HARNESSING ^{68}GA-NOTA-RITUXIMAB IN RADIOIMMUNOTHERAPY (RIT) : REVOLUTIONIZING CANCER TREATMENT

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ABSTRACT

Radioimmunotherapy (RIT) represents a ground-breaking approach in cancer treatment of non-Hodgkin lymphoma (NHL), combining the specificity of monoclonal antibody with radioactive nucleotide. Rituximab, is a chimeric monoclonal antibody directed against B-lymphocyte specific antigen CD20, which is used for the treatment of B-cell malignancies. However, the effectiveness of rituximab is limited partly due to treatment resistance. The aim of this study is to develop the radiolabelled of rituximab to enhance the activity. Rituximab was conjugated with p-SCN-Bn-NOTA (1:10 and 1:50) and the pure conjugated rituximab was collected by using the preparative high performance liquid chromatography (HPLC). The conjugated was radiolabelled with 68Ga and purified by using PD-10 column. The quality control parameters such as pH, radiochemical purity (RCP), stability against time and serum challenge of 68Ga-NOTA-Rituximab were determined and the conditions were optimized. The RCP of ^{68}Ga-NOTA-Rituximab at 1:10 ratio was 97.34 ± 0.16% before purification and increased to 99.63 ± 0.16 % after purification. Likewise, the RCP of rituximab labelled at a 1:50 ratio was 89.02 ± 2.24% before purification and improved to 99.06 ± 0.01% post-purification. Additionally, the labelled rituximab remained stable for up to five hours under both serum and non-serum conditions, maintaining RCP of over 95%. From this study, we conclude that the rituximab was successfully conjugated with the p-SCN-Bn-NOTA and subsequently purified by using preparative HPLC, later radiolabelled with 68Ga. In vitro stability studies with 68Ga-NOTA-Rituximab with and without serum of up to 5 hours exhibited greater than 95% RCP. Further studies in pre-treated animal of NHL would confirm the potential of this 68Ga-NOTA-Rituximab for PET imaging of NHL.

ABSTRAK

Radioimunoterapi (RIT) mewakili pendekatan terobosan dalam rawatan kanser limfoma bukan Hodgkin (NHL), menggabungkan kekhususan antibodi monoklonal dengan nukleotida radioaktif. Rituximab, adalah antibodi monoklonal chimeric yang ditujukan terhadap antigen spesifik B-limfosit CD20, yang digunakan untuk rawatan keganasan sel B. Walau bagaimanapun, keberkesanan rituximab adalah terhad sebahagiannya disebabkan oleh rintangan rawatan. Matlamat kajian ini adalah untuk membangunkan radiolabelled rituximab untuk meningkatkan aktiviti. Rituximab telah dikonjugasikan dengan p-SCN-Bn-NOTA (1:10 dan 1:50) dan rituximab konjugasi tulen dikumpulkan dengan menggunakan kromatografi cecair prestasi tinggi persediaan (HPLC). Terkonjugasi telah dilabel radio dengan 68Ga dan disucikan dengan menggunakan lajur PD-10. Parameter kawalan kualiti seperti pH, ketulenan radiokimia (RCP), kestabilan melawan masa dan cabaran serum 68Ga-NOTA-Rituximab telah ditentukan dan keadaan telah dioptimumkan. RCP ^{68}Ga-NOTA-Rituximab pada nisbah 1:10 ialah 97.34 ± 0.16% sebelum

penulenan dan meningkat kepada 99.63 ± 0.16 % selepas penulenan. Begitu juga, RCP rituximab yang dilabelkan pada nisbah 1:50 ialah 89.02 ± 2.24% sebelum penulenan dan bertambah baik kepada 99.06 ± 0.01% selepas penulenan. Selain itu, rituximab berlabel kekal stabil sehingga lima jam di bawah kedua-dua keadaan serum dan bukan serum, mengekalkan RCP melebihi 95%. Daripada kajian ini, kami menyimpulkan bahawa rituximab berjaya dikonjugasikan dengan p-SCN-Bn-NOTA dan kemudiannya disucikan dengan menggunakan HPLC persediaan, kemudian dilabel radio dengan 68Ga. Kajian kestabilan in vitro dengan 68Ga-NOTA-Rituximab dengan dan tanpa serum sehingga 5 jam menunjukkan lebih daripada 95% RCP. Kajian lanjut dalam haiwan pra-rawatan NHL akan mengesahkan potensi 68Ga-NOTA-Rituximab ini untuk pengimejan PET NHL.

