibodies, proteins, and nanoparticles [5]. Radiopharmaceuticals labelled with ^{64}Cu can be used to diagnose and treat several kinds of malignancies, including prostate, glioblastoma, melanoma, and breast cancers, as well as diseases such as arteriosclerosis and Alzheimer's.

Zirconium-89 Radioisotope

Zirconium-89 is a radioisotope with a half-life of approximately 78.4 hours. Its decay scheme shows a low degree of positron emission (23% β^+) compared to electron capture (77%). ^{89}Zr is usually produced in a biomedical cyclotron via the $^{89}Y(p,n)^{89}Zr$ reaction, then separated and purified with hydroxamate-functionalized resins [5]. In biological systems, ^{89}Zr occurs in the +4 oxidation state and can form complexes with coordination numbers as high as 8. Its favourable half-life makes it completely compatible with the time required to obtain PET images with good spatial resolution using radiolabelled monoclonal antibodies (mAbs) [5]. The most frequently utilised chelator for labelling mAb with ^{89}Zr is deferoxamine (DFO), which coordinates Zr^{4+} in a hexadentate mode, with labelling done via an amide bond.

PET is one of the most popular molecular imaging techniques, providing unique non-invasive and in vivo images that are useful for examining the biological and metabolic processes of subjects during animal model research [6]. The underlying principle of PET is the coincidence detection of a pair of gamma quanta produced by the annihilation of positrons, emitted by the radiotracer injected into the patient's body, with nearby electrons.

2. RADIO-CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF RADIOPHARMACEUTICAL COMPOUNDS

Radio-chromatographic methods are essential for analysing radioactive compounds because they provide critical information on purity, stability, and biodistribution. They are used in a variety of fields, including environmental pollution monitoring, drug development, detailed toxicological analyses, and chemical safety assessment [7]. These methods involve the use of techniques such as gas chromatography (GC), paper chromatography (PC), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC), adapted to the special needs of radiochemistry [8]. Their optimisation and validation are critical to ensuring product quality [9]. Several types of detectors are used, including ionisation detectors (ionisation chambers, proportional detectors, Geiger-Müller detectors), scintillation detectors, autoradiography, and semiconductor detectors [7], [10]. They ensure high sensitivity and specificity, contributing to scientific development and protecting public health and the environment [11].

- *Radio-high-performance liquid chromatography (radio-HPLC)*

Radio-HPLC is a complex analytical technique with high sensitivity that is used to separate, identify, and quantify radiolabelled chemicals from a variety of products. This technology combines HPLC principles with specific radiochemical detection techniques to determine both chemical and radiochemical contaminants [12]. To analyse a radioactive sample with radio-HPLC, a small volume of the sample is injected into the system and transported through the chromatographic column by the mobile phase flow. High-pressure pumps push the mobile phase through the column at a predetermined flow rate (mL/min), transporting the sample. The analyte is slowed as it passes through the chromatographic column due to chemical and physical interactions with the stationary phase [13]. Radio-HPLC systems use two detectors connected in series: (I) a UV-Vis, electrochemical, or refractive index detector for identifying chemical species [13]; and (II) a radiochemical detector (liquid/solid scintillation or semiconductor) for detecting radioactive species, such as detectors [7], [13].

- *Radio-thin-layer liquid chromatography (radio-TLC)*

TLC is a fundamental analytical technique that is widely used in several different fields for the separation and determination of compounds in a mixture [14]. A method specific to the detection of radioactive substances, known as radio-TLC, is a key technique in the quality control of radiopharmaceuticals. This method is based on the migration of radiolabelled compounds on a TLC plate (stationary phase) under the influence of a solvent (mobile phase), performing use of the compound's polarity and solubility differences [13]. When selecting a mobile phase, three key factors are typically considered: solubility, affinity, and resolution. To examine TLC plates, use a scanner with a specific detector, such as a scintillation detector (NaI(Tl)) for gamma-emitting radioisotopes or a beta detector for β particles. The detector captures ionising radiation signals and transfers them to an electronic integrator, which filters, amplifies, and processes the data to provide a precise chromatographic profile indicating the distribution of radiolabelled chemicals. The system includes an automatic scanning mechanism, which moves the TLC plate vertically under the detector, ensuring complete and detailed analysis of the entire surface during a single run [13], [15]. The detector is fitted with interchangeable collimators for different radiation energies, to reduce radiation from surrounding regions. A specific software program analyses the acquired data, allowing the chemicals to be identified and their radioactivity quantified.

- *Gas chromatography (GC)*

Martin and James developed gas chromatography in 1952, and it is now the preferred method to identify and separate analytes from a sample. This technique has several advantages that make it so popular in the field of analytical chemistry, including very high sensitivity, selectivity, and resolution, as well as good accuracy and precision, allowing it to be used at a wide range of concentrations [16].

Gas chromatography can be used to analyse both liquid and gaseous samples. A liquid sample is introduced into the chromatograph via the injection system and transported through the heated chromatographic column, where it is vaporised, and its components are selectively held by the stationary phase. For their elution from the column, it is necessary to pass an inert gas (N2, He) through it. Separation requires a difference in the solubility of the components in the sample. If the components are insufficiently volatile, their boiling points must be lowered by derivatizing the chemical groups. The choice of inert gas supplying the chromatograph depends on the type of

detector used, which in turn depends on the class of compounds analysed. High-purity gases are stored in specially designed cylinders equipped with pressure reducers. These reducers ensure that the pressure remains between 1.5 and 3 atm. A molecular filter is placed in the inert gas path to the chromatograph to prevent water vapour and other contaminants from entering the system [17].

3. SYNTHESIS AND EVALUATION OF ^{64}Cu COMPLEXES: OBTAINING AND QUALITY CONTROL OF RADIOPHARMACEUTICALS RESULTING FROM IRRADIATION OF SOLID TARGETS

3.1. ASSESSMENT OF CHROMATOGRAPHIC PARAMETERS USED FOR QUALITY CONTROL OF ^{64}Cu-LABELED PEPTIDES

3.1.1. ^{64}Cu production at the TR-19 cyclotron and radiochemical processing of [^{64}Cu]$CuCl_2$ solution

The production of the radioisotope ^{64}Cu by the nuclear reaction ^{64}Ni (p,n)^{64}Cu was performed at the Radiopharmaceutical Research Centre using the TR-19 cyclotron (ACSI, Canada) and an automated solid target (ST) post-processing system (Alceo 3.0 COMECER, Italy). The process involved proton irradiation of a solid target of electrodeposited enriched ^{64}Ni, followed by an automated dissolution and purification process. Different concentrations of HCl were used to separate copper ions from ^{64}Ni and cobalt impurities (^{56}Co, ^{57}Co, ^{58}Co, ^{61}Co) using the AG1-X8 ion exchange resin. The final solution of [^{64}Cu]$CuCl_2$ was obtained by elution with 0.5M HCl [18].

3.1.2. Peptide radiolabelling process using the ^{64}Cu radioisotope

Peptide radiolabelling was performed in a reaction vessel at 95°C. 1 mL of [^{64}Cu]$CuCl_2$ solution with a radioactive concentration between 250-1500 MBq/mL and 3.8-4.0 pH (ammonium acetate buffered), was added over 20 nmol of peptide solutions previously dissolved in 50 µL ultrapure water. The reaction took place on a water bath for 25 minutes under continuous stirring at 200 rpm [19]. After 25-30 minutes of reaction time, the solution was purified using the StrataTM-X 33 µm RP cartridge. The radiolabelled peptide was eluted from the cartridge with 1 mL of analytical-grade

ethanol. Subsequently, to remove excess ethanol, the resulting solution was evaporated at 80°C to near dryness. After evaporation, the radiolabelled peptide was recovered with 1 mL saline (0.9% NaCl), and the pH was checked and adjusted with 1M NaOH (if necessary) to have a value between 7.0-7.4 [18], [19].

3.1.3. Quality control of radiolabelled peptides

The quality control requirements for radiopharmaceutical preparations are radiochemical (RCP) and radionuclidic purity (RNP), pH, sterility, pyrogenicity, and residual solvent analysis. These impurities can generate allergic reactions in the human body, toxicological effects, or a decreased image quality. All the mentioned quality criteria were assessed in accordance with European Pharmacopoeia (Ph. Eur.) because the synthesised radiopharmaceuticals described in this work are intended for parenteral administration PET imaging applications [18].

3.1.4. Results and discussion

3.1.4.1. Obtaining and using the [^{64}Cu]$CuCl_2$ solution

Following irradiation of the enriched ^{64}Ni solid target and its post-irradiation processing, an aqueous solution in the form of [^{64}Cu]$CuCl_2$ was obtained with an activity ranging from 10-16 GBq, corrected to end of bombardment (EOB) [20]. This solution was subjected to rigorous quality control to ensure its compatibility with biological and radiobiological applications. Detailed characterization of the [^{64}Cu]$CuCl_2$ solution provided clear and concise information regarding the following specifications: radiochemical purity of 100%, radionuclidic purity of over 99.99%, and a half-life of 12.43 ± 0.2 h [18], [19]. Based on these verifications, the [^{64}Cu]$CuCl_2$ solution was used for *in vitro* studies, to evaluate the interaction between ^{64}Cu and different cell lines, as well as in radiolabelling experiments with peptides, monoclonal antibodies, and nanobodies, which included stability studies both in vitro and in vivo.

