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RESEARCH ARTICLE

^{90}Y-DOTA-Nimotuzumab: Synthesis of a Promising β$^-$ Radiopharmaceutical

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Abstract: ***Background*:** Nimotuzumab is a humanized anti-epidermal growth factor receptor (EGFR) monoclonal antibody, nowadays used for tumour immunochemotherapy. This study aimed to label the conjugate DOTA-nimotuzumab with yttrium-90, in order to provide a β$^-$ emitting radioimmunoconjugate (^{90}Y-DOTA-nimotuzumab) potentially useful to assess the feasibility of a new radio-guided surgery approach.

***Methods*:** The synthesis of ^{90}Y-DOTA-nimotuzumab was performed in two days. Nimotuzumab was conjugated with a 50-fold excess of DOTA and then labelled with $^{90}Y^{3+}$. The ^{90}Y-DOTA-nimotuzumab preparation was optimized considering several parameters such as pH, temperature and reaction volume. Moreover, the ^{90}Y-DOTA-nimotuzumab stability was evaluated in human plasma.

***Results*:** The radioimmunoconjugate ^{90}Y-DOTA-nimotuzumab was obtained with a radiochemical purity greater than 96%, and showed a good stability at 20°C as well as at 37°C in human plasma.

***Conclusions*:** The optimized conditions for a mild and easy preparation of ^{90}Y-DOTA-nimotuzumab joined to a promising stability under physiological conditions suggest to propose this radioimmunoconjugate as a potential diagnostic radiopharmaceutical for β$^-$ radio-guided surgery.

Keywords: ^{90}Y-DOTA-nimotuzumab, radio-guided surgery, molecular probe technique, radiopharmaceutical, monoclonal antibody, immunochemotherapy.

1. INTRODUCTION

Radio-guided surgery guides the surgeon to identify and resect a target tissue like a tumour lesion minimizing the amount of healthy tissue removed [1]. This technique includes the preoperative injection of a radiopharmaceutical followed by the intraoperative identification of the target lesion through a handheld probe that detects radioactive signal [1]. To date, γ emitting radioisotopes like technetium-99m (^{99m}Tc) are mainly used for radio-guided surgery [1, 3-9]. However, γ radiations are more penetrating than both α and β radiations and pass through a large amount of healthy tissues [10]. Thus, the probe reveals photons originated from both tumour lesion (target) and healthy tissue (background) thereby the precision of target localization results was reduced and the background noise increased. To overcome these limits, Faccini and co-workers suggested the use of pure β$^-$ emitting radioisotopes like yttrium-90 (^{90}Y) that penetrates few millimetres of tissue with almost no scatter, rendering the bremsstrahlung contribution negligible [11, 12]. For this purpose, the authors developed a probe able to detect β$^-$ emission for an innovative radio-guided surgical approach. This β$^-$ probe, detecting electrons and operating with low background, enables a clearer delineation of margins of the radioactive tissue and requires administration of very low activity of radiopharmaceutical. Furthermore, it has a small size and thus is easy to handle in the surgical en-