Keywords: PET imaging, Radioimmunotherapy, cancer treatment, radioactive nucleotide

INTRODUCTION

Cancer is the second leading cause of death globally, with lymphoma accounting for approximately half of all blood cancers diagnosed each year. Non-Hodgkin's lymphoma (NHL) ranks as the 5th to 9th most common cancer worldwide, with an estimated 510,000 new cases in 2018 [1]. Despite ongoing efforts to develop effective cancer therapies, treatment selection remains challenging due to the unpredictability of irreversibly dividing cells having the capability of invasion leading to metastasis.

B-cell lymphomas, which account for >90% of NHL, express a large number of CD20 proteins on the outer surface of the affected cells, and this overexpression of CD20 receptor proteins can be considered a tumor marker for the diagnosis and treatment of patients suffering from NHL [2;3] Consequently, targeting CD20 plays a vital role in therapeutic strategies for these cancers. Numerous anti-CD20 monoclonal antibodies have been developed and are now commercially available, offering immunotherapy options for patients with B-cell malignancies [4]. Rituximab, a chimeric monoclonal antibody specific to the CD20 antigen, is widely used as an immunotherapeutic agent in B-cell NHL [5]. However, repeated cycles of rituximab often lead to resistance, reducing tumor response and resulting in relapse in a significant proportion of patients [6].

Hence, the past few decades have witnessed significant advancements in the fields of molecular biology, medicine, and radiochemistry and led to the emergence of radioimmunotherapy (RIT) as a treatment modality for targeting several cancers. RIT, in particular, has shown maximum efficacy in the treatment of NHL, as evidenced by preclinical and clinical studies in patients [7]. The β-emission from radiolabeled antibodies induces cytotoxic effects not only in targeted cancer cells but also through cross-fire and bystander effects. In the development of NHL-specific radioimmunotherapies, CD20 remains the primary target due to its ubiquitous expression on normal B-cells and significant overexpression on malignant B-cells [5].

In preparation for future therapeutic studies utilizing therapeutic radioisotopes, a model metal-chelated immunoconjugate was developed by radiolabeling rituximab with gallium-68 (^{68}Ga). This ^{68}Ga-labelled with rituximab was synthesized to investigate the radiolabeling process and assess the stability of the radiolabeled product, establishing a basis for subsequent research in radioimmunotherapy.

METHODOLOGY

Materials

Rituximab (anti-CD20 monoclonal antibody) was purchased from Roche Inc., Basel, Switzerland and p-SCN-Bn-NOTA 2-S-(4 Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid was obtained from Macrocyclics, Plano, Texas. Chemicals and reagents such as sodium azide, sodium acetate, hydrochloric acid and sodium hydrogen carbonate were purchased from Merck Millipore (Germany), while LC-MS Grade Water and citric acid

were from Fisher Scientific (USA). Phosphate buffer saline (PBS) tablet, sodium hydroxide and acetonitrile were procured from Sigma Aldrich (USA). $^{68}Ge/^{68}Ga$ generator was obtained from Eckert & Ziegler Radiopharma, Germany.

Conjugation of p-SCN-Bn-NOTA to Rituximab

The p-SCN-Bn-NOTA-Rituximab immunoconjugate was synthesized following a protocol similar to Suman *et al.*,[8], with slight modifications. Rituximab (1 mL, 10 mg/mL) was conjugated with p-SCN-Bn-NOTA at two molar ratios: 1:10 and 1:50 (Rituximab: p-SCN-Bn-NOTA). The conjugation reaction was carried out in 0.2 M sodium bicarbonate buffer ($NaHCO_3$), pH 9.0. The mixture was allowed to react for 2 hours at room temperature, followed by overnight incubation at 4°C with continuous shaking.