Radiolabelling process of DOTA-peptide conjugation with ^{64}Cu radioisotope

The [^{64}Cu]CuCl2 solution was used for radiolabelling six peptides derivatized with the DOTA chelator: DOTA-NMN, DOTA-NT(8-13), DOTA-PEG(4)-BBN(7-14), DOTA-NOC, DOTA-NMB and DOTA-cRGDmon. For DOTA-NMN and DOTA-NT(8-13), the [^{64}Cu]$CuCl_2$ solution used had a concentration of ≥ 750 MBq/mL [19], while for the remaining peptides, it ranged between 250-1500 MBq/mL. The labelling parameters, including the volume of [^{64}Cu]$CuCl_2$ solution, temperature, pH, amount of

peptide used, and reaction time, were kept constant for all peptides. Specific details for DOTA-NMN and DOTA-NT(8-13) are presented in Table 1.

Notably, of the six peptides used in the radiolabelling process, only two (DOTA-NMN and DOTA-NT(8-13)) were selected for additional studies, including quality control and *in vitro* stability studies.

Table 1. Radiolabelling parameters for ST experiments [19]

Parameters	Peptides	
	DOTA-NMN	**DOTA-NT(8-13)**
Quantity (nmol)	20	
Radiolabelling pH	3.9 ± 0.1	
[^{64}Cu]$CuCl_2$ volume (mL)	1.00 ± 0.2	
[^{64}Cu]$CuCl_2$ activity (MBq)	1340.4 ± 70.1	
Temperature (°C)	95	
Reaction time (min)	25	
Radiolabelling yield (%)	78.15 ± 3.12	67.04 2.68

3.1.4.2. Radio-HPLC method development and optimization

Initially, the SunFire C18 column (150 × 4.6 mm, 3.5 µm, 100 Å, 16.0% C) and the Hypersil GOLD column (150 × 4.6 mm, 5 µm, 175 Å, 12.0% C).The mobile phase used was a binary mixture of solvents: solvent A – water with 0.1% TFA and solvent B – acetonitrile with 0.1% TFA. The optimal gradient used: 0.01 min/ 95% A/ 5% B, 2 min/ 99% A/ 1% B, 15 min/ 30% A/ 70% B, 20 min/ 99% A/ 1% B, 25 min/ 95% A/ 5% B resulted from the gradual and controlled release of eluent through the column to ensure adequate resolution between two closely eluting components [18].

To optimize the method, parameters such as column temperature and flow rate were adjusted. Analysis using the SunFire C18 column was performed at mobile phase flow rates of 0.5 and 1.0 mL/min and column temperatures of 30°C and 35°C (Fig 1).

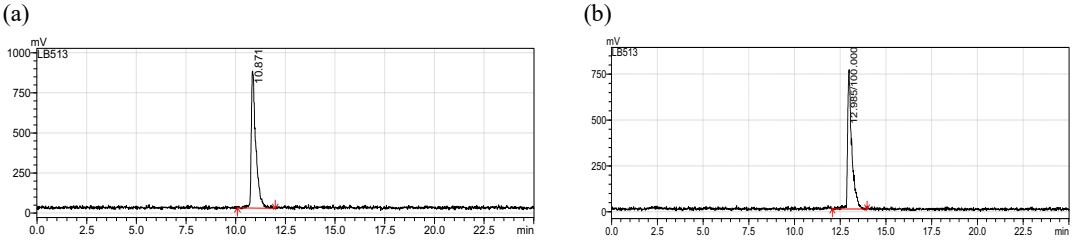


Figure 1. Effect of flow rate on peak form, symmetry, and retention time (a) flow rate of 1 mL/min and (b) flow rate of 0.5 mL/min, keeping the temperature constant at 35 °C

Following the change in column temperature, the difference between the retention time values obtained at 35 °C and those obtained at 30 °C was less than 1% (Figures 1 and 2). This small variation suggests that the proposed method is robust, demonstrating stability and resistance to temperature changes. The method provides reliable and accurate results. The total analysis time for the method used was 25 minutes, which allows the evaluation of samples in a short and well-defined time interval [18].

(a) (b)

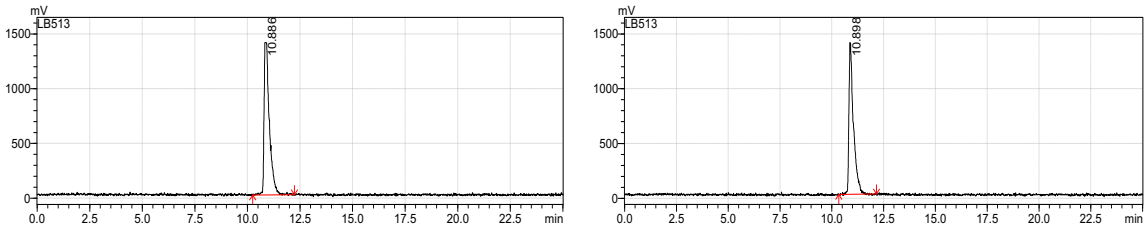


Figure 1. Effect of column temperature on analysis (**a**) 35 °C and (**b**) 30 °C, keeping the flow rate constant at 1 mL/min

To evaluate the performance of the method, four radiolabelled peptides ([^{64}Cu]Cu-DOTA-NMB, [^{64}Cu]Cu-DOTA-PEG(4)-BBN(7-14), [^{64}Cu]-DOTA-NOC and [^{64}Cu]-DOTA-cRGDmonomer) were selected and analysed according to the previously described method. In Table 2, the radiochemical purity values of these are presented.

Table 2. RCP values of radiolabelled peptides, determined by radio-HPLC

Peptides	RCP (%)
[^{64}Cu]Cu-DOTA-NMB	100%
[^{64}Cu]Cu-DOTA-PEG(4)-BBN(7-14)	99.99%
[^{64}Cu]Cu-DOTA-NOC	95.94%
[^{64}Cu]Cu-DOTA-cRGDmon.	92.67%

3.1.4.3. Radio-TLC analysis of synthesized radiopharmaceuticals. Influence of the mobile phase on liquid chromatography

Two mobile phases with significantly different compositions and chemical characteristics were compared for the analysis of [^{64}Cu]$CuCl_2$, [^{64}Cu]Cu-DOTA-NMB, and [^{64}Cu]Cu-DOTA-PEG(4)-BBN(7-14). Radio-TLC analyses were performed using silica gel plates on aluminium support as stationary phase. These plates were scanned using a bismuth germanate detector (BGO) equipped with interchangeable collimators

suitable for different energy ranges. Changes in R_f values were carefully monitored to optimize the separation process and identify chemical species. Mobile phase F1 (migration time 30 minutes) consisted of a solvent mixture of methanol and ammonium acetate (concentration 1 M) in a ratio of 7:3 (vol/vol). While the mobile phase F2 (migration time 15 minutes) was a 0.1 M sodium citrate solution. The chromatograms and ratios generated from the radio-TLC scans are presented in Figure 3.

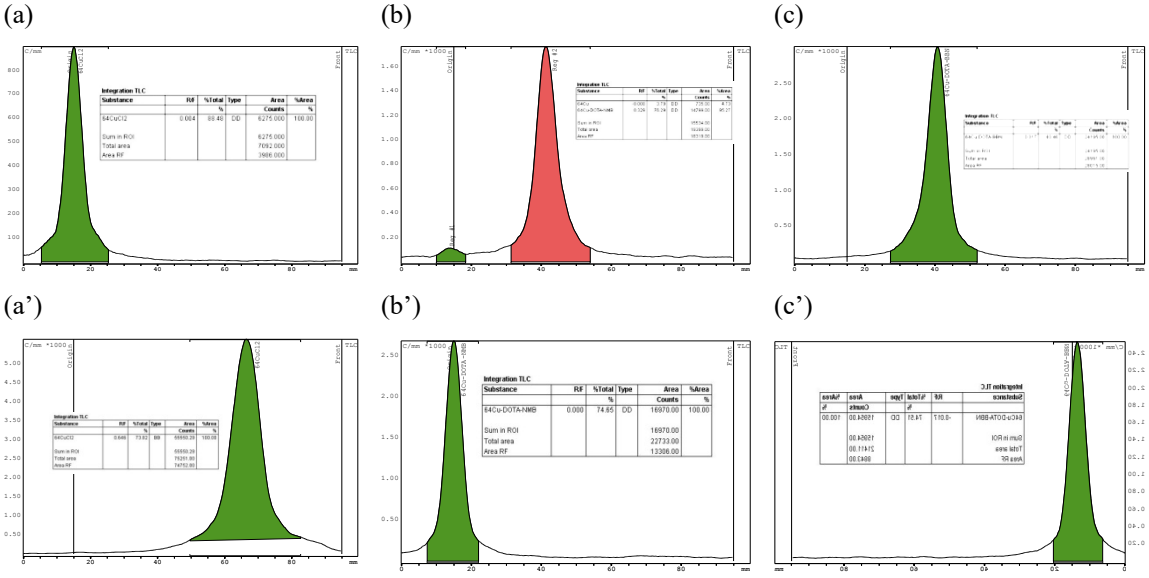


Figure 3. Radio-TLC chromatograms for (a) [^{64}Cu]$CuCl_2$, (b) [^{64}Cu]Cu-DOTA-NMB și (c) [^{64}Cu]Cu-DOTA-PEG(4)-BBN(7-14) in F1 and (a'), (b'), (c') in F2

When the F2 mobile phase was used, the radiolabelled peptides demonstrated a higher affinity for the stationary phase. Radio-TLC analyses revealed that the radiochemical purity of the analysed compounds greater than 95%, which had been confirmed by radio-HPLC. This demonstrates the consistency and precision of the developed methods.