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vironment. The radiation exposure to medical personnel is almost negligible due to the low absorbed dose and the short range of electrons, so allowing a larger number of radio-guided procedures per year [12]. So far, β^- probe has been tested *ex vivo* to detect the tumour margins of meningioma and high-grade glioma using ^{90}Y-DOTATOC as a β^- emitting radiopharmaceutical that, being a somatostatin analogue, binds to somatostatin receptors over-expressed in tumour cells. On the basis of these results, the authors evaluated the potentialities of this innovative radio-guided technique, demonstrating its feasibility [13]. There are until now few available β^- emitting radiopharmaceuticals that are potentially useful for β^- radio-guided surgery. To date, several works are investigating the development of new radiopharmaceuticals for this technique, such as polymeric nanoparticles loaded with ^{90}Y [14]. In particular, we identified ^{90}Y-DOTA-nimotuzumab, already used for tumour radioimmunotherapy, as a possible radiopharmaceutical for β^- radio-guided surgery. This radiopharmaceutical is a monoclonal antibody and binds to epidermal growth factor receptors (EGFR) over-expressed in cancer cells. In detail, nimotuzumab is an IgG subtype 1 kappa monoclonal antibody of approximatively 151 kDa and, is constructed by transplanting the conserved active domain of a murine antibody (anti-EGFR R3) to a human framework, using complementarity-determining region grafting [15]. It is directed against the domain III of the EGFR extracellular region inhibiting growth of tumour cells. It was proposed that the bivalent binding of nimotuzumab inhibited EGFR dimerization on cancer cells, dramatically reducing the signalling that promotes cell proliferation [16]. The method to label a monoclonal antibody with ^{90}Y is indirect and uses a bifunctional metal chelator [17, 18]. The most common bifunctional chelating agent used for labelling nimotuzumab with ^{90}Y is the so called 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-SCN-Bn-DOTA), due to its ability to strongly bind and form stable complexes with a large number of metal radionuclides, including yttrium-90. The present study was aimed at optimizing and improving the labelling procedure of nimotuzumab with yttrium-90, in order to easily provide the β^- emitting radioimmunoconjugate ^{90}Y-DOTA-nimotuzumab as a potential radiotracer suitable for assessing the feasibility of the new β^- radio-guided surgical approach.

2. MATERIALS AND METHODS

2.1. Equipments and Reagents

The monoclonal antibody was nimotuzumab, 5.0 mg/ml in phosphate buffered saline (CIMAher®, Oncoscience AG). The bifunctional chelating agent was *p*-SCN-Bn-DOTA (Macrocyclics). We used Vivaspin 20 and Vivaspin 0.5 with molecular weight cut-off of 30 kDa (Sartorius Stedim Biotech GmbH, Germany) as well as PD-10 desalting columns (GE Healthcare Bio-Sciences GmbH, Germany).

To estimate the protein concentration we used the Bradford assay (Bio-Rad Laboratories, Italy). UV measurements were obtained from an UV/Vis spectrophotometer (DU®530, Beckman Coulter).

We used several reagents (Sigma-Aldrich) for preparation and control of ^{90}Y-DOTA-nimotuzumab, including phosphate saline buffer, ammonium acetate, diethylenetriaminepentacetic acid (DTPA), methanol (MeOH), hydrochloric acid (HCl) and sodium hydroxide (NaOH). Deionized water was obtained from water purification system (Milli-Q Academic, Millipore), which was used to prepare all solutions for all procedures.

Carrier free $^{90}YCl_3$ (Perkin Elmer, Italy) was used. Radiochemical purity was assessed with ITLC-SG strip (Agilent Technologies, Italy) and 10% (w/v) ammonium acetate: methanol (1:1) as eluent system and it was analysed by an autoradiochromatography (Cyclone Plus, Perkin Elmer, Italy) and analysed using OptiQuant image analysis software.

2.2. Preparation of DOTA-Nimotuzumab

The synthesis of DOTA-nimotuzumab was performed according to Beckford-Vera's procedure [17], modifying some relevant steps in order to simplify the preparation, as strongly requested in radiopharmacy. We already improved the DOTA-nimotuzumab characterization by using high performance mass spectrometric techniques [19] and by optimizing the reaction procedures: with MALDI-TOF analysis, we obtained an average number of 20 DOTA and with SDS-PAGE analysis an average number of 28 DOTA. Moreover, in the constant region of Lc and Hc, we found two binding-sites in the Lc and two in the Hc [19].

The synthesis was performed in two days. The first day we conjugated DOTA to nimotuzumab, while the second day we labelled DOTA-nimotuzumab with $^{90}Y^{3+}$.

In particular, the starting solution of 5.0 mg/ml nimotuzumab was concentrated using Vivaspin 20; the protein concentration, evaluated with the Bradford assay, was 24.76 mg/ml ± 10.31. After that, ten milligrams of nimotuzumab were mixed with 50-fold molar excess of *p*-SCN-Bn-DOTA, previously dissolved in 400 µL of phosphate saline buffer (pH 8.5). The final pH of the reaction mixture was adjusted to 8.5 with NaOH 1M. The immunoconjugate DOTA-nimotuzumab was incubated overnight at 4°C. Afterwards, it was purified using a PD-10 desalting column, pre-eluted with ammonium acetate 0.01M. Three fractions were collected and concentrated with Vivaspin 0.5. Protein concentration was determined by Bradford assay and the fractions with highest protein concentration were collected. The immunoconjugate was stored at 4°C until use.

