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RADIOLABELING PARAMETERS OF ^{177}Lu-DOTA-RITUXIMAB

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ABSTRACT

Cancer treatment using radioimmunotherapy (RIT) has been the focus of much research in the last two decades. In RIT, a radioisotope is coupled to a monoclonal antibody (mAb) to form a tumor-specific target agent to improve the cytocidal effect of the mAbs. RIT allows the systemic delivery of radiation to disease target by mAbs while sparing normal tissues. Rituximab® (Mabthera - Roche) is a chimeric mouse-human monoclonal antibody; it selectively binds with high affinity to the CD20 antigen, a hydrophobic transmembrane protein, which is expressed on B-lymphocytes and in more than 90% of B cell non-Hodgkin's lymphomas (NHL). The conjugation and radiolabeling process involve special conditions of pH and temperature, long processes of manipulation and mixing. All this process can damage the antibody structure and compromise its clinical application. Therefore, these parameters must be largely studied. The aim of this work was to evaluate the best radiolabeling conditions of DOTA-rituximab. Briefly, 10 mg of antibody previously purified by ultrafiltration device was conjugated with DOTA-NHS-ester (Macrocyclics) in 50 fold molar excess. The reaction was conducted for 1 hour in phosphate buffer pH 8.0 and gently mixing at room temperature, remaining for 24 hours under refrigeration. The immunoconjugated was purified by size exclusion column and ultrafiltration device. The radiolabeled parameters studied were: immunoconjugated mass, activity of $^{177}LuCl_3$, reaction time, temperature and pH. The radiochemical purity of the preparations was determined using analysis by thin layer chromatography (TLC-SG plates). The best studied condition presented radiochemical purity above 95% and the integrity of antibody was preserved.

1. INTRODUCTION

B-cell non-Hodgkin's lymphomas (NHL) are a heterogeneous group of lymph proliferative malignancies that have different forms of biological behavior and treatment responses. Some B-cell NHL are indolent or grow very slowly, and they are incurable. In contrast, others types are aggressive or very aggressive and rapidly fatal, but they are often curable [1].

Anti-CD20 (Rituximab - Mabthera - Roche) is a genetically engineered chimeric murine monoclonal antibody (mAb) specifically directed against CD20 surface antigen on B lymphocytes [2]. It contains the complementarity determining regions of the murine anti-CD20 antibody 2B8 in conjunction with human kappa and IgG1 heavy-chain constant region sequences. This mAb was approved in 1998 by the U.S. Food and Drug Administration for immunotherapy of CD20 positive Non-Hodgkin's Lymphomas [3-6]. This antibody in combination with chemotherapy has resulted in prolonged remission and improved survival for patients with indolent and aggressive B-cell NHL [1].

Cancer treatment using radioimmunotherapy (RIT) has been the focus of innumerous researches in the last two decades [7]. Two therapeutic radiopharmaceuticals for radioimmunotherapy, anti-CD20 labeled with ^{131}I-tositumomab (Bexxar) and with ^{90}Y-ibritumomab-tiuxetan (Zevalin), have already been approved by FDA for the treatment of indolent, refractory or relapsed NHL. Ibritumomab (Zevalin) is now accepted as first line treatment for follicular lymphoma [3].

The efficacies reported thus far for therapeutic regimens containing ^{90}Y-ibritumomab-tiuxetan and ^{131}I-tositumomab are similar in patients with previously treated and chemotherapy-refractory low-grade relapsed NHL, with overall response rates in 60-83% range and complete response rates ranging from 15% to 52% [8].

The goal of RIT is to target the radiation to the tumor tissue using radiolabeled Mab while limiting the toxicity to normal cells [1, 9]. Radioimmunoconjugates comprising antibodies attached to a bifunctional chelator (BFC) and radiolabeled with a metallic radioisotope provide an excellent way of delivering radioactivity selectively to a tumor target. An appropriate choice of both antibody and radioisotope is essential whether the purpose is imaging or therapy [7, 9, 10].

Lanthanide radioisotopes are β^- emitters that have been demonstrated to be suitable for RIT of tumors. Radiolanthanides are easily produced with a large-scale, relatively low-price, and high specific ativity [7]. Lutetium-177 (^{177}Lu, E_β mean 166 keV, E_γ 113 keV 6.5%, 208 keV 11%, T½ 6.7 days) is a gamma and beta emitter radionuclide. Its gamma emission is used to obtain *in vivo* images of biodistribution and dosimetric studies, while beta emission produces the desired therapeutic effect [3, 11, 12]. ^{177}Lu has several advantages for RIT: low tissue penetration, scarce damage to normal surrounding tissues, low gamma energy radiation, low abundance and adequate life time [3].