3.1.4.4. Quality control of the radiolabelled neuropeptides [^{64}Cu]Cu-DOTA-NMN and [^{64}Cu]Cu-DOTA-NT(8-13)

In this study, a rigorous quality assessment of the compounds [^{64}Cu]Cu-DOTA-NMN and [^{64}Cu]Cu-DOTA-NT(8-13) was performed to test and compare their conformity to the strict standards defined by the Ph. Eur. for radiopharmaceuticals supplied as injectable solutions (Table 3) [18].

Table 3. Quality control results for the ^{64}Cu-labeled compounds [18]

Test	Acceptance criteria	[^{64}Cu]Cu-DOTA-NMN	[^{64}Cu]Cu-DOTA-NT(8-13)
Appearance	Clear, colourless	☑	☑
pH	4.0-8.0	7.5	7.5
Radionuclidic identity ($T_{1/2}$)	$T_{1/2}$= 12.7 h (± 10 %), 24h EOB	12.43 h	12.81 h
Radionuclidic identity	511±1 keV	☑	☑
Radionuclidic purity	> 99.99 % (spectrum γ) 24h EOB	☑	☑
Radiochemical purity	≥ 95 % (radio-TLC)	100 %	100 %
	≥ 95% (radio-HPLC)	100 %	100 %
Residual solvents	Ethanol ≤ 2.5 g/adm.	☑	☑
Endotoxins	≤ 175 EU/mL	☑	☑

3.1.4.5. Stability study

Radio-HPLC was used to examine the peptides stability 72 hours after they had been radiolabelled and purified. To evaluate the RCP status over time, four samples were collected from the same vial (final solution), and injected at different time intervals from the moment the synthesis was completed (Figure 4) [18].

(a) (b)

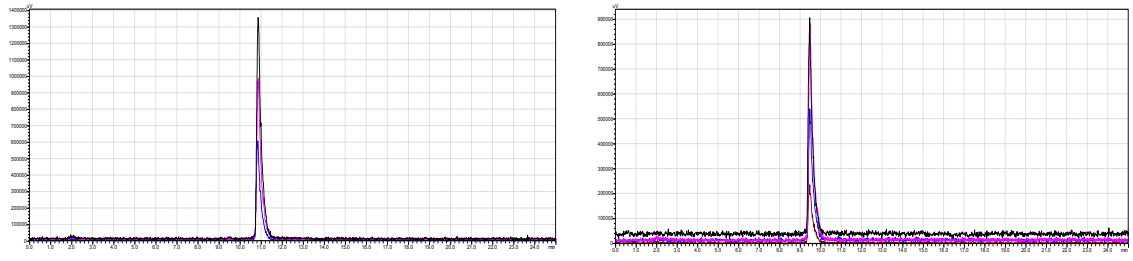


Figure 4. Stability study of radiopharmaceuticals (a) [^{64}Cu]Cu-DOTA-NMN and (b) [^{64}Cu]Cu-DOTA-NT(8-13)

3.2. OPTIMIZATION AND VALIDATION OF A HEADSPACE GAS CHROMATOGRAPHIC METHOD FOR VOLATILE RESIDUAL SOLVENTS IDENTIFICATION IN RADIOPHARMACEUTICAL PRODUCTS

3.2.1. Introduction

This study aims to optimize and validate an improved analytical method of gas chromatography using a flame ionization detector (FID) and a Headspace injection (HS) system, for the quantification of the level of residual solvents frequently used in the synthesis of radiopharmaceuticals, namely: methanol, ethanol, isopropanol, acetonitrile, and acetone [21].

3.2.2. HS-GC method

The residual solvents evaluation was performed using a gas chromatograph, GC Agilent 7890A, equipped with a FID detector, a 7697A Headspace, and an ALS G4513A autosampler. The chromatographic separation was performed on fused silica capillary column (6% poly[(cyanopropyl)-(phenyl)] siloxane and 94% poly(dimethyl) siloxane) with dimensions 30 m × 0.53 mm, 3 μm (Agilent J&W DB-624) [22]. OpenLab CDS software (Agilent Technologies Inc., USA) was used to perform system control, data acquisition, and processing [23].

3.2.3. Results and discussion

3.2.3.1. HS-GC method optimization

The analysis of residual solvents and implicitly the characterization of the method described in this study were performed following the adaptation and optimization of certain parameters of the method following current needs, starting from our previously reported work and the conditions provided by Eur.Ph. (Table 4)[23].

Table 4. HS-GC method parameters used for residual solvent analysis [23]

	Parameters	**Eur. Ph.** [24]	**Initials** [22], [25]	**Optimized**
GC	Carrier gas	He		
	Inlet split ratio	5:1	15:1	20:1
	Carrier flow rate (mL/min)	-	48	33
	Pressure (psi)	-	2.27	7.95

	Oven temperature (°C)	40-240	40-100	35-85
	FID temperature (°C)	250	250	300
	Analysis time (min)	35	7	7
HS	Equilibration temperature (°C)	80 -105	105	100
	Equilibration time (min)	45-60	2	3
	Transfer temperature (°C)	80-110	110	100
	Injection time (min)	-	0.2	0.5

3.2.3.2. Method characterization

The proposed method provides a rapid solution for residual solvents determination, with an analysis time of 7 minutes. All tested radiopharmaceutical batches met the acceptance standards, with residual solvent content below permissible limits, confirming the applicability and reliability of the method. This optimization is essential for radiopharmaceutical applications, where speed and accuracy are essential. Therefore, in this study, a simple, reliable, and efficient HS-GC method was optimized, validated, and implemented for the simultaneous determination of five residual solvents (methanol, ethanol, acetone, isopropanol, and acetonitrile) in radiopharmaceutical products [23].

3.2.3.3. System testing

System testing was carried out to show that the technique can identify and separate residual solvents present in [^{64}Cu]Cu-DOTA-NT sample, with ethanol being the solvent of interest. Compared to the previous method, this method enables earlier detection of solvent peaks, improving the overall efficiency of the process without compromising accuracy [23].

4. SYNTHESIS AND EVALUATION OF ^{89}Zr COMPLEXES: OBTAINING AND QUALITY CONTROL OF RADIOPHARMACEUTICALS RESULTING FROM IRRADIATION OF SOLID TARGETS

4.1. ^{89}Zr production and radiochemical processing of the irradiated target

A ^{nat}Y foil was bombarded with protons at the TR-19 cyclotron (ACSI, Canada) to produce the ^{89}Zr radioisotope, following the nuclear reaction ^{89}Y(p,n) ^{89}Zr,. After

irradiation, the target was dissolved in 2M and 4M HCl at 100°C for approximately 50 minutes. A Zr resin (TrisKem International, France) was used to purify the [^{89}Zr]$ZrCl_4$ solution that was obtained after dissolution. Water and 2M HCl were used to eliminate the impurities from the solution. Finally, ^{89}Zr was eluted from the cartridge using 0.1 M and 0.5 M oxalic acid, yielding [^{89}Zr]Zr-oxalate.

4.2. Conjugation reaction of p-SCN-Bz-DFO chelator with mAb

- *p-SCN-Bz-DFO – anti-HER2*

The anti-HER2/ErbB2 conjugation reaction with p-SCN-Bz-DFO was carried out at a mass ratio of 1:4 (mAb: chelator). To 50 µL or 150 µL of anti-HER2/ErbB2 solution (0.49 ± 0.02 mg/mL), 5 µL or 15 µL of the p-SCN-Bz-DFO stock solution were added. The pH of the conjugation solution was adjusted to 9.20 ± 0.2 using a 0.1M Na_2CO_3 buffer solution. The resulting mixture was incubated at 37 °C for 30 minutes in a Thermoshaker at 300 rpm [26].

- *p-SCN-Bz-DFO –anti-HER2 affibody*

The conjugation of p-SCN-Bz-DFO to the anti-HER2 affibody was performed according to the previously described procedure, with minor modifications to the antibody fragment concentration and reaction time. Two different concentrations of anti-HER2 affibody were tested (0.3 mg/mL and 0.5 mg/mL), and the reaction time was extended from 30 to 60 minutes for both. To maintain a 1:4 mass ratio between the mAb and chelator, 10 µL and 12 µL of p-SCN-Bz-DFO stock solution were added to 100 µL (0.5 mg/mL) and 200 µL (0.3 mg/mL) of anti-HER2 affibody solution, respectively. The conjugation reactions were carried out in a Thermoshaker at 37°C, 300 rpm, and pH 9.20 ± 0.2. The chelator-to-anti-HER2 affibody conjugation ratio was evaluated using mass spectrometry.