All the following tests were performed in triplicate and the results are shown as averages of those measurements (m) and with error bars (ds).

2.3. Radiolabelling and Quality Control of ^{90}Y-DOTA-Nimotuzumab

To optimize the radiolabelling of DOTA-nimotuzumab with $^{90}Y^{3+}$, we performed several tests evaluating the optimal reaction volume and pH. In detail, we tested three different reaction volumes (200 µL, 500 µL and 700 µL), according to literature [17]. In addition, with litmus paper, we per-

formed tests considering three distinct pH values (2.0, 4.0 and 6.0). We did not perform test in alkaline solution, in order to avoid the precipitation of yttrium as $Y(OH)_3$.

A mixture containing 300 µL of ammonium acetate (50 mM, pH 7.0), 20 µL of DOTA-nimotuzumab (240 µg) and 80 µL of $^{90}YCl_3$ (150 MBq) was incubated for two hours at 37°C. The unchelated $^{90}Y^{3+}$ was trapped through the addition of 20 µL of DTPA (50 mM, pH 6.0). The solution was then incubated for 15 minutes at 20°C (room temperature). The radiochemical purity (%R.P.) of the radiolabelled immunoconjugate was determined by using 10 cm ITLC-SG strip and 10% (w/v) ammonium acetate: methanol (1:1) as eluent system. The ITLC-SG strips were analysed by an autoradiochromatography system (Cyclone Plus®). Under these conditions, the Retention factor (Rf) of ^{90}Y-DOTA-nimotuzumab was at the origin (Rf = 0.0-0.1) and the unchelated $^{90}Y^{3+}$ (trapped with DTPA, ^{90}Y-DTPA) was at the front of the strip (Rf = 0.9-1.0) (Fig. **1**).

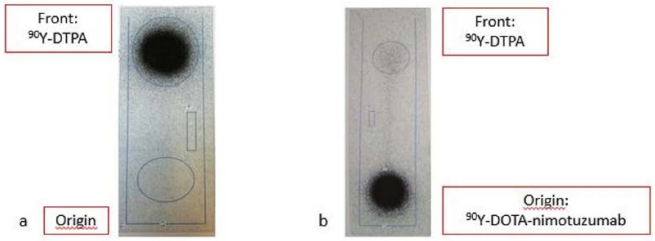

Fig. (1). a) ITLC-SG of ^{90}Y-DTPA complex as reference; **b)** ITLC-SG of the labeling reaction mixture after the addition of DTPA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2.4. Stability Tests of ^{90}Y-DOTA-Nimotuzumab

Stability tests of ^{90}Y-DOTA-nimotuzumab were performed at two different temperatures: room temperature and at 4°C. Samples were analysed at selected intervals of time up to 72 hours after labelling.

^{90}Y-DOTA-nimotuzumab was also incubated in 3 ml of sodium chloride solution (0.9%) and in 3 ml of human plasma at two different temperatures (room temperature and at 37°C, respectively). Both the mixtures were incubated for maximum 72 hours. Samples were analysed immediately and at selected intervals up to 72 hours after labelling.

The stability of radioimmunoconjugate was analysed using ITLC-SG strips and 10% (w/v) ammonium acetate: methanol (1:1) and evaluated by autoradiochromatography system (Cyclone Plus®).

3. RESULTS

3.1. Radiolabelling and Quality Control of ^{90}Y-DOTA-Nimotuzumab

The specific activity of radioimmunoconjugate ranged from 667 to 750 MBq/mg. High radiochemical purity was obtained, with values higher than 96% in each sample. These results were achieved without the purification of the final solution by size-exclusion chromatography on PD-10 desalting column as performed in other preparations [17].

