2009 International Nuclear Atlantic Conference - INAC 2009
Rio de Janeiro,RJ, Brazil, September27 to October 2, 2009
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: 978-85-99141-03-8

STANDARDIZATION OF METHODOLOGY TO DERIVATIZATION AND RADIOLABELING OF THE ANTI-CD20 MONOCLONAL ANTIBODY FROM BIFUNCTIONAL CHELATOR DOTA-NHS-ESTER

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ABSTRACT

Lymphomas are cancers of the lymphatic system, being the most common the non-Hodgkin lymphoma (NHL). The *Radioimmunotherapy (RIT),* that increase the cytotoxic effect of monoclonal antibodies (mAb), therefore labeling these Mab with different radioisotopes. RIT combines the specificity of the antibody and the toxicity of the radionuclides. The mAb anti-CD20 is used for treatment of relapse or refractory NHL. The labeling of anti-CD20 with ^{177}Lu, requires a bifunctional chelating agent that is designed to make a "connect bridge" between the mAb and the radionuclide. The incorporation of the chelating group in mAb structure is called *derivatization.* The aim of this work is to study the derivatization of anti-CD20 antibody with DOTA-NHS-ester chelating group and labeling parameters to produce ^{177}Lu-DOTA-AntiCD20. Five milligrams of anti-CD20 were purified by dialysis against phosphate buffer pH 8.0 and derivatized with DOTA-NHS-ester in 1:250, 1:500 and 1:1000 molar ratios. The reaction was conducted for 1hour in gently mixing at room temperature and remained under refrigeration for 48 hours. The reaction mixture was purified in gel column Sephadex G-50 ; the aliquots that presented greater protein concentration, were mixed and concentrated. The purified antibody conjugated was added to 111-185MBq (3-5mCi) of $^{177}LuCl_3$ diluted in 0.4 M acetate buffer pH 5.5. Radiochemical purity was less than 95% in all the molar ratios, indicating necessity of the purification after the labeling. The mAb derivatized showed stable when stored for to 1 month to 4ºC and 4 days at -20ºC.

1. INTRODUCTION

Lymphomas are cancers that originate from the transformation of a lymphocyte in the lymphatic system. They are generally classified as Hodgkin's disease and non-Hodgkin lymphoma, the latter being the most common [1].

Non-Hodgkin lymphomas can occur at any age and are often marked by lymph nodes that are larger than normal, fever, and weight loss. There are many different types of non-Hodgkin lymphomas. These types can be divided into aggressive (fast-growing) and indolent (slow-growing) types, and they can be formed from either B-cells or T-cells [2].

The American Cancer Society [3] estimates that in 2009, 65,890 new cases will arise only in the U.S. and the number of deaths may reach 19,500.

The NHL can spread to other organs. The prognosis depends on the histologic type, stage, and treatment.

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The NHL can be divided into two prognostic groups: the indolent lymphomas and the aggressive lymphomas. The indolent NHL have a relatively good prognosis with a median survival as long as 10 years, but they usually are not curable in advanced clinical stages. The aggressive type of NHL has a shorter natural history, but a significant number of these patients can be cured with intensive combined chemotherapy regimens. The risk of late relapse is higher in patients with a divergent histology of both indolent and aggressive disease [4].

Patients with aggressive forms of NHL may have sustained complete remissions with combined chemotherapy regimens or aggressive consolidation with marrow or stem cell support [5, 6].

1.1. Rituximab and Radioimmunotherapy

CD20, is a hydrophobic transmembrane protein with a molecular weight of approximately 35kD located on pre-B and mature B lymphocytes. The antigen is expressed on most B-cell non-Hodgkin's lymphoma but is not found on stem cells, pro-B cells, normal plasma cells or other normal tissues. Plasma blasts and stimulated plasma cells may express CD20. CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation, and possibly functions as a calcium ion channel. CD20 is not shed from the cell surface and does not internalize upon antibody binding. Free CD20 antigen is not found in the circulation; thus a drug that reacts with CD20, such as an antibody, would not be neutralized before binding to its target cell [7].

Rituximab, a chimeric murine/human monoclonal antibody (mAb), approved in the United States only for the treatment of refractory or relapsed B-cell lymphomas, reacts with the CD20 antigen. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids with a molecular weight of 145 kDa. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM, which is similar to the parent murine antibody, 2B8 [7].