4.3. Purification of the p-SCN-Bz-DFO-mAb conjugation

After 30 and 60 minutes, respectively, the unconjugated chelator was removed by size exclusion chromatography using a PD-10 column and 0.9% NaCl saline solution [27]. The conjugate solutions were diluted with 0.9% NaCl to a final volume of 1 mL before eluting on the column. The unbound chelator was removed by washing with 1.5 mL of 0.9% NaCl, and the chelator-mAb conjugate that was retained on the column was eluted with 2.5 mL of 0.9% NaCl.

4.4. ^{89}Zr radiolabelling of the p-SCN-Bz-DFO-mAb conjugation

This study examined the radiolabelling of two types of immunoglobulins, anti-HER2/ErbB2 and anti-HER2 affibody, using ^{89}Zr. These immunoglobulins were initially conjugated to the bifunctional chelator p-SCN-Bz-DFO before being radiolabelled at 37°C for 60 minutes with a [^{89}Zr]Zr-oxalate solution using a Thermoshaker set to 550 rpm. The [^{89}Zr]Zr-oxalate solution was neutralized with 2M Na_2CO_3 to a physiological pH of 7.20 ± 0.2 before being added to the conjugates [28]. The p-SCN-Bz-DFO–anti-HER2 and p-SCN-Bz-DFO–anti-HER2 affibody immunoconjugates were radiolabelled with 0.5–1.0 mL of [^{89}Zr]Zr-oxalate solution. To optimize the reaction and assess the impact of pH on labelling, the pH was varied between 7.0 and 9.2. After a 60-minute reaction, the solutions were left to cool at room temperature and subsequently purified using a PD-10 column.

4.5. Characterization of synthesized ^{89}Zr(IV) complexes

As with any radiopharmaceutical used in molecular imaging and described in the European Pharmacopoeia, the product must undergo rigorous quality control procedures. In this study, the following quality control parameters were assessed: appearance, pH, radiochemical purity, radionuclide purity and identity, stability, and reaction yield.

Radiochemical and radionuclidic purity

The radiochemical purity was determined using TLC plates composed of silica gel on plastic support with 0.1M sodium citrate as the mobile phase. Also, for the solutions of [^{89}Zr]$ZrCl_4$ and [^{89}Zr]Zr-oxalate, both types of stationary phases (cellulose vs. silica gel) were evaluated with four distinct mobile phases.

The purity and radionuclide identification were determined by measuring the half-life of the radioisotope ^{89}Zr with a dosage calibrator. This was determined after three successive assessments of the [^{89}Zr]Zr-oxalate sample's activity over 24 hours. Further, the characteristic ^{89}Zr peak at 909 keV was detected using a gamma-ray spectrometer with an HPGe detector.

Stability studies of ^{89}Zr(IV) complexes in physiological, human and rat serum

The stability of ^{89}Zr(IV) complexes was evaluated in physiological, human, and rat serum. To prepare the sample, a radioactive substance with an activity of 5.5-10

MBq was used and 200 µL of serum was added. The samples were incubated at 37 °C with periodic shaking for up to 140 hours. The samples stability was assessed via a radio-TLC method.

4.6. Results and discussion

4.6.1. ^{89}Zr production, post-irradiation processing and physicochemical characterization of the [^{89}Zr]Zr-oxalate solution

Following ^{nat}Y foil irradiation to the TR-19 cycle, a mean activity of 2.67 ± 0.81 GBq/series was obtained. The [^{89}Zr]$ZrCl_4$ solution, obtained after dissolving the target in HCl, was passed through an ion exchange column pre-loaded with a fenamic acid derivative (R-CO-NH-OH). To remove metallic impurities, the column was washed with 2M HCl and water. Finally, ^{89}Zr was eluted as oxalate using 0.1M and 0.5M oxalic acid solutions. After dissolving and purification of the irradiated target, a [^{89}Zr]Zr-oxalate solution with an activity of 1.95 ± 0.7 GBq was obtained, corrected to EOB.

The resulting gamma spectrum (Figure 5) revealed the presence of only the specific ^{89}Zr peaks (909 keV and 511 keV), without other contaminated radioisotopes. These results indicate a RNP of ≥ 99.9% for [^{89}Zr]Zr-oxalate solution.

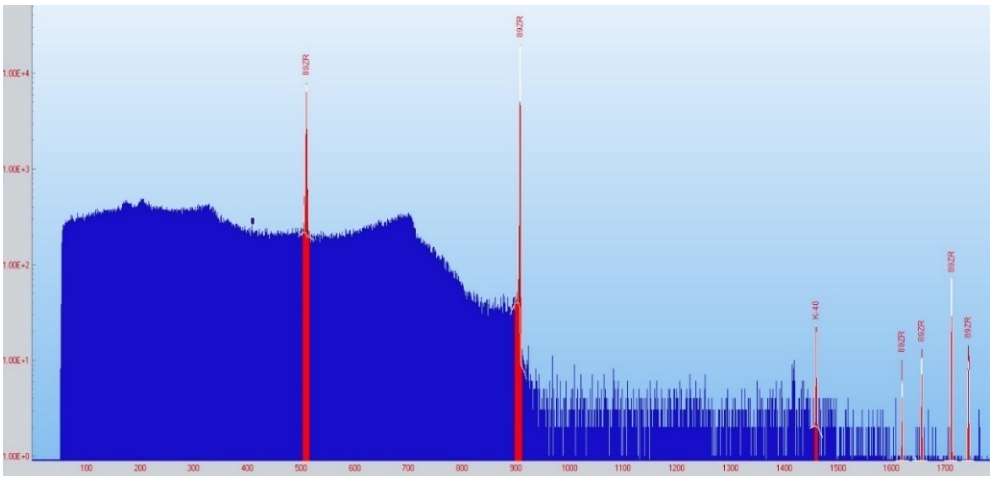


Figure 5. Gamma spectrum of [^{89}Zr]Zr-oxalate solution

The radio-TLC/0.1M sodium citrate/silica gel method was used to assess the radiochemical purity of the [^{89}Zr]Zr-oxalate solution during the purification and pH adjustment processes. The final [8^{9}Zr]Zr-oxalate solution used in the labelling process had a pH of 7.2 ± 0.2, and a radiochemical purity ≥ 95%.

4.6.2. Adaptation and optimisation of the radio-TLC method for the analysis of ^{89}Zr-labelled compounds

This study aimed to find an appropriate, fast, and efficient method for identifying and separating different ^{89}Zr complex molecules. Four different mobile phases were compared in this experiment with two types of stationary phases (silica gel and cellulose). The mobile phases compared in the study were: (Method 1) 0.1 M sodium citrate, (Method 2) a mixture of methanol and 1 M ammonium acetate in a molar ratio of 7:3 (vol/vol), (Method 3) 20 mM citric acid buffered with 2M Na_2CO_3 at a pH of 5.0, and (Method 4) a mixture of methanol and water acidified with 4% TFA in a molar ratio of 1:1. All radio-TLC tests were performed with a sample volume of 3 µL.

Based on the results, Method 1, which employs a 0.1 M sodium citrate mobile phase and a silica gel stationary phase, proved to be the most efficient and reproducible approach for separating [^{89}Zr]Zr(IV). This method generated distinct peaks, indicating well-controlled sample migration. Consequently, this mobile phase was used in all subsequent experiments, with a solvent migration time of approximately 15 minutes on the plate.

4.6.3. Radiolabelling evaluation of [^{89}Zr]Zr-oxalate immunoconjugates

- *[^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2*

For the radiolabelling of the anti-HER2/ErbB2 monoclonal antibody, two [^{89}Zr]Zr-oxalate solutions with different radioactive concentrations (0.23 and 0.43 MBq/µL) were used. The radiolabelling efficiency was assessed by radio-TLC. In the first experiment, 1 mL of [^{89}Zr]Zr-oxalate (0.43 MBq/µL) was used to label 0.181 nmol of antibody, obtaining a yield of 2.16%. To improve this yield, the antibody quantity and the [^{89}Zr]Zr-oxalate activity were adjusted. In the second experiment, increasing the antibody quantity to 0.543 nmol and reducing the [^{89}Zr]Zr-oxalate activity to 0.23 MBq/µL significantly enhanced the reaction yield to 12.73%. The resulting [^{89}Zr]Zr–p-SCN-Bz-DFO–anti-HER2 solutions were clear and colourless, with a radionuclidic purity (RNP) of ≥ 99.9% and a radiochemical purity (RCP) ranging from 3.41% to 42.51%. RCP was evaluated before and after purification using a PD-10 column.

- *[^{89}Zr]Zr –p-SCN-Bz-DFO –anti-HER2 affibody*

In these experiments, different volumes of [^{89}Zr]Zr-oxalate solution (0.5-1.1 mL) were used for radiolabelling different quantities of anti-HER2 affibody (6.89 and

8.25 nmol), while maintaining a constant temperature (37°C), reaction time (1 h), and labelling pH within the range of 7.0-9.0 (Table 5). This method permitted the evaluation of radiolabelling efficiency under different conditions, which was essential for optimising the overall process.