The pH and reaction volume influenced the radiolabelling yield. We tested the radiolabelling efficiency of ^{90}Y-DOTA-nimotuzumab evaluating three pH values: at pH 2.0, we obtained a %R.P. of 48.6%, at pH 4.0 the %R.P. was 62.9% and at pH 6.0 was 96.1% (Fig. **2**). In consideration of that, at pH ranged from 5.5 to 6.0, we performed tests with different reaction volumes: in a final volume of 200 µL, %R.P. was 53.9%, in 500 µL was 96.1% and in 700 µL was 54.8% (Fig. **3**). Therefore the optimal conditions to obtain a stable radioimmunoconjugate were a value of pH = 6.0, and the reaction volume =500 µL.

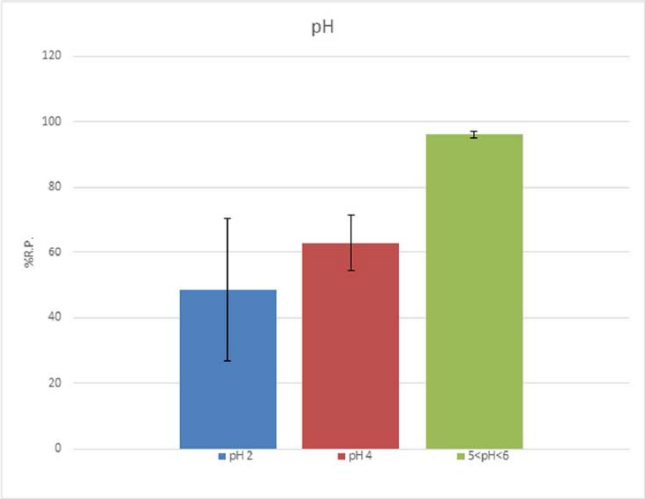

Fig. (2). ^{90}Y-DOTA-Nimotuzumab preparation at different pH values. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

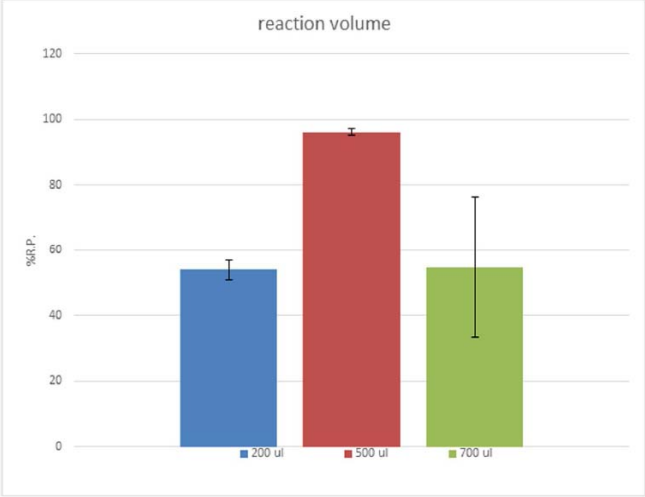

Fig. (3). ^{90}Y-DOTA-Nimotuzumab preparation in different final reaction volumes. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Stability Tests of ^{90}Y-DOTA-Nimotuzumab

The stability of the complex in the final solution decreased from a mean value of 97.0% at T0 to 88.0% at T72 when the storage was at room temperature, whereas when the storage was at +4°C the stability decreased from a mean value of 98.0% at T0 to 92.7% at T72 (Fig. **4**); the results obtained at 0h, 2h, 24h, 48h and 72h are reported in Table **1**. In both conditions of experimental incubation (in 0.9% NaCl and human plasma, at 37°C), the initial %R.P. was greater than 95%. ^{90}Y-DOTA-nimotuzumab was almost as stable in human plasma (from mean value of 95.2% at T0 to 81.9% at T72) as in 0.9% NaCl (from mean value of 95.6% at T0 to 70.9% at T72) (Fig. **5**). The results obtained at 0h, 2h, 24h, 48h and 72h are reported in Table **2**.