Post-incubation, the conjugate was purified using preparative HPLC equipped with a size-exclusion column (SEC 3.5µm; 7.8 x 150mm) (Waters Alliance). The mobile phase consisted of 0.05 M phosphate-buffered saline (PBS) containing 0.05% sodium azide (NaN_3), pH 6.8, at a flow rate of 0.5 mL/min. Purification of conjugated rituximab was performed to remove the excess of p-SCN-Bn-NOTA. The purified fractions containing the conjugated Rituximab were pooled and stored at 2-8°C for subsequent radiolabelling.

Radiolabelling And Purification Of Immunoconjugate With ^{68}Ga

Gallium-68 Chloride ($^{68}GaCl_3$) with an activity range of 141-170 MBq was eluted from a $^{68}Ge/^{68}Ga$ generator using 5.0 mL of 1 M HCl. The eluted $^{68}GaCl_3$ was quantified using a dose calibrator (Capintec™, New Jersey, USA) and was subsequently used to radiolabel the p-SCN-Bn-NOTA-Rituximab conjugate.

For the radiolabelling, approximately 10 mg of the conjugated p-SCN-Bn-NOTA-Rituximab in 1 M sodium acetate was reacted with 1 mL of $^{68}GaCl_3$. The pH of the reaction mixture was adjusted to 4.0-4.5. The reaction was incubated at room temperature for 15 minutes. The labelling procedure was repeated for the p-SCN-BN-NOTA-Rituximab with different molar ratio.

The radiolabelled Rituximab was purified using a PD-10 column packed with Sephadex™ G-25 Fine gel, with phosphate-buffered saline (PBS) used as the eluent. To perform the purification, 500 µL of the reaction mixture was applied to the column, and the activity was determined by dose calibrator. The collected tubes with the labelled Rituximab were mixed in a pool. The radiochemical purity (RCP) of the preparation was determined before and after purification by Instant Thin Layer Chromatography (ITLC).

Radiochemical purity determination (RCP)

Radiochemical purity (RCP) of the purified and non-purified ^{68}Ga-NOTA-Rituximab immunoconjugates was assessed using Instant Thin Layer Chromatography (ITLC). ITLC-SG strips were employed in solvent comprising 20 mM citric acid and 10% acetonitrile (ACN). The solvent facilitated the migration and separation of components based on their polarities. The RCP and retention factor (Rf) were analyzed using the AR-2000 Radio-TLC Imaging Scanner (Eckert & Ziegler Radiopharma, Germany).

Determination of the stability of radiolabelled p-SCN-Bn-NOTA in the presence of human serum

The stability of the ^{68}Ga-NOTA-Rituximab conjugate was evaluated by incubating the radiolabelled conjugate in 1 mL of 1% bovine serum albumin (BSA) for up to 5 hours. Stability was monitored and the RCP reading was assessed over time to evaluated for any degradation or dissociation of the radiolabel from the conjugate.

RESULTS AND DISCUSSIONS

Determination of retention time (RT) of p-SCN-Bn-NOTA and Rituximab with preparative- High

Performance Liquid Chromatography

The separation and retention times of the Rituximab and the chelator p-SCN-Bn-NOTA were assessed using preparative HPLC. The chromatographic separation was performed using a mobile phase of 0.05M phosphate-buffered saline (PBS) at pH 6.8, containing 0.05% sodium azide. The flow rate was maintained at 0.5 mL/min, and detection was performed at a wavelength of 280 nm. Figure 1 presented for HPLC profile of Rituximab and p-SCN-Bn-NOTA, showing their retention times (RT) under specific conditions. A series volumes of Rituximab from 5-100 µl has been injected to the HPLC and the RT appeared at 10.157- 10.168 minutes, while p-SCN-Bn-NOTA was appeared at 14.099 minutes. These results confirm that the two substances have distinct retention times, allowing for the effective separation of rituximab from the chelator p-scn-bn-nota during the labeling process. These different RT are indicative of efficient chromatographic separation of the Rituximab and the p-SCN-Bn-NOTA, which is crucial for subsequent labeling with the radionuclide, ensuring that free chelator and unbound RItuximab are minimized. The differences of rt for both components served as a reference point of good separation, purification and collection of conjugated of p- SCN-Bn-NOTA to rituximab that being used for radiolabeling with ^{68}Ga.