The conjugation and radiolabeling process involve special conditions of pH and temperature, long processes of manipulation and mixing. All this process can damage the antibody structure and compromise its clinical application. Therefore, these parameters must be largely studied. The aim of this work was to evaluate the best radiolabeling conditions of DOTA-rituximab. These best conditions must provide high radiochemical purity and the preservation of antibody's structural integrity.

2. MATHERIALS AND METHODS

2.1. Preparation of immunoconjugated (DOTA-rituximab)

The parameters of immuconjugation have been adapted from published data [7, 9, 12-17]. Briefly, 10 mg of monoclonal antibody (rituximab, Mabthera-Roche), previously purified by ultrafiltration device, was conjugated with DOTA-NHS-ester (Macrocyclics) in 50 fold molar excess. The reaction was conducted for 1 hour in phosphate buffer pH 8.0 and gently mixing at room temperature, remaining for 24 hours under refrigeration. The immunoconjugated was purified by size exclusion column (Sephadex G25 column - Pharmacia Biotech) using sodium acetate buffer 0.4 M pH 5.5 - 7.0 and concentrated with ultrafiltration device (Amicon Ultra-50 - Millipore, MWCO 10,000). The purification efficiency of the immunoconjugate was

evaluated by HPLC (Shimatzu) using molecular exclusion column BioSep SEC-S 3000 (300 x 7,8 mm, 5 μm - Phenomenex, EUA), flow rate of 1 mL/minute of sodium phosphate buffer 50 mM pH 7 for 15 minutes.

Besides the evaluation of purification efficiency, this method allows the evaluation of the conjugation process was successful by evaluating the increase in the retention time of the antibody.

2.2. Specific activity study

For the study, 0.1 - 1 mg of immunoconjugate was labeled with 18,5 - 185 MBq (0.5 - 5 mCi) of [^{177}Lu]$LuCl_3$ (IDB - Netherlands). The resulting solution was incubated for 60 min at 43 ºC.

Radiochemical purity controls were performed by TLC-SG (Pall Corporation) using as mobile phase citrate/citric acid buffer 0.1 M pH 5.0.

2.3. Stability study

^{177}Lu-DOTA-rituximab was storage at 2 - 8 ºC for 72 hours. At the end of specific time intervals, an aliquot of ^{177}Lu-DOTA-rituximab was collected and submitted to radiochemical purity control as described above.

2.4. Radiolabeling study: variation of temperature, time and pH

The best and intermediate conditions found on the study of specific activity were applied to study the labeling parameters: time (30 min - 2 hours), temperature (43 ºC and 50 ºC) and pH (5.5 and 7.0). The radiochemical purity control was performed as described above.

2.5 Statistical analysis

Statistical analysis was performed using the statistical program GraphPad Prism 5.00® (GraphPad Software, Inc., San Diego, CA, USA). All the data were submitted to normality test (Shapiro-Wilk test). Nonparametric data were expressed as median ± interquartile with 95% confidence interval (CI) while parametric data were expressed as mean ± standard error of mean (SEM) with 95% CI.

To compare until two parametric groups (paired sample or not), the Student's t test was used with two-tailed distribution. For the non-parametric groups, the Wilcoxon test was used.

For comparing three or more groups the two-factor variance test (two-way ANOVA) was used. When necessary, the data were subjected to post-test (Bonferroni test or Newman-Keuls multiple comparison test).

3. RESULTS AND DICUSSION

RIT has been successfully used against CD-20 lymphoma [12] and rituximab labeled with radionuclides may provide a better therapeutic effect than rituximab alone in patients with relapsed or refractory B-cell NHL. Macrocyclics chelator molecules such as DOTA need to be coupled to rituximab to enable labeling with radiometals [3, 13]. It is an agent widely used in the synthesis of radioconjugates because it forms very stable complexes with many metals. Compared with other bifunctional agents such as DTPA, DOTA provides more stable complex *in vivo* and *in vitro* [3, 18].

In the HPLC analysis of rituximab and conjugated rituximab, a change in the retention time (RT) was observed, showing an RT of 7.19 for the rituximab and 6.67 for the conjugated antibody. This was an expected result, since a molecular exclusion column was employed and the molecular weight of the antibody increased after conjugation (Table 1).

Table 1: Time retention (TR) of conjugate and unconjugate antibody (n ≥ 3).

TR of unconjugate antibody	TR of conjugate antibody
7.19 ± 0.057	6.67 ± 0.026

The difference between unconjugate and conjugate antibody is significant ($P<0.0001$), indicating that there was conjugation.

