THE RELIABILITY OF THE ANALYTICAL METHODS FOR DETERMINATION OF CONJUGATED ANTIBODIES AFTER FINAL PREPARATION IN SERUM SAMPLES OF RAT

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Abstract: Understanding the pharmacokinetics and biodistribution of conjugated antibodies is one of the critical steps in enabling their successful development and use. Their complex structure requires the introduction of multiple bioanalytic methods to define their structure, stability, behavior and fate of both components in vivo.
Appropriate analytical methods should provide insight into the half-life and stability of the blood after application, biodistribution in the body, binding to a specific receptor, time and stability of binding, and the presence of fragments as a result of their destruction.
In our paper we will focus on the method of obtaining conjugated antibodies (rituximab) as well as the use of the HPLC method for their identification in serum samples after intravenous administration. The paper gives us the first indications how the obtained experimental data can be used in the computer simulation of the pharmacokinetic behavior of the same immunoconjugates, to be characterized in biological matrices and to discuss the technical challenges and limitations.
Rituximab (Mabthera®) is an anti-CD20 monoclonal antibody that has demonstrated efficacy in patients with various lymphoid malignancies, including indolent and aggressive forms of B-cell non-Hodgkin s lymphoma (NHL) and B-cell chronic lymphocytic leukaemia (CLL). With the aim of to improve the cytocidal effect of the monoclonals antibodies (mAbs), was introduced the radioimmunotherapy (RIT), where a radioisotope is coupled to a mAb. For labeling mAb with metal and lanthanides radioisotopes, conjugation was previously required in order to introduce a chelating group (p-SCN-Bn-1B4M-DTPA) in the protein chain.
All chemicals and reagents required for experiments were of analytical grade. The antibody solution was previously purified by dialysis or Sephadex gel column. 5-10 mg of antibody was dialyzed against phosphate buffer pH 8.0 and buffer was changed 3 times in 24 hours. The absorbance of a sample of the protein was measured at 280nm in a UV spectrophotometer and protein concentration was calculated in mg/mL.
A rat model was used to monitor the pharmacokinetics of the antibodies and to see their stability after injection. Serum samples were analyzed using the HPLC method and were the first indicator of the possibility that this method would be the first step in their identification.
The structural differences and the behavior of the conjugated antibodies obtained are a major indicator that the use of appropriate analytical techniques, as well as their proper validation and use, is a critical parameter for their development and use as potential therapists.
Only if valid data obtained through appropriate analytical methods can be used as data in computer programs and used for modeling. The HPLC technique is one of the most appropriate, which provides data on the behavior of

conjugated monoclonal antibodies in the blood after their administration and the possibility of using them as a parameter for the role of animal models in translational medicine.
Keywords: rituximab, antibody conjugates; pharmacokinetics, biodistribution; animal model, high performance liquid chromatography-HPLC,

1. INTRODUCTION

Antibody therapy represent a broad and rapidly growing group that has a significant impact on the results obtained in the treatment of various malignancies. Their strength is in the existing potential to activate the immune system to specifically target malignant cells without causing many side effects which is quite different in comparison with many conventional chemotherapeutics (1).

Antibodies, also known as immunoglobulins, are B-cell molecules with a molecular weight of about 150 kDa. They are composed of 4 polypeptide chains (2 heavy and 2 light chains). Antibodies can circulate as soluble, or can bind to the B-cell membrane as part of the B-cell receptors. Both heavy and light chains have a constant moiety (c), which is identical in immunoglobulins of the same isotype, and unique variable moieties (v) that contain antigen binding site, located on both light chain molecules. (2).

The multiple acting mechanism of monoclonal antibodies is important for explaining the antitumor activity of tumor antigen-specific antibodies. In recent publications, most attention has been focused on the ability of antibodies to interact with a critical signaling pathways that maintain the malignant phenotype and to trigger an anti-tumor antigen-specific immune response. (3,4).

The European Medicines Agency and the US FDA have registered several native monoclonal antibodies for the treatment of various solid tumors and hematological malignancies. There are a large number of clinical studies involving antibodies, and not a small number of those in preclinical trials. Due to the great interest and the obtained results, monoclonal antibodies can be used as native (unconjugated) or conjugated with various drugs, toxins and radioisotopes to improve the specificity and pharmacological response and eliminate side effects (5-8).