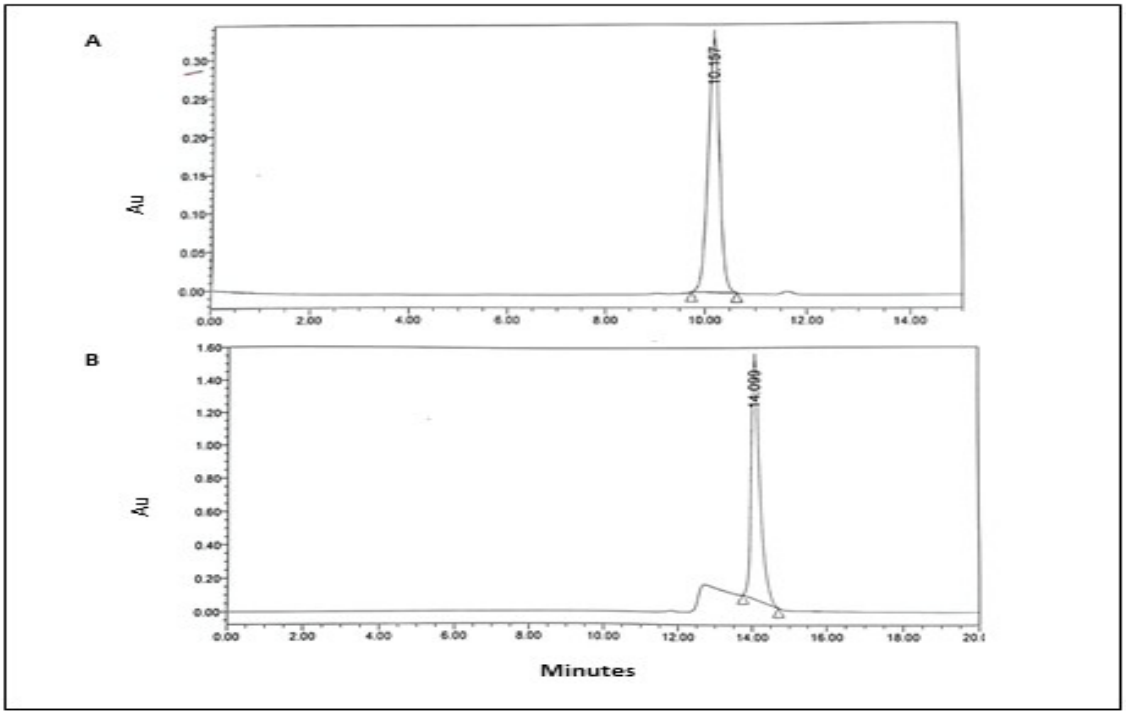

Figure 1. HPLC profile (UV, 280nm) showing A) The retention time (RT) of injected 5µl of Rituximab (10mg/ml) at 10.157 minutes and B) retention time (RT) of injected 5µl of p-SCN-Bn-NOTA at 14.099 minutes. (Flow rate 0.5 ml/min; Solvent 0.05M PBS (pH 6.8) containing 0.05% sodium azide)

Determination of Radiochemical Purity (RCP %) of purified and non-purified radiolabeling Rituximab

The radiochemical purity (RCP) of the radiolabeled product, ^{68}Ga-p-SCN-Bn-NOTA-Rituximab, was determined before and after purification using a PD-10 column. The purification was performed at two different ratios; 1:10 and 1:50 As shown in Figure 2, the RCP values of 1:10 before purification, were 97.33 ± 0.16 % and 89.02 ± 2.24% for the 1:50. These values indicate that, Rituximab was successfully labelled with ^{68}Ga, yet there were still a significant number of impurities or free ^{68}Ga in the impurified samples.