Radionuclides emitting short-range β-particles are an important part of radiotherapeutic armamentarium [19]. Radiolabeling of antibodies conjugated to DOTA with trivalent metals as ^{177}Lu and ^{90}Y has usually been accomplished by prolonged incubation of conjugate with radiometal solution at room temperature. Exposure of a mAb to a relativity concentrated solution of the high-energy β^- for an extended period of time could result in significant radiolysis of the protein. Therefore, rapid and efficient corporation of radiometal into immunoconjugate is desirable [18].

Thinking about it, the standard method used to perform the labeling, was adapted from a condition developed by Lewis and colleagues (1994). Thus, the standard condition was 1 hour at 43 ° C, pH 5.5, final volume of 100 µL with constant stirring at 350 rpm.

Different specific activities were studied as well as the stability of radioimmunoconjugate at 2-8 ºC. These results were expressed in the FIG. 1, 2 and 3.

To analyze these results, the data were subjected to tests of normality (Shapiro-Wilk test) and the P values were lower than 0.05 indicating that all data has a nonparametric distribution.

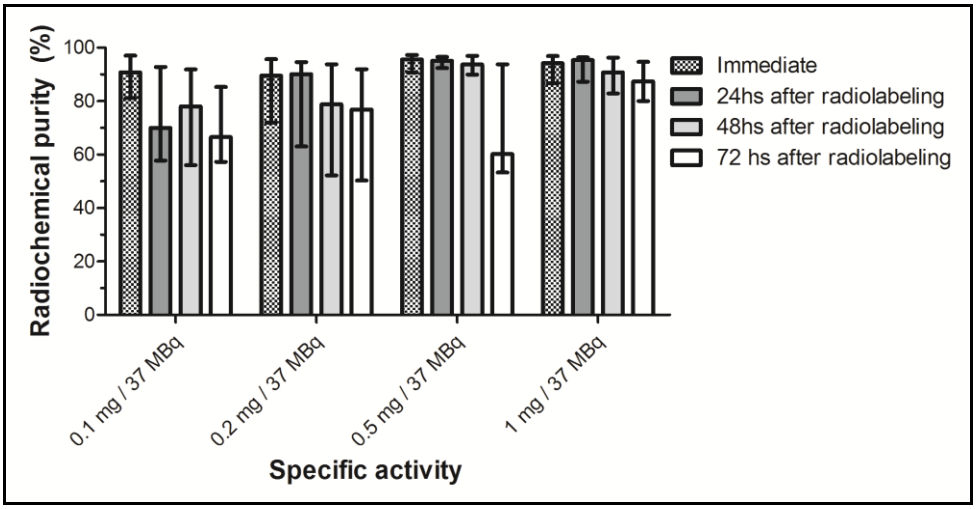

Figure 1: Radiochemical purity of immunoconjugate radiolabeled in different specific activity (mass variation) for 1 hour at 43 ºC, pH 5.5, 350 rpm. Results expressed in median ± interquartile with 95% CI (n ≥ 3).

The four groups did not showed significant difference (P = 0.800), but when the radiochemical purity was analyzed as a function of time (stability), a significative difference was observed (P = 0.040).

The conditions that presented better results were 0.2 mg/37 MBq, 0.5 mg/37 MBq, 1 mg/37 MBq. However, only the masses of 0.2 and 0.5 mg were radiolabeled with different activities (Fig. 2 and 3).