Table 5. Parameters used in the radiolabelling process of the anti-HER2 affibody with ^{89}Zr

Parameters	[^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody					
	Exp.1	***Exp. 2***	***Exp.3***			
Quantity of affibody (nmol)	6.89	8.25	8.25			
Quantity of p-SCN-Bz-DFO (nmol)	265	318.8	318.8			
pH labelling	7.2 ± 0.2	7.3 ± 0.2	7.0-7.5	7.5-8.0	8.0-8.5	9.0
[^{89}Zr]Zr-oxalate volume (µL)	1040 ± 106.1	1050	500 ± 0.2			
[^{89}Zr]Zr-oxalate activity (MBq)	265.5 ± 91.2	460	112.1 ± 8.2			
Temperature (°C)	37	37	37			
Reaction time (min)	60	60	60			
Radiochemical yield (%)	75.09 ± 7.2	33.83	78.41	45.45	14.59	11.96
Molar activity (MBq/nmol)	26.5 ± 4.4	18.86	11.45	5.97	2.07	1.49
RCP (%)	97.96	42.05	87.96	50.99	16.35	13.41

The initial experiment assessed the radiolabelling process of the p-SCN-Bz-DFO-anti-HER2 affibody immunoconjugate using a small amount of anti-HER2 affibody (6.89 nmol) and a [^{89}Zr]Zr-oxalate solution with a radioactive concentration of approximately 0.25 ± 0.06 MBq/µL. This experiment served as a reference for establishing the baseline conditions for the labelling procedure, considering the parameters adjusted in experiments 2 and 3. The labelling yield was approximately 75%, indicating relatively high efficiency under the given experimental conditions. The radiochemical purity before and after purification exceeded 97% (Figure 6).

(a) (b)

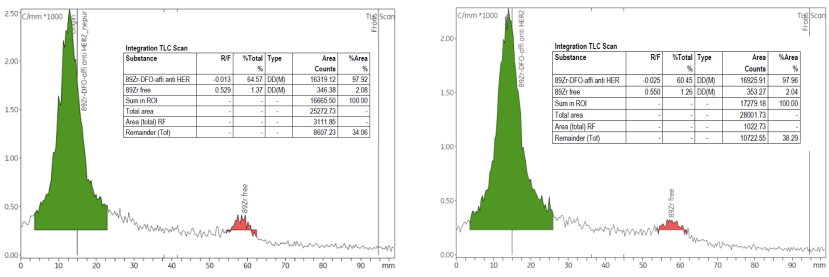


Figure 6. Radio-TLC chromatograms for the [^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody complex (Experiment 1): (a) before purification and (b) after purification

In experiment 2, 8.25 nmol of anti-HER2 affibody was radiolabelled with a radioactive concentration of 0.44 MBq/µL from [^{89}Zr]Zr-oxalate solution. The labelling yield obtained in experiment 2 increased from 34% to approximately 68% within 12 hours after the start of the labelling reaction, indicating a value comparable to the results obtained in experiment 1, but still suggesting a lower labelling reaction efficiency. The [^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody compound radiochemical purity increased significantly from 42% to 84.62% (Figure 7).

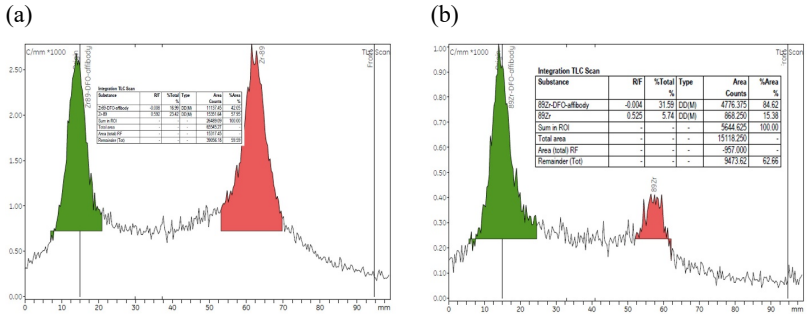


Figure 7. Radio-TLC chromatogram resulting from the analysis of the [^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody solution from Experiment 2 after (a) 1 hour from the initiation of the reaction and (b) overnight at 37°C

Based on the results of experiment 2, four different pH values (7.0-9.0) were used in experiment 3, while the quantity of anti-HER2 affibody (8.25 nmol) and chelator (318.8 nmol) were kept constant. Radio-TLC was used to assess the progress of the labelling processes at 1, 2, and 12-hour intervals after initiation. The results indicated a continuous increase in radiochemical purity after 2 hours, demonstrating that the process had not reached saturation and would continue to evolve (Figure 8).

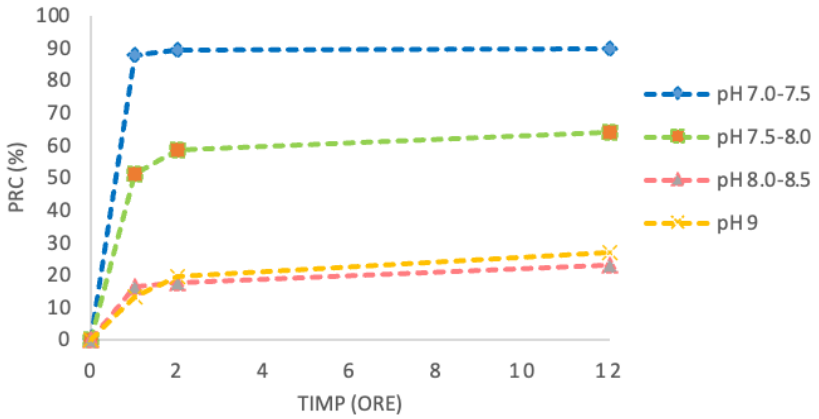


Figure 8. Influence of labelling pH on radiochemical purity over time

After 1 hour of labelling at pH 7.0 - 7.5, radiochemical purity increased to over 85% and remained stable over time. These results demonstrate that this pH range is optimal for both the radiolabelling process and the compound stability.

4.6.4. Stability Study

The stability of the ^{89}Zr(IV) complexes was investigated in both saline (0.9% NaCl) and biological conditions, including human and rat serum. The radiolabelled antibody fragment decreased the RCP by 7.1% in saline solution after 140 hours of incubation. However, the RCP increased to 53.42% in human serum and 35.63% in rat serum (Figure 9). These results indicate that, despite the labelled antibody fragment short plasma half-life, its stability in human and rat serum can be optimised for PET imaging applications.

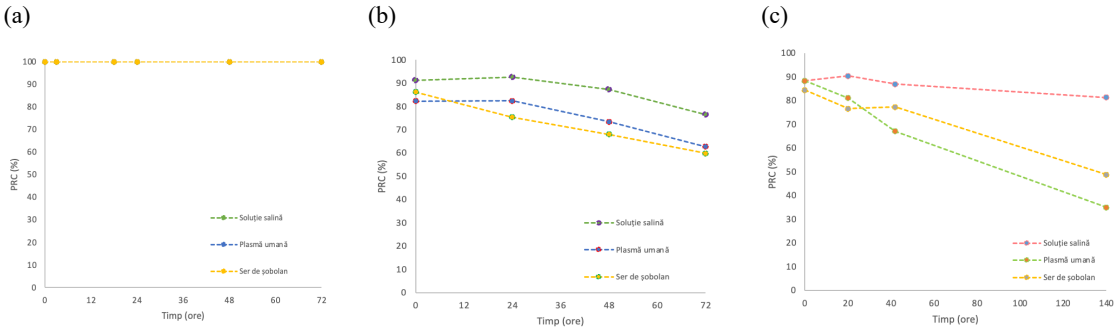


Figure 9. Stability study of compounds (a) [^{89}Zr]Zr-oxalate, (b) [^{89}Zr]Zr-p-SCN-Bz-DFO and (c) [^{89}Zr]Zr –p-SCN-Bz-DFO – anti-HER2 affibody in saline, human serum and rat serum

Other applications

In vitro, studies were conducted on breast tumour cell lines (MCF-7 and BT-474) to evaluate the uptake and retention of the [^{89}Zr]Zr–p-SCN-Bz-DFO–anti-HER2 affibody compound. The results demonstrated uptake of the compound in all cell lines analysed, with significantly higher retention in HER2-positive cell lines, suggesting increased affinity for this type of cell. This behaviour underscores the potential of the compound as a molecular imaging agent for HER2-positive tumours, enabling more precise identification.

5. EVALUATION OF RADIOLABELING AND CHARACTERIZATION PROCESSES OF RADIOPHARMACEUTICALS BASED ON ^{64}Cu AND ^{89}Zr, OBTAINED AFTER IRRADIATION OF LIQUID TARGETS

5.1. Production of ^{64}Cu and ^{89}Zr radioisotopes from liquid targets: post-irradiation processing and characterization

Experiments to obtain medicinal radioisotopes ^{64}Cu and ^{89}Zr were carried out at the KIUBE cyclotron (IBA, Louvain-la-Neuve, Belgium), located within the Institute of Nuclear Sciences Applied to Health (ICNAS) in Coimbra, Portugal. To produce ^{64}Cu, a liquid target (LT) of dissolved nickel-64 was irradiated [19], whereas ^{89}Zr was produced using a solution of yttrium nitrate.