After purification, both ratios showed significant improvements in RCP value, with values reaching 99.63 ± 0.16% for the 1:10 ratio and 99.06 ± 0.01% for the 1:50. This demonstrates that the purification process efficiently removed the free gallium and other impurities, yielding a highly pure radiolabelled rituximab and it showed can be used for in-vivo applications. This is consistent with Adriana *et al.*, [9], that showed the radiochemical yield of the labelled antibody is higher with the purification process with PD-10 column at 1:10, 1:20, 1:50 and 1:100. The slight difference in RCP between the two ratios after purification suggests that a larger elution volume (1:10 ratio) may be slightly more efficient in achieving higher purity, although both conditions yielded results well within acceptable limits for clinical use. The data suggest that the purification method effectively enhances the RCP, thereby indicating the potential for increased clinical reliability. The ITLC analysis showed RCP values more than 99% post purification with PD10 column for both ratio 1:10 and 1:50.

In the present chromatography system, the radiolabelled rituximab retained at the origin, whereas free ^{68}Ga and ^{68}Ga-p-SCN-Bn-NOTA migrated with the solvent front. The retention factor (Rf) of ^{68}Ga-p-SCN-Bn-NOTA-Rituximab was determined to be 0.08-0.15 (Figure 3A-3D), whereas Rf of free ^{68}Ga was 1.04-1.05 (Figure 3E). This findings was in the line with Thakral *et al.,* [11] that performed TLC to separate unlabeled Lutetium-177, ^{177}Lu-DOTA and labelled ^{177}Lu-DOTA-SCN-Rituximab (BioSim). In 20mM citric acid: 10 per cent ACN as mobile phase and Silica gel strips as stationary phase, ^{177}Lu-labelled biosimilar mAb was retained at the origin whereas the undesirable impurities (^{177}Lu and ^{177}Lu-DOTA) migrated along the solvent. RCP is an important parameter to determine the efficacy of radiolabeling of ^{68}Ga with NOTA-Rituximab.

Conducting RCP assessment of free ^{68}Ga before proceeding with the RCP assessment of radiolabeled ^{68}Ga-NOTA-Rituximab is crucial for identification of free ^{68}Ga. The observation that the free ^{68}Ga peak appears at the solvent front shows that free ^{68}Ga is not strongly retained by the stationary phase of the ITLC strip. Instead, it moves with the solvent, indicating its unbound state. This is consistent with the expected behavior of unbound or free species in chromatographic separations. By assessing the purity of free ^{68}Ga, it can confirmed that the starting material for radiolabeling is devoid of any significant impurities or contaminants that could interfere with the subsequent steps or compromise the quality of the radiolabeled product. These findings indicate that the radiolabelling process has been successful and meets the required specifications (> 95%) for RCP, ensuring the suitability of the radiopharmaceutical for further use in preclinical or clinical applications.