Radioimmunotherapy is a cancer treatment modality that combines the targeting power of monoclonal antibodies with the cell-damaging ability of localized radiation. These treatments are made by linking monoclonal antibodies – engineered in a laboratory to recognize and attach to substances on the surface of certain cells - to radioactive isotopes. When infused into a patient, these radiation-carrying antibodies circulate in the body until they locate and bind to the surface of specific cells, and then deliver their cytotoxic radiation directly to the cancerous cells. The first radiolabeled monoclonal antibody for B-cell lymphoma approved for human use was Zevalin, a murine IgG1 anti-CD20 monoclonal antibody radiolabeled with Indium-111 (^{111}In) and Yttrium-90 (^{90}Y) [8].

The lanthanide radionuclide ^{177}Lu is available commercially for research and investigational purposes as a diagnostic and radiotherapy agent in the treatment of several malignat tumors. The nuclear properties of ^{177}Lu are advantageous compared to other therapeutic radionuclides, e.g., ^{90}Y, which is paired with an antibody or peptide by the same techniques

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as ^{177}Lu. The maximum β^- energy 498.3 of ^{177}Lu and the 130 keV average, provide a short range of 0.25 mm, which is effective in destroying small malignant tumors, but with less damage to healthy cells. Another advantage of ^{177}Lu is the low exposure by gamma-rays, due to the low energy and emission probability of the main transitions: 113 keV and 208 keV. This gamma-emission is suitable for direct imaging by gamma scintigraphy cameras, thus eliminating the need for a surrogate such as ^{111}In. Another advantage is the relatively longer and more convenient half-life of 6.65 days [9].

To produce radiolabeted mAb using radionuclides like ^{90}Y and ^{177}Lu it is necessary to introduce in mAb structure, a chelator with affinity for the radionuclide. This process "called" derivatization, must be conducted under studied conditions in order to produce labeled antibodies with high radiochemical yield and without lost of immunoreactivity.

2. MATERIALS AND METHODS

2.1. Antibody Derivatization

The chelator (DOTA-NHS-ester - Macrocyclics®) was conjugated to the antibody (Anti-CD20 - Rituximab -Mabthera®) based on procedures previously described [10-17], using 5mg of antibody previously desalted into phosphate buffer pH8.0. The reaction was conducted for 1 hour and gently mixing at room temperature and remained overnight under refrigeration. The molar mAb:chelator ratio employed were 1:1000, 1:500 and 1:250. One aliquot of 50µg of mAb were separated to determine the average number of chelators per mAb. After derivatization, the sample was purified by Sephadex G50 (Pharmacia Biotech®) column to remove the excess of chelator and change the buffer (0.2M acetate buffer pH 5.5). The fractions of 1 mL containing the Antibody were identified by UV-visible spectrophotometry (280 nm). Protein fractions (pool) were concentrated by Vivaspin 30.000MWCO (Pharmacia Biotech®).

2.2. Determination of Average Number of Chelates per Antibody

The method used to determine the average number of chelator per antibody was previously described [15]. Briefly, 50 µg of mAb conjugator reaction mixture, prior to Sephadex purification, were added to 200 µL of 0.2M acetate buffer pH 5.5, and finally, to 111-185MBq (3-5mCi) of $^{177}LuCl_3$ solution were added into the reaction mixture; which was incubated at 43ºC for 1h with constant mixing.

Two different instant chromatography methods were used to determine the percentge of free ^{177}Lu, unconjugated chelator ^{177}Lu-DOTA and the product ^{177}Lu-Anti-CD20-DOTA contained within the reaction mixture [15].
To separate ^{177}Lu-DOTA-mAb from the free ^{177}Lu and ^{177}Lu-DOTA, duplicate plates of Whatman nº 1 were developed with 10mM EDTA-buffer pH 4.5. To determine the amount of ^{177}Lu-DOTA, duplicate plates were developed with 9% NaCl, 10mM NaOH solution. The percent of each of the three species were then determined by subtraction, according to Equation 1 [15].

$$DOTA/mAb = \frac{\%originEDTA}{(\%originEDTA + \%solventFromNaOH)} \qquad (1)$$

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2.3. Labeling of the Immunoconjugate with ^{177}Lu

The derivatized and purified antibody was added to 111-185MBq (3-5mCi) of $^{177}LuCl_3$ diluted in 0.4 M acetate buffer pH 5.5. The resulting solution was incubated for 1h at 43ºC. At the end of the reaction, 10mM of DTPA was added to the mixture and left to react for more 15 minutes in order to chelate any free radiometal.