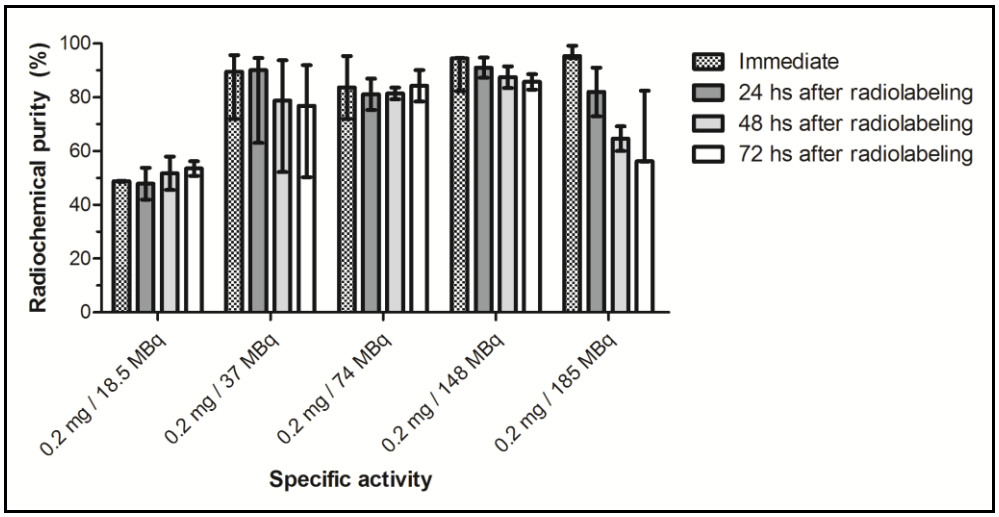

Figure 3: Radiochemical purity of immunoconjugate radiolabeled in different specific activity (activity variation) for 1 hour at 43 ºC, pH 5.5, 350 rpm. Results expressed in median ± interquartile with 95% CI (n ≥ 3).

Good radiochemical purity was observed when 0.2 mg of immunoconjugate was radiolabeled with 37, 74, 148 and 185 MBq of ^{177}Lu (FIG. 2). The four groups showed significant difference ($P<0.0001$), mainly due to the low radiochemical purities observed in lower specific activity (0.2 mg / 185.5 MBq).

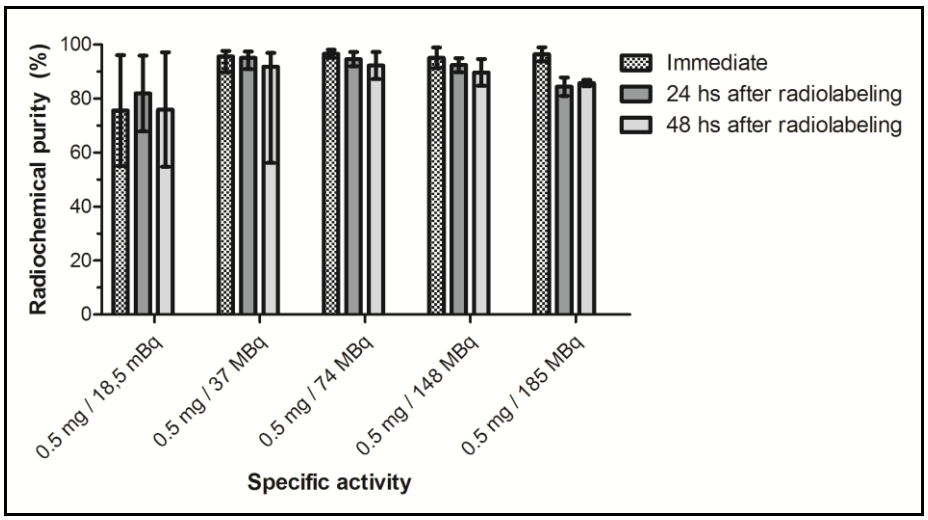

Figure 3: Radiochemical purity of immunoconjugate radiolabeled in different specific activity (activity variation) for 1 hour at 43 ºC, pH 5.5, 350 rpm. Results expressed in median ± interquartile with 95% CI (n ≥ 3).

When a larger mass of DOTA-rituximab (0.5 mg) was radiolabeled, the radiochemical purity increased just after labelling (immediate) and better stability was observed when labeled antibody was stored at 2-8 ºC for most activities studied (FIG. 3). However, this increase was not significant ($P = 0.4661$).

Thus, in order to improve the radiochemical yield, the condition which results in good radiochemical purity and higher specific activity (0.2 mg / 185 MBq) was scheduled for radiolabeling with different times, temperatures and pH (FIG. 4).

The data indicated a significant difference in the conditions of radiolabeling ($P=0.0198$).

The increase of temperature (50 ºC) provided radiochemical purity greater when the pH was maintained at 5.5 ($P<0.05$). However, this increase did not occur when the pH was maintained at 7.0. Although many studies have reported good radiochemical yield at neutral pH [9, 13-15, 17], this was not observed in any condition studied.

Using lower pH, best results were observed at 43 ºC ($P<0.05$), which were not observed at 50 ºC ($P>0.05$), probably due to the low yields found in the later times (1h30 min and 2h). These low yields were probably due to the denaturation of the antibody.

Although rituximab is a robust antibody [13], it is known that exposure of proteins to high temperatures for prolonged periods, promotes its denaturation.