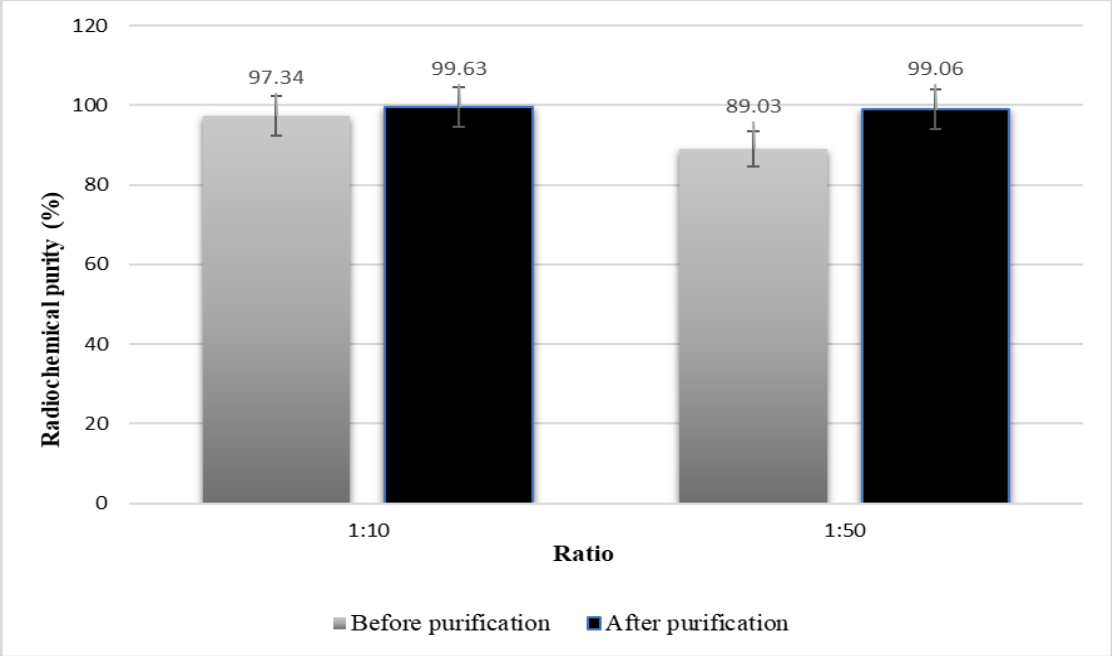

Figure 2. Radiochemical purity (%) of ^{68}Ga-p-SCN-Bn-NOTA-Rituximab before and after purification in PD-10 column at ratio 1:10 and 1:50