Monoclonal antibodies: Rituximab and other antibodies

Rituximab is a type I anti-CD20 chimeric IgG 1 kappa TMA, bearing human derived constant regions and mouse derived variable regions. It has a molecular weight of 143859.7Da and molecular formula of $C_{6416}H_{9874}N_{1688}O_{1987}S_{44}$. It is composed of two light chains of 213 amino acids each and two heavy chains with 451 amino acids each as follows (9):

Rituximab heavy chain chimeric

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKGLEWIGAIYPGNGDTSYNQKFKGKATLTAD
KSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGT
AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV
DKKAEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIETISKAKGQPREPQVYTLPPSRDE
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGVSCSVMH
EALHNHYTQKSLSLSPGK

Rituximab light chain chimeric

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGTSYSLTI
SRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

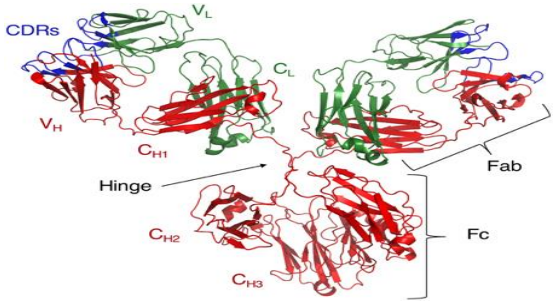


Figure 1. Graphical representation of the structure of a monoclonal IgG antibody (10).

Points to consider before clinical use

Although human monoclonal antibodies or chimeric antibodies containing human constant region domains are attractive from a conceptual standpoint, for intravenous applications, they might not be ideal for targeted radiotherapeutic use, particularly with longer half-life radionuclides such as ^{177}Lu. The reason is that the clearance of human IgG from the blood pool is slow, with biological half-lives of 20-24 days generally observed. For this reason, when radiolabeled human constant region bearing constructs have been administered at therapeutic doses, hematological toxicity has been dose limiting.

Regulatory agencies governing the granting of permission to do clinical trials in different countries likely will have different criteria. Nonetheless, several types of measurements should be performed to obtain the data necessary to permit clinical investigation.

Radioimmunotherapy (RIT) combines the targeting advantage of a monoclonal antibody with the radiosensitivity of non-Hodgkin lymphoma (NHL) cells. The unlabeled antibodies have indeed added efficacy, but many patients still relapse. One method of enhancing the cytotoxic potential of monoclonal antibodies is to attach them to a radionuclide to form a radioimmunoconjugate (RIC). The use of an RIC has the advantage of targeting not only the cell to which the antibody is bound but also the surrounding tumor cells and microenvironment (11).

The investigation of target-specific radiopharmaceuticals based on monoclonal antibodies (mAb), their fragments and peptides for therapy and molecular imaging is increasing due to the availability of new radioisotopes and biomolecules with improved characteristics. Unfortunately, the success of radioimmunotherapy (RIT) in hematologic disease has not been translated to solid tumours yet. Nevertheless, treatment of minimal residual disease, locoregional applications and pretargeted RIT has shown some advances (12).

Radionuclides for antibody labeling

The choice of the radionuclide to be used in the development of radiopharmaceuticals depends on the radionuclide's availability and also its physical properties, chemical properties and biological properties; all of which should be matched with the intended clinical use of the radiopharmaceutical. Physically, the radionuclide should have a high yield of particle radiations of appropriate energy. The half-life, especially for antibody-based radiopharmaceuticals, should be matched appropriately with the in vivo pharmacokinetics of the targeting antibody.

Biodistribution of conjugated antibodies

Biodistribution is an important parameter for the use of radiolabeled conjugated antibodies. A major contributor to molecular failure is often incomplete or poor understanding of pharmacokinetics (PK) and disposition profiles that lead to limited or reduced efficacy. Increased and fundamental characterization efforts aimed at disseminating mechanisms affecting PK and disposition of mabs and peptides may help to improve the design for their intended pharmacological activity, and thus their clinical success. The factors of PK and disposition for mabs and peptides are largely influenced by the target dispositions mediated by drugs and authorization mechanisms associated with nontarget related to the interaction between the structure and physiochemical properties of mabs and peptides with physiological processes (13)

The role of animal models in defining the pharmacokinetic parameters of conjugated antibodies (14)

Pharmacokinetic models are used to describe the temporal disposition and absorption of a substance in a living system. For medical purposes, pharmacokinetics can be used to assess optimal drug dosing regimens in a variety of therapeutic situations.