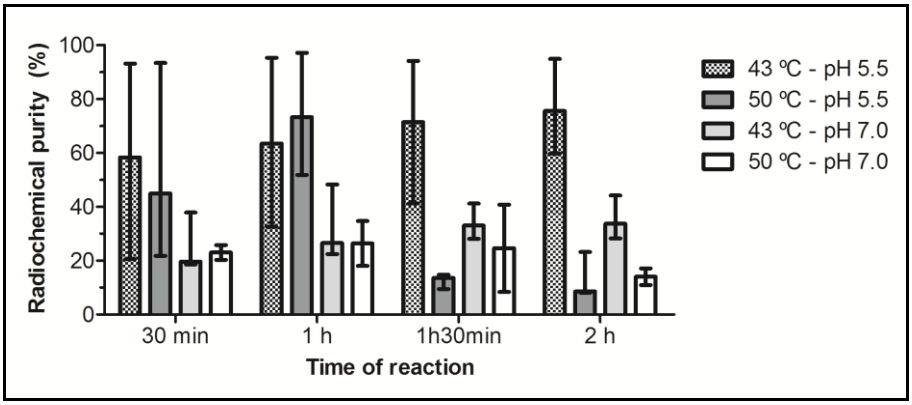

Figure 4: Radiochemical purity of immunoconjugate radiolabeling in different time, temperature and pH for 0.2 mg of immunoconjugate, 185 MBq (5 mCi) of $^{177}LuCl_3$, 350 rpm. Results expressed in median ± interquartile with 95% CI (n = 3).

The activity of $^{177}LuCl_3$ incorporated by µg or mg of antibody, in order words, the radiopharmaceutical specific activity, is important property from the point of view of development of new radiopharmaceuticals [20]. Low specific activity radiopharmaceuticals have low value in terms of clinical application, since unlabelled molecules compete with the labeled for binding receptor *in vivo*, as well as increase the toxicity due to the physiological effects of this binding. In contrast, very high specific activity can promote protein denaturation due to the radiolysis effect [20]. The TAB. 2 correlates the radiochemical yield and the specific activity of the labeled antibody.

Table 2: Specific activity of ^{177}Lu-DOTA-rituximab (n ≥ 3).

Mass of rituximab-DOTA (mg)	Activity of $^{177}LuCl_3$ (MBq / mCi)	% Radiochemical purity of ^{177}Lu-DOTA-rituxumab (CCD)*	Specific activity of ^{177}Lu-DOTA-rituximab (MBq/µg)	Specific activity of ^{177}Lu-DOTA-rituximab (MBq/mg)
0.1	37 / 1	90.75 ± 97.68; 73.64	0.34	335.78
0.2	18,5 / 0.5	48.77 ± 48.99; 48.61	0.05	45.11
0.2	37 / 1	89.58 ± 97.12; 67.53	0.17	165.72
0.2	74 / 2	83.71 ± 95.35; 72.07	0.31	309.73
0.2	148 / 4	94.53 ± 94.73; 82.30	0.70	699.52
0.2	185 / 5	95.35 ± 99.23;94,68	0.88	881.99
0.5	18,5 / 0.5	75.63 ± 96.14; 55.11	0.03	27.98
0.5	37 / 1	95.59 ± 97.74; 89.75	0.07	70.74
0.5	74 / 2	96.64 ± 98.14; 95.14	0.14	143.03
0.5	148 / 4	95.10 ± 98.93; 91.27	0.28	281.50
0.5	185 / 5	96.47 ± 99.07; 93.87	0.36	356.94
1	37 / 1	94.23 ± 97.01; 84.93	0.03	34.87

* Median ± upper limit; lower limit with 95% CI

The best specific activity that resulted in high radiochemical yield was 881.99 MBq/mg. This value is higher than that found by Audicio et al. (111-370 MBq/mg) in their preclinical studies [3].

A preliminary dosimetric study conducted by Forrer et al. (2009), with ^{177}Lu-DOTA-rituximab in two patients in a therapeutic setting demonstrated effective delivery of radiation absorbed dose to the tumors by specific binding of the antibody to the tumors. The activity administered of ^{177}Lu was 1,480 MBq (5 mg of antibody). The specific activity found by them was 296 MBq/mg.

In comparison, the higher specific activity observed in this study can probably promote good therapeutical effect considering a future clinical application.

3. CONCLUSIONS

Rituximab was coupled to DOTA with satisfactory efficiency, the radioimmunoconjugate was produced in high specific activity and showed good stability at 2-8 ºC.

The best radiolabeling condition was 0.2 mg of immunoconjugate, 185 MBq of ^{177}Lu, 43 ºC with pH 5.5. This condition conferred stability to the antibody even in later times (1-2 hours).

Studies of specific binding to tumor cells should be performed in order to evaluate the influence of specific activity on receptor binding.

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