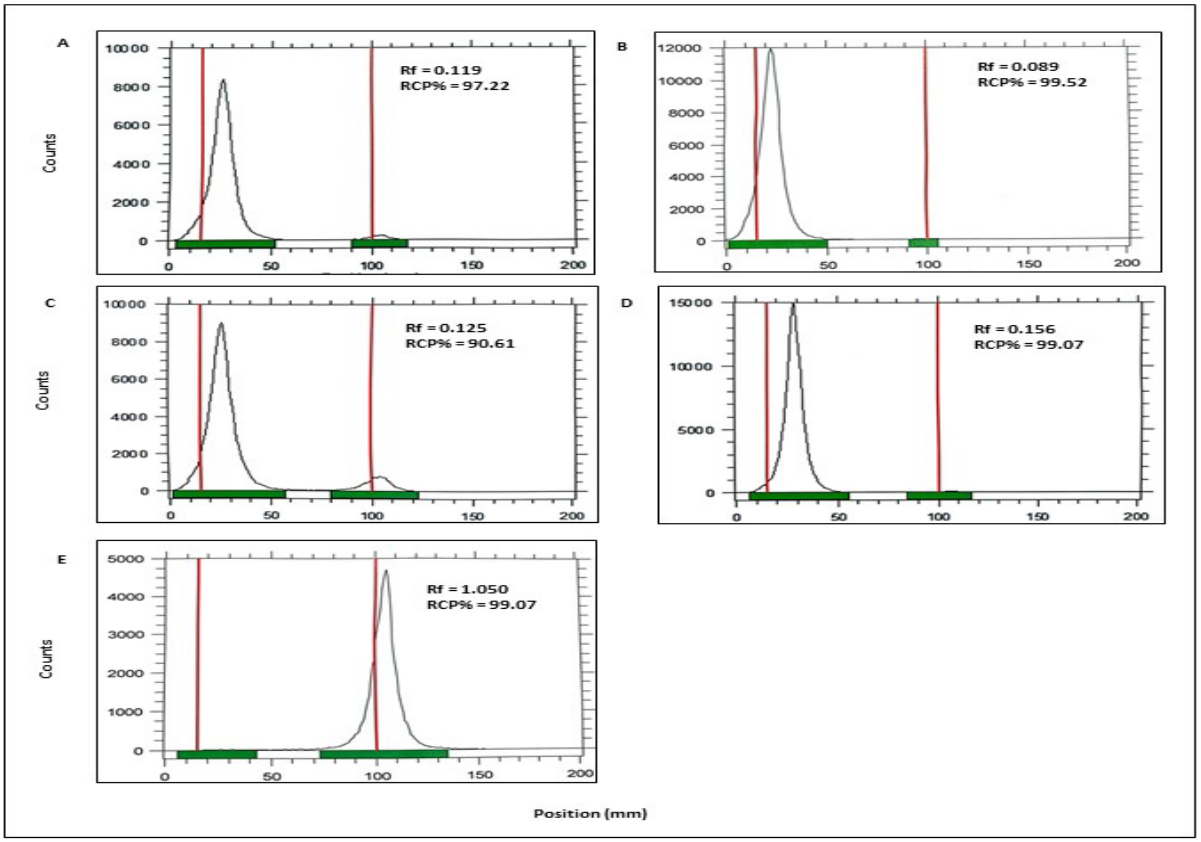

Figure 3. Thin layer chromatography (ITLC) of the non-purified radiolabelled of 1:10 and 1:50 (A and C); purified radiolabelled of 1:10 and 1:50 (B and D); and free ^{68}Ga (E). *Rf= Retention factor; RCP= Radiochemical purity*

Determination of RCP % of radiolabeled Rituximab in hourly and in serum challenged

In vitro stability of the ^{68}Ga-p-SCN-Bn-NOTA-Rituximab was tested by ITLC by periodic sampling showed that metal ion was intact with the immunoconjugate under physiological conditions. Stability was found to be >95% at multiple time points up to five hours (Figure 4). Apart from that, after incubation of radiolabeled Rituximab with fetal bovine serum (FBS), the RCP values showed to be >98% up to five hours, with no evidence for either degradation of ^{68}Ga intact (Figure 5). These values consistently exceed the specified threshold (95%) with or without serum challenged. This stability indicates that the radiolabelled product maintains its integrity and efficacy over time under controlled conditions, which is essential for its potential use in various applications, including molecular imaging and targeted therapy. This results were in line with Suman *et al.*, [10] that showed that % RCP for both 68Ga-NOTA-F (ab′)2-rituximab and 68Ga-NOTA-F (ab′)-rituximab greater than 95% when incubated in saline/serum up to 2 hours at 37°C.

The findings contribute to the characterization and possible clinical application of ^{68}Ga-NOTA-Rituximab by offering insightful information about its stability profile under physiological conditions. The stability were tested up to five (5) hours due to short half-life of ^{68}Ga, which is 68 minutes. As it decays, the propotion of radioactive component decrease, causing the chemical changes, that lead to a reduction in the RCP %.