- *Characterization of [^{64}Cu]$CuCl_2$ and [^{89}Zr]Zr-oxalate solutions*

Quality control was performed immediately after the purification step. The parameters monitored for each sample were: sample appearance, pH, radionuclidic identity, radiochemical and radionuclidic purity [19]. The purity and radionuclidic identity of the [^{64}Cu]$CuCl_2$ and [^{89}Zr]Zr-oxalate solutions were assessed by measuring the half-lives of the radioisotopes, using a dose calibrator, as well as by gamma-ray spectrometry, a method similar to that applied to solid targets [19]. Additionally, radiochemical purity was evaluated by radio-TLC, using 0.1 M sodium citrate as the mobile phase.

5.2. Radiolabelling of neuropeptides with ^{64}Cu(II) resulting from LT irradiation

The binding of the ^{64}Cu radiotracer to the DOTA-NMN and DOTA-NT(8-13) peptides was performed maintaining the same temperature and reaction time conditions as in the experiments with solid targets. However, for every 20 nmol of peptide, a double volume of approximately 2 mL of [^{64}Cu]CuCl2 solution (pH 3.8-4.0), with a radioactive activity of 861.8 ± 17.3 MBq/mL was used [19]. Following the radiolabelling process, the solution was purified using the SPE Cartridge, Oasis HLB Plus Extraction. The labelled peptide was then eluted from the cartridge with 1 mL of

ethanol and evaporated to near dryness at 80 °C. After evaporation, it was recovered in 1 mL of saline solution, and the pH was adjusted, if necessary, to a range of 7.0-7.5 [19].

5.3. Characterization of neuropeptides labelled using the [^{64}Cu]$CuCl_2$ solution obtained by LT irradiation

The radiochemical purity of the radiolabelled peptides, [^{64}Cu]Cu-DOTA-NMN and [^{64}Cu]Cu-DOTA-NT(8-13), was evaluated using an Agilent 1260 Infinity II radio-HPLC system equipped with a UV-VIS detector and an ELYSIA Raytest gamma radiation detector. The chromatographic column used was an Agilent Eclipse XDB-C18, 4.6 x 150 mm, with a particle size of 5 µm. The mobile phase consisted of water with TFA (solvent A) and acetonitrile (solvent B). The analysis was performed using a linear gradient at a flow rate of 3.5 mL/min. The analysis time was 5 minutes [19].

5.4. Labelling of HER2 receptor-specific vector with the ^{89}Zr radioisotope obtained by LT irradiation

- *Conjugation of the chelator to the carrier biomolecule*

To maximise labelling reaction yield, the p-SCN-Bz-DFO chelator was conjugated with biological molecules following a well-established methodology. 188 µg/47 µL of Trastuzumab (concentration 4 mg/mL) was incubated with 9.4 mg/10 µL p-SCN-Bz-DFO for 90 minutes in a Thermoshaker at 37 °C, establishing a 1:50 ratio between the two. The reaction was carried out in a total volume of 530 µL, including 453 µL 0.9% NaCl and 20 µL 0.1M Na_2CO_3. For the anti-HER2 affibody, 141 µg/300 µL (concentration 0.5 mg/mL) was mixed with 600 µg/30 µL of p-SCN-Bz-DFO, achieving a 1:4 ratio (chelator: biomolecule). The pH was adjusted to 9.0 using 50 µL 0.1 M Na_2CO_3, and the solution was incubated for 30 minutes. The immunoconjugates were purified using the PD-10 preparative column, to remove excess uncoupled chelator.

- *Radiolabelling of Trastuzumab with [^{89}Zr]Zr-oxalate solution*

To label Trastuzumab, 950 µL of [^{89}Zr]Zr-oxalate solution (33.29 MBq) was used. The pH was adjusted to 7.00 using 315 µL of 0.1 M Na_2CO_3 buffer. The [^{89}Zr]Zr-oxalate solution was treated with 500 µL of 0.5M HEPES (pH 6.8) and 500 µL of p-SCN-Bz-DFO-Trastuzumab solution. The reaction was incubated in a Thermoshaker at

37°C for 1 hour. Following this, the resultant solution was purified using a PD-10 column.

- *Radiolabelling of anti-HER2 affibody with [^{89}Zr]Zr-oxalate solution*

3 mL of [^{89}Zr]Zr-oxalate solution (98.09 MBq) was added to 2.5 mL of purified p-SCN-Bz-DFO-anti-HER2 affibody. The reaction was incubated for 3 hours at 37°C in a Thermoshaker, followed by purification using a PD-10 column. The parameters for the labelling process were adjusted to meet the experimental conditions for liquids while following the main steps outlined for solid experiments.

5.5. Characterization of ^{89}Zr(IV) complexes synthesized by LT irradiation

Radio-TLC analysis was performed on cellulose plates (15 x 1.5 cm), using a 0.1M sodium citrate solution (pH 5.0) as the mobile phase. A volume of 10 µL of the sample was spotted on each plate, and the analysis time was 5 minutes.

Radio-HPLC analysis of the [^{89}Zr]Zr-p-SCN-Bz-DFO-Trastuzumab complex was performed using the same system for the labelled neuropeptides but with a Superdex 200 10/300 GL column. The mobile phase consisted of a mixture of 13.8 g sodium dihydrogen phosphate, 14.2 g disodium hydrogen phosphate, 17.4 g sodium chloride and 1.3 g sodium azide, all dissolved in 2 L of water, with a pH between 6.2 and 7.0. The mobile phase flow rate was 0.5 mL/minute, and the column was maintained at 26 °C. A sample volume of 20 µL was injected, and the analysis time was 1 hour.

5.6. Results and discussion

5.6.1. [^{64}Cu]$CuCl_2$ solution production and characterization

Following the production of the medical radioisotope ^{64}Cu at the Kiube cyclotron, within the ICNAS Institute in Portugal, and the post-irradiation processing, an average radioactive activity of 3.7 ± 0.2 GBq (corrected at EOB) was obtained. This value is significantly lower than the 16.2 ± 0.8 GBq obtained by irradiating solid targets [19]. During quality control, the [^{64}Cu]$CuCl_2$ solution had a clear, colourless appearance, a radiochemical purity of 100% (determined by radio-HPLC and radio-TLC), a radionuclidic purity of over 99.99% (determined by gamma spectrometry) and a half-life of 12.4 ± 0.2 hours, measured by the dose calibrator [19].

5.6.2. Neuropeptide radiolabelling with [^{64}Cu]$CuCl_2$ obtained by LT

The [^{64}Cu]$CuCl_2$ solutions resulting from post-irradiation processing by each of the two methods (ST and LT) were subjected to similar radiolabelling procedures. These procedures involved concentration by evaporation and pH adjustment to 3.8-4.0 using ammonium acetate [19]. Depending on the production method, different volumes of [^{64}Cu]$CuCl_2$ solution were used for radiolabelling the same quantities of neuropeptide (20 nmol). The LT experiments used a [^{64}Cu]$CuCl_2$ solution with a radioactive concentration of 492.5 ± 9.8 MBq/mL and a volume of 1.75 mL, compared to the ST experiments that used a concentration of 1340.4 ± 70.1 MBq/mL and a volume of 1 mL [19]. The radiolabelling parameters for the LT experiments are presented in the table below.

Table 6. Radiolabelling parameters used in liquid target experiments (LT) [19]

Parameters	**Peptides**	
	DOTA-NMN	**DOTA-NT(8-13)**
Quantity (nmol)	20	
Radiolabelling pH	3.9 ± 0.1	
[^{64}Cu]$CuCl_2$ volume (mL)	1.75 ± 0.25	
[^{64}Cu]$CuCl_2$ activity (MBq)	861.8 ± 17.3	
Temperature (°C)	95	
Reaction time (min)	15	
Radiolabelling yield (%)	61.22 ± 2.45	63.24 ± 2.53

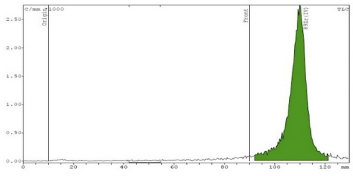
The neuropeptides DOTA-NMN and DOTA-NT(8-13) were successfully radiolabelled with the radioisotope ^{64}Cu, resulting in high radiochemical purity of over 99% and molar activities of 27.2 and 26.4 GBq/µmol for the LT experiments, compared to 45 and 52 GBq/µmol for the ST ones. The Ligand Tracer technique was used to determine the specificity of the synthesised radiopharmaceuticals on various colon and prostate cancer tumour cell lines. Following the evaluation of this in vitro study, a significant interaction between [^{64}Cu]Cu-DOTA-NT(8-13) and the colon cancer tumour lines (HT29 and HCT116) was discovered.

5.6.3. [^{89}Zr]Zr-oxalate solution production and characterization

Zirconium-89 was produced by irradiating liquid targets at the IBA Kiube cyclotron, with a radioactive activity of approximately 200 MBq, corrected to EOBafter 5 hours of irradiation. After irradiation, the solution was purified using an in-house manufactured cartridge containing resin like that used in the ST experiments to remove

metallic impurities. The [^{89}Zr]Zr-oxalate solution was then eluted from the column with 1 M oxalic acid. The radiochemical purity of the [^{89}Zr]Zr-oxalate solution was evaluated by both radio-TLC and radio-HPLC, and the final purity was over 99.9%.