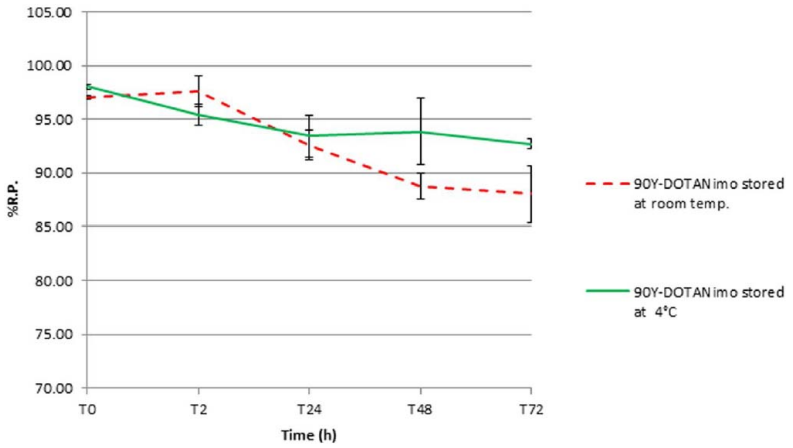

Fig. (4). *In vitro* stability studies of ^{90}Y-DOTA-nimotuzumab stored at room temperature and 4°C. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

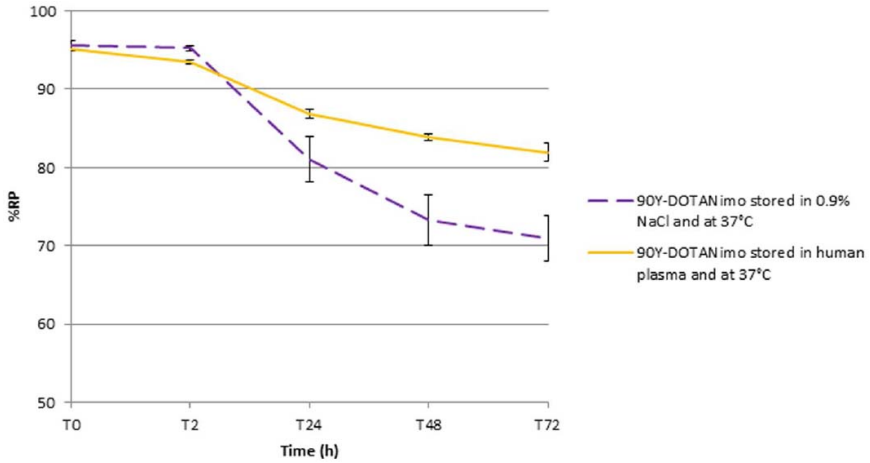

Fig. (5). *In vitro* stability studies of ^{90}Y-DOTA-nimotuzumab stored at 37°C in 0.9% NaCl and in human plasma. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Values of %R.P. of ^{90}Y-DOTA-nimotuzamab stored at room temperature (RT) and 4°C. (t test, p<0.05).

-	RT	4°C	p
T0	97	98	0.04
T2	97.6	95.4	0.53
T24	92.6	93.4	0.54
T48	88.8	93.9	0.07
T72	88	92.7	0.11

Table 2. Values of %R.P. of ^{90}Y-DOTA-nimotuzumab stored at 37°C in 0.9% NaCl and in human plasma. (t test, p<0.05).

-	Human plasma - 37°C	0.9% NaCl - 37°C	p
T0	95.2	95.6	0.47
T2	93.5	95.2	0.004
T24	86.8	81.7	0.07
T48	83.9	73.3	0.03
T72	81.9	70.9	0.03

Table 3. Parameters used to label the DOTA-nimotuzumab.