Understanding the PK and PD of monoclonal antibodies and their biological and mechanical basis are key in:

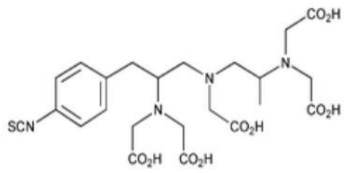
- enabling their design and selection,
- designing appropriate efficacy and toxicity studies,
- translation of PK / PD parameters in humans
- optimizing the dose and mode of use in order to increase the success of the clinical application

In vivo stability of the antibody conjugate should be assessed in normal rodents (rats or mice). It is recommended that the biodistribution of the isotope (radioactive or non-radioactive) antibody conjugate be determined at several time points including 1 and 3 days after injection with 3 mice per group of different samples.

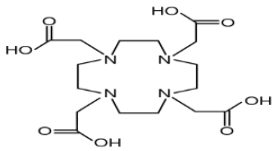
Bifunctional chelating agents are compounds that contain a strong metal chelating group at one end and a reactive functional group at the other end that is capable of binding to proteins. When conjugated to monoclonal antibodies, these agents act as radiometal carriers for tumor targeting (15).

Numerous techniques for modifying biomolecules have been developed. Conjugation groups facilitate the binding of chelators to biomolecules and the most commonly used are: anhydride, bromo- or iodoacetamide, isothiocyanate, N-hydrosuccinamide ester and maleimide groups. These groups, whether they are a natural part of the biomolecule, or are artificially introduced, can serve as "handles" for conjugating bifunctional chelating agents (BFCA) with the biomolecule (16,17)

Chelators that can hold high-stability radiometals under physiological conditions are essential to avoid excessive damage to healthy cells. Acyclic chelators based on the DTPA structure and macrocyclic chelators based on the DOTA structure are the most commonly used agents in RIT.



p-SCN-Bn-1B4M-DTPA



DOTA

Figure 2. Commonly used bifunctional chelating agents

Liquid chromatography with detection in the ultraviolet region of the spectrum

Chromatographic techniques, especially size exclusion HPLC are one of the most commonly used and important as appropriate controls of conjugated antibodies in serum, as well as the eventual presence of degradation products.

These can be protein standards bracketing (above and below) the 150 kDa of IgG molecules or another antibody that itself has been evaluated with regard to its purity. Ideally, the size distribution of the antibody-chelate conjugate before and after labeling should be evaluated. The results should be expressed as percentage of protein present as monomeric IgG, dimers, aggregates as well as lower molecular weight impurities.

HPLC-UV is a popular analytical technique used to separate, identify and quantify each component of a given mixture. It is also known as an advanced column chromatography technique. The solvent or mobile phase in liquid column chromatography usually flows through the column by gravity. In this technique, the mobile phase moves through the column under different pressures depending on the nature of the test sample and therefore this method is much faster than other chromatographs on the column. (18,19).

In this technique, the pumps are of great importance because they allow the mobile phases together with the sample to enter a column filled with solid material, known as the stationary phase. The interaction of each component of the sample will be different with the mobile or stationary phase and this allows a difference in the flow rate of each component, leading to their separation (20).

Size exclusion chromatography or gel filtration chromatography is one of the HPLC separation methods. The column used is filled with a material that contains many pores. When dissolved molecules of different sizes enter the column, smaller dissolved molecules pass more slowly through the column because they penetrate deep into the pores, while large dissolved molecules pass quickly through the column because they do not enter the pores. Therefore, larger molecules elute from the column earlier and smaller molecules later, which effectively sorts the molecules by size. This is the principle of separation of size exclusion chromatography. Various types of columns are available, filled with absorbents of different molecule sizes and the nature of their structure (22).

The choice of stationary and mobile phases play an important role in the process of sample separation (22, 23).

The basic mobile phases used include any mixture of water with different solvents, however some HPLC systems are used without aqueous mobile phases composed of some acid or salt which help to separate the sample component. (24).