2.4. Quality Control of Labeled mAb

Chromatographic profile of ^{177}Lu, ^{177}Lu-DOTA and ^{177}Lu-DOTA-Anti-CD20 were determined using as stationary phase Whatman nº 1 paper (Whatman , Maidstone, Uk), and Instant Thin Layer Chromatographic (ITLC-SG) (Pall Corporation); various mobile phases were used like methanol : ammonium acetate 0.4M (v/v), 0,9% NaCl ,10mM NaOH and 10mM EDTA-buffer pH 4.5. Samples of the labeled antibody preparations was spotted on the stationary phase and developed in mobile phases. Radiochemical purity was determined by thin-layer chromatography, using ITLC-SG and Whatman nº 1 strips, eluted with methanol: ammonium acetate 0.4 M 1:1 (v/v) as solvent [10].

3. RESULTS AND DISCUSSION

3.1 Chromatographic Profile

The chromatographic profiles of components of the reaction are presented in Table 1. There was no difference in the profiles when using ITLC-SG or paper chromatography.

Table 1. Chromatographic profile (Rf)

	9% NaCl, 10mM NaOH	10mM EDTA-buffer pH 4.5	Methanol: ammonium acetate 0.4 M
^{177}Lu-DOTA-Anti-CD20	0	0	0
Free ^{177}Lu	0-0.1	1.0	0.9-1.0
^{177}Lu-DTPA or ^{177}Lu-DOTA	1.0	1.0	0.9-1.0

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3.2 Determination of average number of chelating group per antibody

This study was realized after three different at conditions of derivatization: 1h at room temperature with mild agitation, 24hs and 48hs (4ºC), to determine the influence of time, temperature and molar mAb:chelator ratio on the number of chelating groups per antibody molecule (Table 2).

Table 2. Number of chelating group per antibody

Molar ratio	1h derivatization	24hs derivatization	48hs derivatization
1:1000	6.9	2.6	13
1:500	9	3.2	2.7
1:250	4.5	3.6	5.8

(N=2)

These preliminary results showed no increment in the number of chelating groups/molecule of mAb, when increasing the reaction time. So, 24 hs was used as derivatization time in the next experiments.

3.3 Labeling of the immunoconjugate with ^{177}Lu and quality control

The antibody conjugated at molar ratio of 1:1000 and conjugation time of 48 hs showed a radiochemical purity superior than 75 % after labeling and decreased after 24 and 48 hs (Table 3).
The results were similar when the mAb was labeled 15 days after derivatization, showing that the derivatization kept stable when stored at 4ºC.

Table 3. Radiochemical purity of the conjugated antibody (1mg) at molar ratio of 1:1000

Radiolabeling after purification (%)			Radiolabeling after 15 days of the purification – stored at 4ºC (%)		
Immediately	24hs after labeling	48hs after labeling	Immediately	24hs after labeling	48hs after labeling
78.02±7.9	69.53±1.2	65.13±1.65	79.47±6.2	65.17±7.1	58.80±2.6

(N=2)

The excess of chelating groups in the structure of the antibody molecule can promote the damage in the protein molecule leading to the reduction of its immunoreactivity. To prevent this, different derivatization molar ratios were performed (1:500 and 1:250).

ISBN: 978-85-99141-03-8

When aliquots of 0.5mg and 1.0mg of the conjugated antibody derivatized at molar ratios of 1:500 and 1:250 was labeled, radiochemical purity showed similar results when compared with 1:1000 ratio, immediately after labeling. However, after 24 hs, the radiochemical purity decreased, specially in the case of 1:250 ratio (Table 4).

Table 4. Radiochemical purity of the conjugated antibody labeled after purification

Molar ratio	Immediately		24hs after labeling	
	0.5mg	1mg	0.5mg	1mg
1:250	57.83±2.12	48.19±2.14	29.28±14.51	6.68±1.02
1:500	63.42±1.67	52.60±0.1	56.38±9.83	17.29±5.79

(N=2)

These conjugated antibody when labeled after stored for 15 days at -20ºC, showed a decreasing in radiochemical purity (Table 5).