Authors	Radionuclide used for the labelling	Purification of raw materials (YES/NOT)	Final reaction volume used for the labelling (μ)	Final pH used for the labelling	Final purification of ^{90}Y-DOTA-nimotuzumab (YES/NOT)	Radiochemical Purity (%) after purification
Alvarez *et al*., 2011	Yttrium 90	n.d	225 - 228	n.d.	YES	> 97%
Beckford Vera *et al*., 2013	Yttrium 90	YES	51 - 130	n.d.	YES	> 98%
	Luthetium 177	YES	n.d.	n.d.	YES	> 98%
Martinez *et al*., 2014	Yttrium 90	n.d	≈210	n.d.	YES	> 98%
Beckford Vera *et al*., 2011	Luthetium 177	YES	46 - 603	n.d.	YES	> 97%
Barta *et al*., 2013	Luthetium 177	n.d.	83 - 192	n.d.	YES	> 99%
Beckford Vera *et al*., 2012	Luthetium 177	YES	83 - 192	n.d.	YES	> 98%
Caldaza *et al*., 2012	Luthetium 177	n.d	n.d.	n.d.	YES	n.d.
Martalena *et al*., 2012	Luthetium 177	NOT	> 1000	5.5	YES	> 98%
Pandey *et al*., 2019	Luthetium 177	n.d	≈400	6.0	YES	> 98%

4. DISCUSSION

In the process to provide a suitable radiopharmaceutical to be used for our innovative approach of radioguided surgery, we first identified ^{90}Y-DOTA-nimotuzumab, then we characterized the immunoconjugate in the paper by Martelli *et al*. [19]: briefly, with gel electrophoresis (SDS-PAGE) analysis, we found that from 0 to 4 DOTA bind the light chain (Lc) and from 0 to 24 the heavy chain (Hc) of nimotuzumab. As in the SDS-PAGE analysis, with MALDI-TOF analysis emerged that nimotuzumab bind from 0 to 35 DOTA molecules. Moreover, after separation of DOTA-nimotuzumab Lc and Hc followed by trypsin digestion, we identified a total of 4 DOTA-binding sites: two in the constant region of Lc and two in the constant region of Hc. Probably, many DOTA bind the variable region of nimotuzumab [19].

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In the present study, we evaluated the possibility to simplify and improve the results of the synthesis of radioimmunoconjugate ^{90}Y-DOTA-nimotuzumab. The mainly used protocol to label nimotuzumab with β^- radionuclides has been described by Beckford Vera and co-workers. They synthetized ^{177}Lu-DOTA-nimotuzumab as radiopharmaceutical for cancer radio-immunotherapy [17]. Other authors reported their results in the labelling procedure of DOTA-nimotuzumab with ^{90}Y or ^{177}Lu (Table **3**). In our study, several modifications of the Beckford Vera procedure have been tested in order to achieve a fast and easier labelling procedure, more suitable to be performed in hospital radiopharmacies.

Firstly, in our work, ^{90}Y has been used instead of ^{177}Lu. The main reason is that ^{90}Y is a pure β^- radionuclide, preferentially suitable for the β^- radio-guided surgery approach: indeed the probe developed by Faccini and co-workers [11] is more efficient with the higher energy β^- emission of ^{90}Y than that of ^{177}Lu; moreover, the concomitant γ emission of ^{177}Lu is an undesired source of background.

Secondly, in our optimized preparation the radioimmunoconjugate has been obtained with a radiochemical purity greater than 96% without purifying the starting materials with a chelating ion exchange resin. On the contrary, in the

Beckford Vera and Martinez's studies the starting materials have been purified for reducing the possible metal contamination [17, 18]; this costs time and resources, and, as a consequence, is less easy to be performed in the clinical routine. We hypothesize that this result may be due to the use of a deionized water obtained from Milli-Q system purification in the preparation of all solutions. The Milli-Q system has a resistivity value of pure water of 18.2 MΩ/cm (at 25°C); this value ensures that the overall concentration of ions is below 1μg/L (< 1ppb). Only two other studies labelled the antibody with ^{90}Y, using the same simpler water purification system with results similar to ours [18, 21]; whereas one study with ^{177}Lu reported similar results to ours [25].