This study was designed to predict the pharmacokinetics of previously prepared conjugated monoclonal antibodies (rituximab) to a rat animal model (Wistar) by taking blood samples. Pharmacokinetics of native monoclonal antibodies will be monitored.

The serum obtained from the blood samples was analyzed by the HPLC-UV method to determine the presence of conjugates, to determine their structure, as well as their stability by monitoring for possible decomposition products.

Through the obtained chromatograms we want to determine that they can be used as a control of computer modeling to assess human pharmacokinetic parameters and to contribute with our results in the development of translational medicine.

2. MATERIAL AND METHODS

Preparation of solutions

Water preparation

Water used either for conjugation or for preparation of reagent solutions was purified from metal impurities.

Phosphate buffer preparation

Solution A was prepared by adding of 17.9 g Na_2HPO_4 x $12H_2O$ into 500 ml of purified water (pH 9.4). Solution B was prepared by adding of 6.8 g NaH_2PO_4 x H_2O into 500 ml of purified water (pH 4.5). Phosphate buffer 0.1 M solution for conjugation was prepared by adding of 23 ml of solution B to solution A. The pH of the buffer should be in the range of 8.2-8.3. In the case of lower pH value, 2 M NaOH was used to set up of the pH. Prepared buffer was then filtered through the Millipore filter 0.22um. After filtration, the pH of the prepared buffer was checked again.

Ammonium acetate buffer preparation

Acetate buffer (0.5 M) was prepared by dissolving of 9.64 g of ammonium acetate in 250 ml of water (pH 6.8). After 24 hours, solution was filtered through a Millipore filter 0.22um, diluted to a concentration of 0.05 M and pH=7.0 set up using 25% ammonium hydroxide.

Purification of rituximab from MabThera®

2ml of the commercial product (MabThera®) was loaded into Amicon 2ml 30kDa ultrafilters and ultrafiltered at 5000 rpm in 4 cycles of one hour per cycle at 2-8°C. 2ml of 0.1M phosphate buffer pH 8.0 was added to the ultrafilter at the end of every cycle. The ultrafiltrate was discarded. After the 4th cycle, the purified rituximab was recovered by reverse filtration at 5000rpm for one hour. The product was reconstituted to 2ml with 0.1M pH 8.0 phosphate buffer and stored at 2-8°C temperatures in 15ml falcon conical centrifuge tubes.

Conjugation

2ml of the rituximab purified product was pipetted into a 15ml Falcon centrifugation tube, into which 1.54mg in 0.154ml of 1b4mDTPA in 0.1M phosphate buffer pH 8.0 was added to make a rituximab-to-1b4mDTPA mole ratio of 1:20. The mixture was then mounted on an orbital shaker in 2-8°C and left to react overnight (for 18 hours) at shaker speed of 50rpm.

Purification of immunoconjugate

The reaction mixture was loaded onto 2ml Amicon 30kDa ultrafilters and ultra-filtered at 5000 rpm in 2-8°C for 4 one-hour cycles, washing with 2ml of 0.1M pH 8.0 phosphate buffer in each cycle. After every cycle, the absorbance of ultrafiltrate at 280nm wavelength in a uv-vis spectrophotometer was determined and the ultrafiltration continued until the ultrafiltrate absorbance was zero (after the 4th cycle).

The purified immunoconjugate was recovered from the ultrafilters by reversing the ultrafilters and centrifuging at 500rpm for one hour.

Concentration determination of antibody immunoconjugate by UV-VIS spectrophotometry

Concentration of the antibody/immunoconjugate was determinate using UV spectrophotometer (Jenway UV/VIS spectrophotometer 6715), and semi-micro UV polypropylene tubes with 0,1M PBS pH=8.0, at 280nm in trilicate.

The purified immunoconjugate was reconstituted to 1ml using 0.1M phosphate buffer pH 8.0. An aliquot of 0.100ml was drawn therefrom, reconstituted to 1ml with 0.1M phosphate buffer pH 8.0 and its concentration determined using UV-VIS spectrophotometry at 280nm wavelength (Jenway UV/VIS spectrophotometer 6715), and semi-micro UV polypropylene tubes at 280nm in triplicate.