Table 5. Radiochemical purity of the conjugated antibody labeled after stored for 15 days at -20ºC

Molar ratio	Immediately		24hs after labeling	
	0.5mg	1mg	0.5mg	1mg
1:250	35.07±0.41	36.03±0.32	6.65±3.08	15.65±0.83
1:500	26.75±5,81	26.51±2.08	5.74±1.33	5.69±4.73

(N=2)

The conjugated antibody derivatized at molar ratios of 1:500 and 1:250 were both stored at 4ºC and -20ºC for 4 days, and then labeled. The respective radiochemical purity showed in Table 6. Radiochemical purity of the same conjugated compounds 24 hours after labeling of these preparations are showed in Table 7.

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Table 6. Radiochemical purity of the conjugated antibody labeled after 04 days of storage at 4ºC and -20ºC

Conditions of storage	Immediately			
	0,5mg		1mg	
	4ºC for 4 days	-20C for 4 days	4ºC for 4 days	-20ºC for 4 days
1:250	56.69±1.98	56.95±3.51	48.56±2.96	86.85±13.23
1:500	57.17±0.45	57.58±1.87	63.80±0.24	64.99±6.30

(N=2)

Table 7. Radiochemical purity of the conjugated antibody 24 hs after labeling (4 days of storage at 4ºC and -20ºC)

Conditions of storage	24hs after labeling			
	0,5mg		1mg	
	4ºC for 4 days	-20ºC for 4 days	4ºC for 4 days	-20ºC for 4 days
1:250	49.03±8.50	47.61±3.63	39.03±8.50	47.61±3.63
1:500	45.67±16.34	40.46±11.56	52.31±13.03	53.70±12.51

(N=2)

When aliquots (1mg) of the same conjugated antibody derivatized at same molar ratio stored for 30 days at 4ºC was subjected to two different labeling conditions: 1) standard temperature and labeling time 1h/43º, 2) reduced standard labeling time (30min/43ºC). Radiochemical purity of the compound derivatized at molar ratio of 1:250 was superior than 61% and superior than 80% respectively, and the compound derivatized at molar ratio of 1:500 demonstrated radiochemical purity superior than 77% and superior than 71% respectively (Table 8).

Table 8. Radiochemical purity of the conjugated antibody stored for 30 days at 4ºC, subjected to two different labeling conditions

Molar ratio	Immediately		After 24hs of radiobeling
	30'/43ºC	1h/43ºC	1h/43ºC
1:250	80.45±22.3	61.45±9.3	43.58±16.4
1:500	71.28±23.9	77.87±1.2	75.23±0.7

(N=2)

***ISBN:* 978-85-99141-03-8**

4. CONCLUSIONS

The chromatographic profile of the conjugated antibody observed in this work was compatible with those described in literature [10, 15] and easy to accomplish through ascending paper chromatography (Whatman nº1) and Instant Thin Layer Chromatographic (ITLC-SG).

According to Nikula et. al. [15], the method to determine the number of chelating groups per mAb described might not produce the precise numbers of chelating that had really attached to the antibody molecule. Moreover, he suggest further studies and application of other methods. This can explain the increase of correlation between chelating : mAb ratio and the number of chelating group/mAb.

The conjugated antibody derivatized at the molar ratio of 1:1000 presented higher radiochemical purity than those derivatized with 1:500 and 1:250 when radiolabed immediately after the purification. Even though, derivatization at this molar ratio produced multiple results considering the number of chelating groups conjugated to the antibody molecule which might lead to protein structural damage. However, derivatization at 1:500 and 1:250 achieved radiochemical purity lower than 95% that indicates the necessity of a purification step in order to remove the excess of free radionuclide.

Moreover, results from radiochemical purity observed in this study appears compatible with the antibody/chelate molar ratio used, since recent published datas showed the use of lower molar ratios (1:2 – 1:20), which might suggest the study of derivatizations with lower molar ratios.

The derivatized immunoconjugates stored for 30 days at 4ºC and those stored for four days at -20ºC showed good radiochemical purity results, which might indicate that these conditions are favorable for immunoconjugates derivatized storage [10, 16].

Further studies are in development to determine the labeled antibody immunoreactivity and ability to recognize CD20 receptor.

ACKNOWLEDGMENTS

The authors wish to thank to CAPES and Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN-SP for the financial support to this project.

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