Thirdly, the ^{90}Y-DOTA-nimotuzumab has not been purified from "free ^{90}Y" (not chelated with DOTA-nimotuzumab), in order to reduce the exposition to β^- radiations of the operator and to avoid any chance of radioactive contamination. In all previous experiences, the authors always reported the need to add a purification step after the labelling procedure of either ^{90}Y or ^{177}Lu with DOTA-nimotuzumab [17, 18, 20-26]. Only few of them reported the purification step as not mandatory but strongly recommend when the radiochemical purity was less than 95% [20, 23]. To the best of our knowledge, our study is the first report showing that the ^{90}Y-DOTA-nimotuzumab purification could be avoided without any impact on the radiochemical purity of the radioimmunoconjugate (mean % R.P. higher than 97%). Despite our simplified radiolabelling procedures, the radiochemical purity results were in line with the results of Beckford Vera *et al*.; they showed a higher than 97% radiochemical purity but obtained after the step of purification through size exclusion chromatography PD-10 column with PD-10 column [17], which is a time expensive and cumbersome procedure.

We have also verified that the main factors influencing the radiolabelling protocol are the reaction volume and the pH value. Final reaction volumes previously reported by some authors were widely variable (mean 295.5 μL, range from 46 μL to more than 1000 μL) [17, 18, 20-26], whereas the pH values for the reactions were reported by only a few of them (mean pH = 5.75, range from 5.5 to 6.0) [24, 25] (Table **3**). In our work, we performed 3 tests for each condition of reaction volume (range 200 – 700 μL) and pH (range 2.0 – 6.0), to obtain the highest radiochemical purity. The best results were achieved using the final reaction volume of 500 μL and the pH of 6.0. Such data are at variance with what reported by Beckford Vera and co-workers who stated that reaction volumes and pH Values do not influence the labelling results.

Furthermore, we studied ^{90}Y-DOTA-nimotuzumab in human plasma to evaluate its stability until 72 hours. To the best of our knowledge, we found only one study which evaluated the stability of DOTA-nimotuzumab in a mouse serum rather than with human plasma. Caldaza *et al*. [22] tested the stability of DOTA-nimotuzumab, labelled with ^{177}Lu, in mouse serum at 37°C: the radioimmunoconjugate was stable over a 24 h. In our work, we performed the stability test in human plasma at 37°C, to better simulate the physiological human conditions: the radioimmunoconjugate resulted stable up to 72 h in all tests.

There were some limitations in our study. First, our paper focuses only the radiolabelling procedure of ^{90}Y-DOTA-nimotuzumab; we have not yet determined the immunoreactivity and the *in vivo* studies that would require an Investigational Medicinal Product Dossier (IMPD) and would be performed according to the GMP (annex 1). Subsequently we will investigate the accuracy of the radiopharmaceutical, its biodistribution and its feasibility in β^- radio-guided surgery. Furthermore, it is a good laboratory practice rule to test the stability of a new radiopharmaceutical until 2 half-lives of the radionuclide; in our study, we limited the test only until 72 hours because the final endpoint of our project is to perform β^- radio-guided surgery (based on ^{90}Y), at 24 hours after the labelling procedure and patient injection.

CONCLUSION

In this study we clearly show that our labelling procedure of ^{90}Y-DOTA-nimotuzumab is fast, easy, reproducible and safe for the operators. Therefore, ^{90}Y-DOTA-nimotuzumab is suitable for the synthesis in a hospital radiopharmacy. Further studies will be anyway required to investigate the biodistribution of ^{90}Y-DOTA-nimotuzumab in *vivo* and its suitability for β^- radio-guided surgery.

AUTHORS' CONTRIBUTIONS

TS: guarantor of integrity of entire study, performed the research and wrote the paper. TS, DM, AC, IB, AG: performed the research. All authors: contributed to conception and design, or analysed and interpreted data, drafted the manuscript, or critically contributed to or revised the manuscript, or enhanced its intellectual content.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

The datasets used and/or analysed during the current study are available from the corresponding author, on reasonable request.

FUNDING

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CONFLICT OF INTEREST

Francesco Collamati, Valerio Bocci and Riccardo Faccini are listed as inventors on an Italian patent application (RM2013A000053) entitled "Utilizzo di radiazione beta2 per la identi□cazione intraoperatoria di residui tumorali e la corrispondente sonda di rivelazione" and on the PCT patent application (PCT/IT2014/000025) entitled "Intraoperative detection of tumour residues using beta2 radiation and corresponding probes," covering the method and instruments described in this paper.

ACKNOWLEDGEMENTS

Declared none.

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