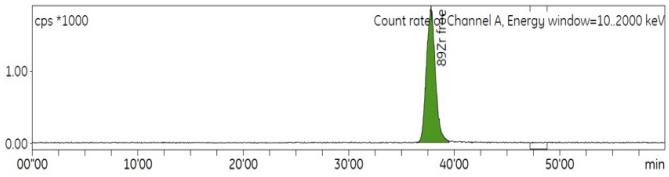
(a)



Integration TLC

Substance	R/F	%Total %	Type	Area Counts	%Area %
89Zr(IV)	1.249	90.50	DD	20358.27	100.00
-	-	-	-	-	-
Sum in ROI	-	-	-	20358.27	100.00
Total area	-	-	-	22494.51	-
Area (total) RF	-	-	-	656.08	-
-	-	-	-	-	-
Remainder (Tot)	-	-	-	2136.24	9.50

(b)



Integration Count rate of Channel A, Energy window=10..2000 keV

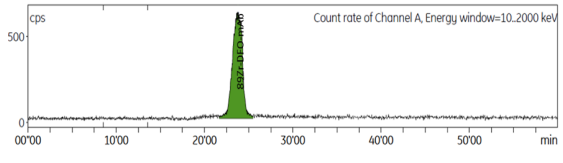
Substance	R/T s	Type	Area Counts	%Area %
89Zr free	37'46	DD(M)	110909.2	100.00
Sum in ROI	-	-	110909.2	100.00
Area (total)	-	-	112556.0	-
BKG1 (CPS)	-	-	14.34	-
Remainder	-	-	1646.83	1.46

Figure 10. (a) Radio-TLC and (b) radio-HPLC chromatograms of the [^{89}Zr]Zr-oxalate solution resulting from LT irradiation

5.6.4. Radiolabelling of Trastuzumab and anti-HER2 affibody with [^{89}Zr]Zr-oxalate obtained by LT irradiation

Radiolabelling of p-SCN-Bz-DFO-Trastuzumab and p-SCN-Bz-DFO-anti-HER2 immunoconjugates with [^{89}Zr]Zr-oxalate solution resulted in yields of over 60% for the LT experiments, compared to 12% ([^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2) and over 65% ([^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody) for ST experiments under similar conditions (labelling temperature and pH). The radiochemical purity of the radiolabelled compounds was determined using radio-TLC and radio-HPLC techniques. The RCP for anti-HER2 affibody (LT and ST) and Trastuzumab (LT) was ≥ 85%, while the RCP for anti-HER2 (ST) was around 44%.

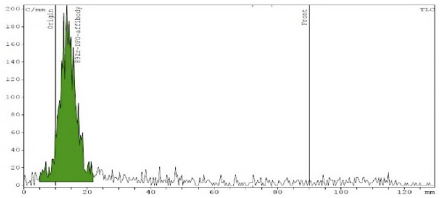
(a)



Integration Count rate of Channel A, Energy window=10..2000 keV

Substance	R/T s	Type	Area Counts	%Area %
89Zr-DFO-mAb	23'45	DD(M)	55754.05	100.00
Sum in ROI	-	-	55754.05	100.00
Area (total)	-	-	929969.64	-
BKG1 (CPS)	-	-	23.125	-
Remainder	-	-	874215.59	94.00

(b)



Integration TLC

Substance	R/F	%Total %	Type	Area Counts	%Area %
89Zr-DFO-affi	0.050	79.91	DD	1091.167	100.00
-	-	-	-	-	-
Sum in ROI	-	-	-	1091.167	100.00
Total area	-	-	-	1365.500	-
Area (total) RF	-	-	-	1095.278	-
-	-	-	-	-	-
Remainder (Tot)	-	-	-	274.33	20.09

Figure 11. Chromatogram (a) radio-HPLC generated for the [^{89}Zr]Zr-p-SCN-Bz-DFO-Trastuzumab and (b) radio-TLC for the [^{89}Zr]Zr-p-SCN-Bz-DFO-anti-HER2 affibody

GENERAL CONCLUSIONS

The research described in this doctoral thesis focused on the development and characterisation of radiopharmaceuticals that had specific targeting using the radioisotopes ^{64}Cu and ^{89}Zr. Analytical methods for quality control were developed, optimized and validated, according to the standards of the European Pharmacopoeia, ensuring their efficiency and quality in preclinical and clinical applications. Also, the processes of obtaining the two radioisotopes by irradiating solid and liquid targets were compared, and the results allowed the optimization of the biomolecule labelling processes, to obtain high yields and RCPs and long-term stability.

The principal objective of Chapter 1 of this thesis was to obtain ^{64}Cu through ST irradiation at the TR-19 cyclotron and to evaluate the peptide's radiolabelling process using this isotope. The $[^{64}Cu]CuCl_2$ solution met all of the European Pharmacopoeia's standards, with an RNP of over 99.99%, a RCP >95%, and excellent stability. Peptide radiolabelling resulted in yields >65% and a RCP >95%, demonstrating the process efficiency. Also, three chromatographic methods (radio-HPLC, radio-TLC, and HS-GC) were developed and optimised, showing precision, reproducibility, and sensitivity, allowing for rapid and accurate analysis of radiolabelled substances. These results illustrate the feasibility of using ^{64}Cu for peptide radiolabelling and the potential for PET imaging studies.

Chapter 2 focused on the evaluation and optimisation of the radiolabelling processes of anti-HER2 and anti-HER2 affibodies with ^{89}Zr, aiming to identify the optimal conditions for producing high-quality radiopharmaceuticals for HER2+ breast cancer imaging. The studies showed that, although mAb labelling resulted in low yields, the radiolabelling of the anti-HER2 affibody was successfully optimized, achieving yields >75% and RCP >80%. Also, the radio-TLC method using 0.1 M sodium citrate/silica gel proved to be the most efficient for separating $[^{89}Zr]Zr(IV)$ species, ensuring rapid and reproducible analysis. *In vitro* studies on breast cancer cell lines (BT-474 and MCF-7) demonstrated a promising potential of the radiolabelled compound for specific targeting of HER2+ cells, supporting its applicability in PET imaging.

The final experimental chapter of this thesis established the feasibility of producing ^{64}Cu and ^{89}Zr radioisotopes using liquid targets, illustrating both the advantages and limits of these methods when compared to irradiating solid targets.

Although the specific activities obtained were lower, the synthesized solutions complied with the requirements of the European Pharmacopoeia, allowing for the efficient radiolabelling of biomolecules of interest. Adapting the reaction conditions depending on the radioisotope and the carrier molecule was essential for optimizing the yield and radiochemical purities, demonstrating the applicability of both methods in the development of radiopharmaceuticals for PET imaging.

In conclusion, this thesis significantly contributes to the understanding and improvement of knowledge regarding the optimisation of radiopharmaceutical synthesis and analysis processes, providing a solid foundation for future research in this field. The results obtained bring new perspectives on the use of medical radioisotopes ^{64}Cu and ^{89}Zr in molecular imaging and personalized therapy, as well as contributions to the development of radiolabelling and chromatographic analysis methods in nuclear medicine.

ORIGINAL CONTRIBUTIONS AND PROSPECTS FOR FUTURE

The results described in this doctoral thesis represent the original contributions obtained from the research carried out during the doctoral studies. The study focuses on the development, optimisation and characterisation of biologically active compounds labelled with ^{64}Cu and ^{89}Zr radioisotopes, with applicability in PET molecular imaging and targeted therapy. The personal contributions found both in the synthesis process and in the characterisation of radiopharmaceuticals are detailed below:

- ✓ Development and optimisation of the synthesis processes of highly specific biomolecules (peptides, monoclonal antibodies and antibody fragments), using the medical radioisotopes ^{64}Cu and ^{89}Zr, aimed at precisely targeting receptors overexpressed in colon, prostate and breast cancer (HER2+);
- ✓ Implementation of efficient and reproducible radiolabelling methodologies, ensuring the obtaining of high-quality compounds and long-term stability in biological environments);
- ✓ Development, optimisation and implementation of advanced quality control techniques (radio-HPLC, radio-TLC, GC) for the assessment of radiochemical purity, as well as for determining the stability of compounds in different

environments similar to the human system (saline solution, human serum and rat serum);

- ✓ Validation of quality control methods according to international standards;
- ✓ Comparison of the proposed methodology with existing approaches, highlighting the advantages of its use;
- ✓ Evaluation of the *in vitro* potential of radiopharmaceuticals on cell lines specific to colon, prostate and breast tumours.

Prospects

The thesis has made significant contributions to the optimisation of radiolabelling processes of biomolecules with medical radioisotopes ^{64}Cu and ^{89}Zr, providing solutions for improving the efficiency and safety of radiopharmaceuticals used in PET imaging. In the future, further optimisation of experimental methodology and expansion of preclinical studies will facilitate the integration of these radiopharmaceuticals into medical practice. These compounds could lead to the precise localisation of tumours and monitoring of their long-term evolution. Also, future studies could focus on *in vivo* stability analysis, evaluating the amount of ^{64}Cu and ^{89}Zr released into the bloodstream under the influence of biological factors. The use of rigorous chromatographic methods in quality control will ensure reproducible and accurate results, essential for the development of this field.