Labeling of immunoconjugate with non-radioactive lutecium

Labeling of the immunoconjugate with the non-radioactive isotope was performed by dissolving the immunoconjugate in sterile 0.9% NaCl solution. 1.0 µL of LuCl3 solution with a concentration of 1.0709 µg / µL [equivalent radioactivity 177Lu of 4377.1 MBq, corresponding to the MTD code of the patient] will be added to the dissolved lyophilisate. Solutions p-SCN-Bn-1B4M-DTPA-Tr were incubated at room temperature for up to 30 minutes.

Administration of conjugated antibodies to rats

Administration of conjugated rat antibodies was performed in the caudal vein, in a volume of 0.5 ml. The samples used in this study were taken 1 hour after caudal vein injection. The serum obtained after blood centrifugation was used for HPLC analysis.

Figure 3. Intravenous administration of conjugated antibodies in rat tail vein

Determination of rituximab-immunoconjugate integrity and presence of decay products in serum using HPLC-UV method

Integrity injectable rituximab-immunoconjugate in which the antibody-ligand ratio was 1:20, as well as the possible presence of decomposed product determined by the HPLC-UV method, using the Zorbax Bio Series GF-250 column. Specific volume (20 μL) of serum containing bio-conjugate and labeled rituximab-immunoconjugate with non-radioactive lutecium bio applied to a chromatographic column using a mobile phase phosphate buffer and a flow rate of 1 mL. The elution process was sent with a UV detector at a wavelength of 280 nm.

3. RESULTS

Purification of rituximab from commercial product MabThera®

Rituximab used in my work was obtained from commercial product MabThera®. Because this commercial product was dedicated for the application in the patients contained some additives and stabilizer. For realize our experiments we needed the pure antibody. For that reason we purified commercial product using ultrafiltration.

During the process of ultrafiltration as is described in methods, we wanted to eliminate all other substances and fragments of the antibody obtained during the process of ultrafiltration.

Size exclusion chromatography was used (SEC) to confirm the purity and presence of the whole rituximab (25)

Conjugation of rituximab with 1b4m-DTPA

The process of conjugation as described in the methods section above, was performed using the ligand 1b4mDTPA in 0.1M phosphate buffer pH 8.0 added to the purified antibody. The ratio of the antibody and ligand in the mixture of the complex rituximab-to-1b4mDTPA was 1:20. After the incubation period obtained, the immunoconjugate mixture containing rituximab-1b4mDTPA was purified.

Purification of immunoconjugate

We used the same method of ultrafiltration for purification of the immunoconjugate as we used for the antibody, as a more convenient method that does not necessarily damage the antibody.

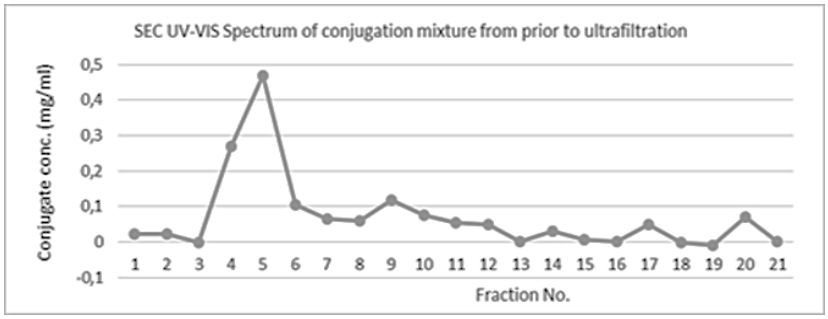


Figure 4. SEC spectrum of a sample of the non-purified conjugation mixture

As we can see from the figure 4 above, the immunoconjugate rituximab-to-1b4mDTPA is the biggest of the multiple present peaks. After purification, as shown in the figure 5 below, the smaller peaks were eliminated, demonstrating the effectiveness of the purification process.