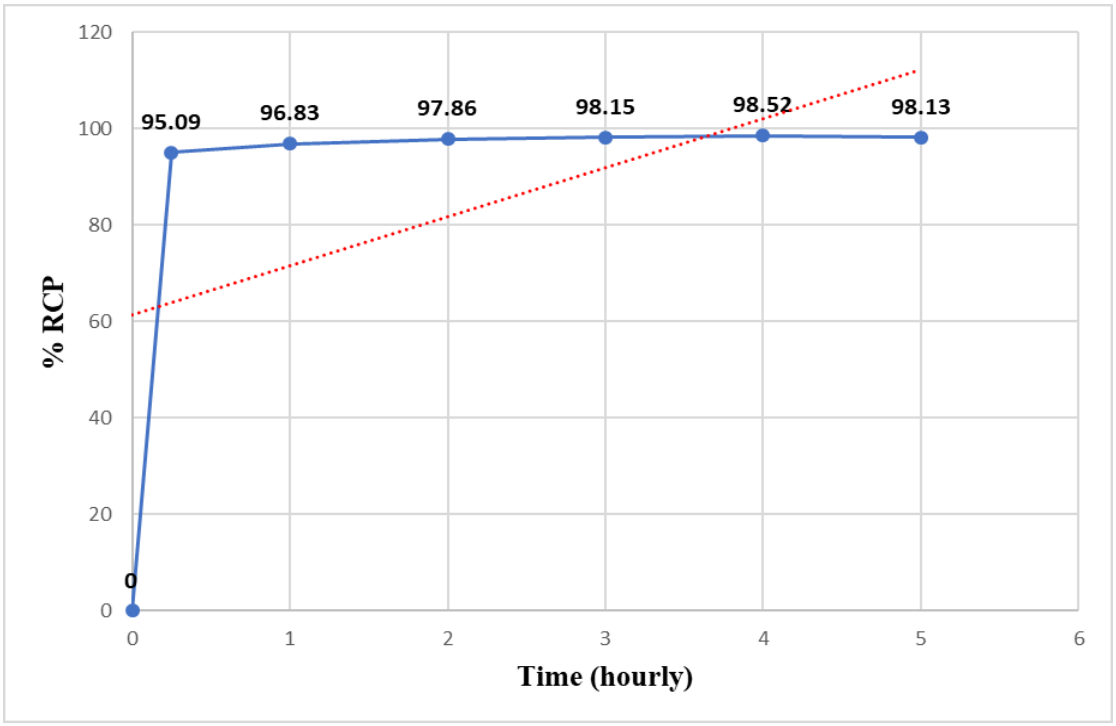

Figure 4: RCP % for stability study of radiolabeled ^{68}Ga-NOTA-Rituximab at time interval

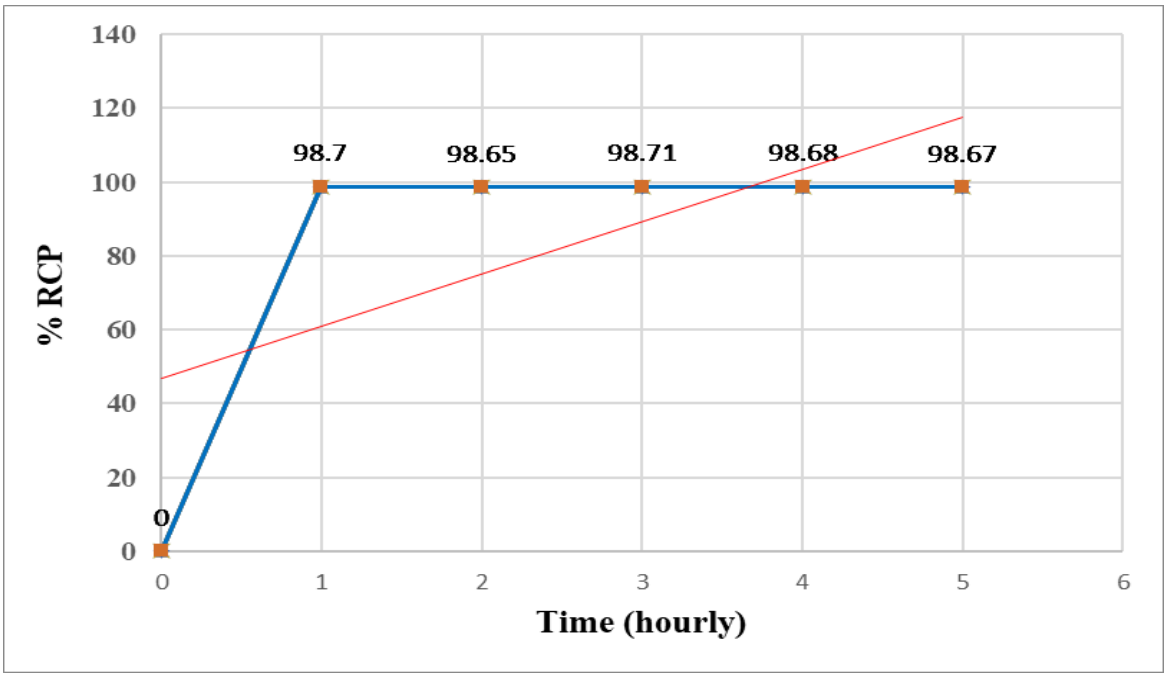

Figure 5: RCP % for stability study of radiolabeled ^{68}Ga-NOTA-Rituximab at time interval with serum challenged

CONCLUSION

In conclusion, this work reported the optimization of the conjugation of p-SCN-Bn-NOTA to Rituximab and radiolabelling with ^{68}Ga that resulting in high radiochemical yield and high specific activity labelled Rituximab, with stable RCP value in serum condition for hours. Thus, this research represents a significant step forward in the development of radiopharmaceuticals for cancer management, highlighting the successful radiolabelling of Rituximab (MabThera) and its potential for improving patient outcomes in oncology. Hence, it suggested the potential for application in preliminary study. In this way, further experimentation in preclinical trials for RIT purposes need to be explored with further formulation of radiolabelling and stability study.

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