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DISSEMINATION OF THE RESULTS OBTAINED WITHIN THIS DOCTORAL STUDY

I. Articles published in ISI Clarivate Analytics Web of Science (AIS) indexed and cited journals containing the results of the thesis

1. **M.R. Tudoroiu-Cornoiu**, E. L. Chilug, D. Cocioaba, S. Baruta, R. Șerban, A. C. Ion and D. Niculae (2024), "Assessment of chromatography separation parameters in the quality control of copper-64-labeled neurotensin-like peptides", Journal of Radioanalytical and Nuclear Chemistry, 333 (9), 1-13 (AIS = 0.24, IF = 1.5, SRI = 0.723, Q3).
   https://doi.org/10.1007/s10967-024-09694-1
2. **M.R. Tudoroiu-Cornoiu**, E. L. Chilug, D. Niculae, A. C. Ion (2024), "Validation of a headspace gas chromatography method for volatile residual solvents identification in radioactive drugs", University Politehnica of Bucharest Scientific Bulletin series B-chemistry and materials science, 86(4) (AIS = 0.043, IF = 0.3, SRI = 0.105, Q4)
3. D. Cocioabă, A.I. Fonseca, R. Leonte, I. Hrynchak, **R. Tudoroiu-Cornoiu**, S.J.C. do Carmo, B. Burghelea, S. Băruță, A.R. Almeida, R. Șerban, A. Dinischiotu, A.J. Abrunhosa & D. Niculae (2024), "Neurotensin (8-13) and Neuromedin N Neuropeptides Radiolabelling with Copper-64 Produced on Solid or Liquid Targets",
   Molecules, 29(6), 1390. (AIS = 0.676, IF = 4.2, SRI = 1.653, Q2).
   https://doi.org/10.3390/molecules2961390
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   https://doi.org/10.3389/fmed.2023.1197846
5. I. Hrynchak, D. Cocioabă, A.I. Fonseca, R, Leonte, S.J. do Carmo, **R. Cornoiu**, A. Falcão, D. Niculae & A.J. Abrunhosa, (2023), "Antibody and Nanobody

Radiolabeling with Copper-64: Solid vs. Liquid Target Approach", Molecules, 28(12), 4670. (AIS = 0.676, IF = 4.2, SRI = 1.653, Q2). https://doi.org/10.3390/molecules28124670

TOTAL SCORE = 2.495 AIS and 13.3 IF (SRI = 6.74)

II. Articles submitted for publication in ISI Clarivate Analytics Web of Science (AIS) indexed and cited journals containing the results of the thesis

1. D. Cocioabă, S. Băruță, L. Crăciun, R. Leonte, A. Necșoiu, **M.-R. Tudoroiu-Cornoiu**, A. Jipa and D. Niculae, (2025), "Optimized production of ^{89}Zr as a medical radioisotope on a variable energy cyclotron and external beam-line", EJNMMI Physics. (AIS = 1.008, IF = 3.0, SRI = 1.744, Q1).

III. Papers presented at international conferences (2021-2024)

1. Diana Cocioabă, Radu Leonte, **Roxana Tudoroiu-Cornoiu**, Bogdan Burghelea, Radu Șerban, Dragoș-Andrei Niculae, Simona Băruță, Andrei Necșoiu and Dana Niculae (2024), "^{64}Cu and ^{89}Zr – emerging radioisotopes produced at Radiopharmaceutical Research Centre @IFIN-HH", The 6th Romanian Congress of Nuclear Medicine together with the 2nd edition of International Conference of Applying Radionuclides in Therapy (ART), March 21 – 23, Brașov, Romania *(poster presentation).*
2. Andrei Necșoiu, Diana Cocioabă, Simona Băruță, **Roxana Tudoroiu-Cornoiu**, Radu Leonte, Dana Niculae (2024), "Production, characterization and translation of radioisotopes of medical interest at the Radiopharmaceuticals Research Center (IFIN-HH, CCR)", The 6th Romanian Congress of Nuclear Medicine together with the 2nd edition of International Conference of Applying Radionuclides in Therapy (ART), March 21 – 23, Brașov, Romania *(poster presentation).*
3. Dana Niculae, Radu Șerban, Livia Chilug, Dragoș Andrei Niculae, Diana Cocioabă, **Maria-Roxana Cornoiu**, Anca Dinischiotu, Ionela Neagoe, Gina Manda (2023), "Investigation of therapeutic effect, associated hypoxia and antioxidant signaling induced by copper-64 in colon carcinoma", 25th iSRS, May 22-26 Honolulu, Hawaii *(oral presentation).*

4. Ivanna Hrynchak, Diana Cocioabă, Radu Leonte, Magda Silva, **Roxana Cornoiu**, Dana Niculae, Antero Abrunhosa (2023), "GMP Production of Copper-64 for antibody and nanobody radiolabeling: liquid vs solid target", 25th iSRS, May 22-26 Honolulu, Hawaii, *(poster presentation).*
5. Diana Cocioabă, Radu Leonte, Bogdan Burghelea, **Roxana Cornoiu**, Radu Șerban, Alina Raicu, Simona Bărută, Andrei Necșoiu, Dana Niculae (2023), "Pharmaceutical grade processing of 89Zr and 64Cu medical radioisotopes", 10th Balkan Congress of Nuclear Medicine together with 5th Romanian Congress of Nuclear Medicine, March 15th – 18th, Bucharest, Romania *(poster presentation+ oral presentation).*
6. Radu Șerban, Dragos Niculae, Ionela Neagoe, **Roxana Cornoiu**, Diana Cocioabă, Mihaela Temelie, Gina Manda, Anca Dinischiotu, Dana Niculae (2023), "*In vitro* assessment of cellular response to the internal radiotherapy delivered by Auger-electron and beta emissions of Copper-64", 10th Balkan Congress of Nuclear Medicine together with 5th Romanian Congress of Nuclear Medicine, March 15th – 18th, Bucharest, Romania *(poster presentation+ oral presentation).*
7. **Roxana Cornoiu**, Livia Chilug, Radu Șerban, Radu Leonte, Diana Cocioabă, Bogdan Burghelea, Alina Raicu, Dana Niculae (2023), "Radiolabeling of peptides with copper-64 as part of drug development process", 10th Balkan Congress of Nuclear Medicine together with 5th Romanian Congress of Nuclear Medicine, March 15th – 18th, Bucharest, Romania *(poster presentation+ oral presentation).*
8. Diana Cocioabă, **Roxana Cornoiu**, Radu Leonte, Bogdan Burghelea, Dana Niculae (2022), "Optimization of the cyclotron radioisotopes production and purification process using an automated solid targets irradiation system", European Nuclear Physics Conference (EuNPC), Oct 24 – 28, Santiago de Compostela, Spain *(poster presentation).*
9. Diana Cocioabă, Radu Leonte, Alexandra Fonseca, Bogdan Burghelea, **Roxana Cornoiu**, Livia Chilug, Antero Abruhnosa and Dana Niculae (2022), "Comparative study on ^{64}Cu production on variable energy cyclotrons TR-19 (ACSI) /KIUBE (IBA) using solid and liquid targets", European Symposium on Radiopharmacy and Radiopharmaceuticals *(oral presentation).*

10. Livia Chilug, Dana Niculae, Radu Leonte, Gina Manda, Radu Serban, **Maria-Roxana Cornoiu** (2022), "Preclinical assessment of nanoparticles conjugated with ^{64}Cu", 24th International Symposium on Radiopharmaceutical Sciences, May 29 - June 2, Nantes, France *(poster presentation).*
11. Radu Serban, Dana Niculae, Gina Manda, Ionela Neagoe, **Roxana Cornoiu**, Dragos Niculae, Mihaela Temelie, Anca Dinischiotu (2021), "Assessment of cellular response to internal radiotherapy delivery by Auger-electrons emissions of Cu-64", Adaptation of the tumour and its ecosystem to radiotherapies, September 22nd-25th, Le Bono, West of France *(oral presentation).*
12. Radu A. Leonte, Diana S. Cocioabă, Ramona D. Dusman, Bogdan G. Burghelea, Simona I. Baruta, **Roxana M. Cornoiu**, Liviu S. Crăciun, Dana Niculae (2021), "Production of Cu-64 on an automated irradiation and processing system – Process validation", Ninth International Conference on Radiation in Various Fields of Research (RAD), June 14-18, Herceg Novi, Montenegro *(oral presentation).*

IV. Papers accepted for presentation at international conferences (2025)

1. Dana Niculae, **Maria-Roxana Tudoroiu-Cornoiu,** Adina Gabriela Puiu, Radu Anton Leonte, Diana Cocioabă, Radu Șerban, Dragoș Andrei Niculae, Alina Catrinel Ion (2025), "^{89}Zr-radiolabeling of p-NCS-Bz-DFO-anti-HER2 affibody immunoconjugate and *in vitro* assessment of its potential in HER2+ breast cancer imaging", 26th iSRS, May 11-15, Australia *(oral presentation).*