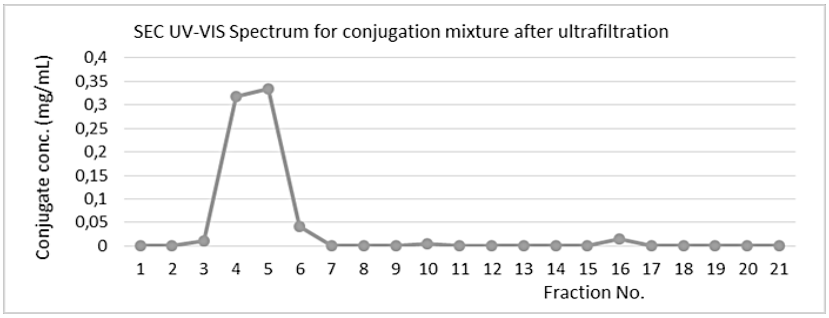


Figure 5. SEC spectrum with UV-VIS detection for the purified immunoconjugate

Concentration determination by UV-VIS spectrophotometry

After purification, we determined the concentration of the purified immunoconjugate as described in the methods section above. Then, the bulk immunoconjugate was reconstituted with 0.1M phosphate buffer pH 8.0 into a 13.7ml solution of 1mg/ml and stored at 2-8°C in a 15ml falcon conical centrifuge tube.Purified and labeled non-radioactive isotope immunoconjugates were injected into rats in a caudal vein (Figure 3)

Blood samples (0.5 ml) were taken from rat tail after 1 hour in Ependorff polypropylene tubes

Serum obtained by centrifugation was used to introduce a test method using HPLC-UV size exclusion chromatography

Determination of the integrity of rituximab-immunoconjugate and the presence of decay products in serum using HPLC-UV size exclusion chromatography

Chromatographic techniques are one of the most commonly used for examining the presence of conjugated antibodies in serum, as well as the eventual presence of degradation products.

Integrity injectable rituximab-immunoconjugate in which the antibody-ligand ratio was 1:20, as well as the possible presence of decomposed product determined by the HPLC-UV method, using the Zorbax Bio Series GF-250 column. Specific volume (20 μL) of serum containing bio-conjugate and labeled rituximab-immunoconjugate with non-radioactive lutecium bio applied to a chromatographic column using a mobile phase phosphate buffer and a flow rate of 1 mL. The elution process was sent with a UV detector at a wavelength of 280 nm.

The figures 6 and 7 represent the HPLC profile of the conjugated antibody and a sample of serum containing a conjugated antibody 1 hour after injection into a healthy rat.

The HPLC-UV method was to be a method for monitoring the pharmacokinetics of injected retiuximab conjugates.

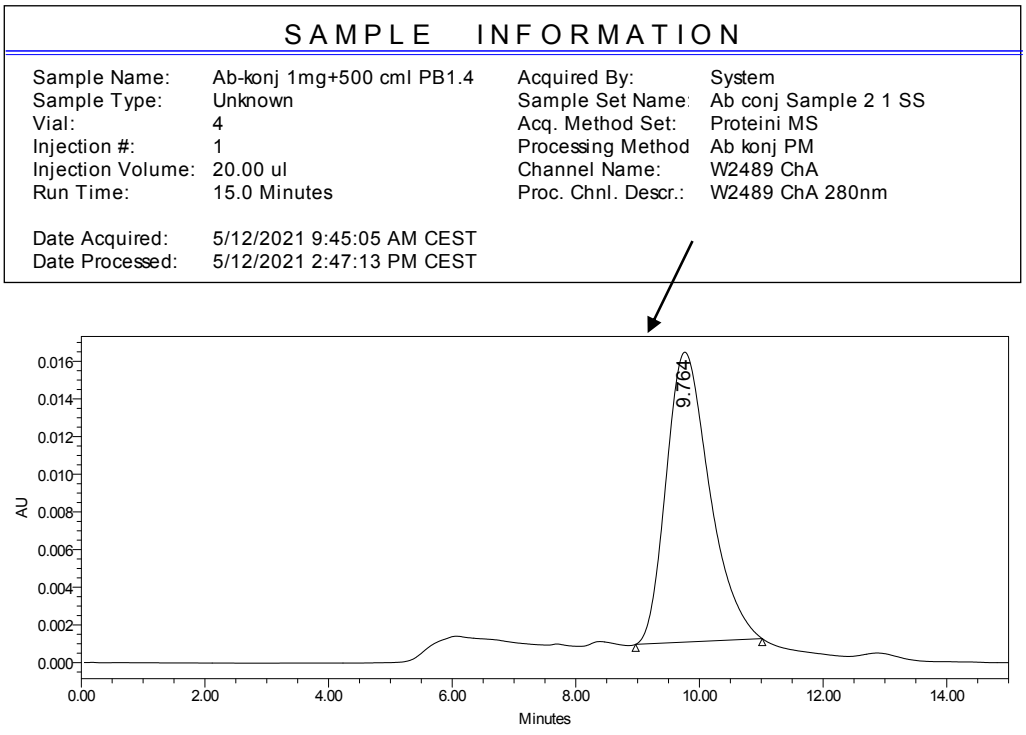


Figure 6. HPLC profile of conjugated antibody (rituximab- p-SCN-Bn-1B4M-DTPA)

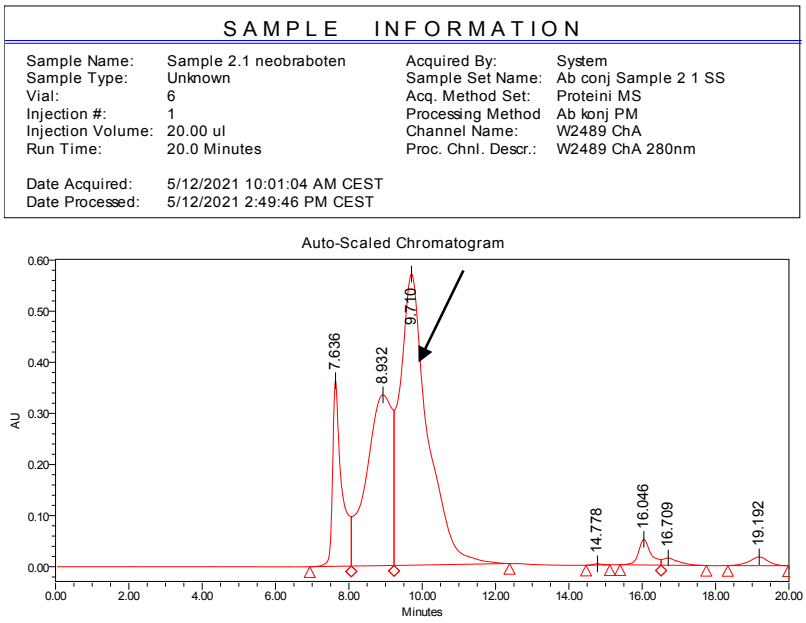


Figure 7. HPLC profile serum sample containing conjugated antibody (rituximab- p-SCN-Bn-1B4M-DTPA) 1 hour after injection

The first tests were aimed at confirming the method and to see if the obtained HPLC profiles from the serum samples will be able to give us information about the presence of conjugates, determine their structure, as well as their stability by monitoring possible decay products and at the same time to computer simulations are used to examine the pharmacokinetics and pharmacodynamics of injected antibodies.
The same program should be used to give us a prediction of the human pharmacokinetics of conjugated monoclonal antibodies.
Preliminary results show that the conjugated antibody is stable and that the purification and conjugation procedure does not affect its stability.
The chromatogram obtained from the immunoconjugate in rat serum 1 hour after injection (picture) clearly shows the presence of the immunoconjugate, but the peaks obtained with time ..... are an indication that an additional procedure should be developed for processing the sample for analysis. Namely, the presence of additional peaks can be an indicator of impurities obtained from serum proteins.
The resulting chromatograms as such can not be used as data to monitor the pharmacokinetics of the immunoconjugate at different times, but can be of great importance in the introduction and standardization of the HPLC method for the determination of conjugated antibodies, which has always been a major problem in biological preparations and their biosimilar formulations.

4. CONCLUSION

Our preliminary obtained results confirm the thesis that the introduction of new preparations is a long-term process and that each step must be scientifically adapted using the most appropriate analytical tools and sensitive tests to detect small product-related differences between the biosimilar and the reference product.
We confirmed that the purification method of ultrafiltration that had been used previously for similar studies was still suitable for purification of rituximab antibody form its excipients in MabThera without significant damage to the antibody. The purification method is therefore recommended for use in similar experiments.
The structural differences and the behavior of the conjugated antibodies obtained are a major indicator that the use of appropriate analytical techniques, as well as their proper validation and use, is a critical parameter for their development and use as potential therapists.
Only valid data obtained through appropriate analytical methods can be used as data in computer programs and used for modeling.

The HPLC technique is one of the most appropriate, which provides data on the behavior of conjugated monoclonal antibodies in the blood after their administration and the possibility of using them as a parameter for the role of animal models in translational medicine.

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