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Original

Labelling of the anti-III-neurotubulin monoclonal antibody by ^{99m}Tc and its binding to responsible antigen

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Abstract

BACKGROUND: β-III-neurotubulin is a characteristic degradation protein of cellular cytoskeleton of nervous tissues, which originates with disorders that lead to the loss of peripheral neurons or neurons of the central nervous system. For the diagnostics of neuropathic and neurodegenerative processes, β-III--neurotubulin is therefore the goal structure, and radioactive labelled antibody TU-20 ^{99m}Tc could theoretically enable diagnostic application *in vivo*.

MATERIAL AND METHODS: In our case, were selected three ways of labelling monoclonal antibody IgG1 — TU-20, which shows a high affinity towards β-III-neurotubulin with ^{99m}Tc. The indirect labelling was ensured through the bifunctional chelator HYNIC, the direct labelling by electrolytic means, and the direct labelling of antibody TU-20 reduced by 2-mercaptoethanol. To observe each single feature, the appropriate chemical and biochemical control methods were used. Chemical purity was ensured by gel filtration and, together with chemical stability, it was checked by paper chromatography. To control the biological stability, SDS electrophoresis was used. The immunoreactivity was checked using ELISA-tests.

RESULTS: The results have shown that the optimal method for labelling the antibody TU-20 is indirect labelling through the bifunctional chelator HYNIC, and the least effective method of labelling the antibody TU-20 is reduced by 2-mercaptoethanol.

DISCUSSION: The results of labelling the monoclonal antibody towards antigen TU-20 with ^{99m}Tc confirmed that structure of the monoclonal antibody is destroyed by 2-mercaptoethanol, and in the case of electrolytic labelling, there are not enough binding places for radionuclide ^{99m}Tc on the monoclonal antibody.

Key words: monoclonal antibody, TU-20, ^{99m}Tc, HYNIC, 2-mercaptoethanol, radiolabelling.

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Introduction

The radionuclide technique of labelling is a part of development of radiodiagnostic and radiotherapeutic methods, which can be uncovered. Appropriately radiolabelled monoclonal antibody TU-20 with ^{99m}Tc could be used as a radiodiagnostic for the diagnosis of neurodegenerative disorders as Parkinson's or Huntington's diseases [1, 2]. β-tubulin has seven isotypes but only β-III--neurotubulin is run from in neurons exclusively [1, 2]. Neurodegenerative disorders and processes could be characterized by the amount of anti-monoclonal antibodies towards β-III-neurotubulin in celiolymph. Radionuclide ^{99m}Tc is used for direct binding proteins through thiolate groups to rising bonds ^{99m}Tc-cysteinyl-protein. Chemical reduction of disulphide bonds in proteins leads to an increasing of number of binding places [3]. Reduction of disulphide bonds of the monoclonal antibody could be made by a suitable reduction reagent (2-mercaptoethanol). However, this could destroy the higher structure of the monoclonal antibody, and it is not dependent on the added amount of reduction reagent. Electrolytic process is used as a direct method of labelling. There is an electrolysed ^{99m}Tc of the generator for reduction of ^{99m}Tc (VII) to lower oxidation degrees in which radionuclides have a higher affinity to the monoclonal antibody. However, use of this method is incorrect because the monoclonal antibody is not reduced and there are not enough binding places for ^{99m}Tc on TU--20. In addition, indirect methods were used for labelling through bifunctional chelator hydrazinonicotinamide (HYNIC), which makes bonds between the radionuclide and monoclonal antibody. We used a method on which the monoclonal antibody is radiolabelled with ^{99m}Tc using HYNIC with tricin as a co-ligand [4–9].

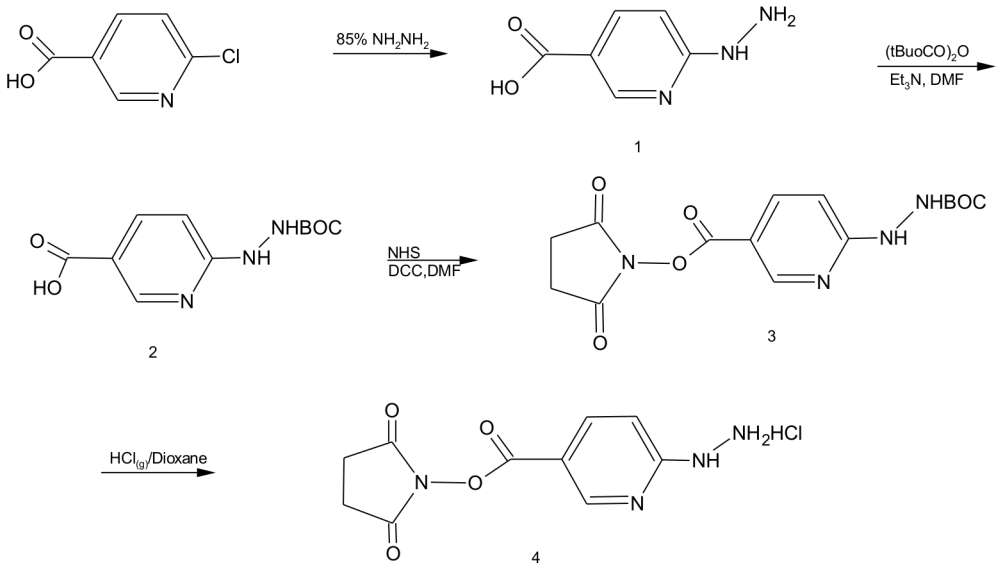

Figure 1. Reaction scheme for the preparation of succinimidyl 6-hydrazinopyridine-3-carboxylate hydrochloride.

Material and methods

Indirect labelling

One of method of labelling of the monoclonal antibody with radionuclides is labelling in the presence of a bifunctional chelator; its imposition makes bonds between the monoclonal antibody and radionuclide. In our case, we used HYNIC as a bifunctional chelator. HYNIC was prepared in ÚJV NRI, inc. a.s. Preparation of HYNIC (Figure 1). Conjugate monoclonal antibody — HYNIC originates from binding HYNIC to the amino group of molecules of lysin. A stock solution of HYNIC was interfused with monoclonal antibody and the reaction volume was complemented to 1 ml. Reaction time was 4, 24 hours. Conjugate was purified by gel filtration (Sephadex G 25) and separated to 6 fractions. High-molecular fraction was detected by spectrophotometry (280 nm). $SnCl_2.2H_2O$ to reduction ^{99m}Tc from generator was interfused with Tricin, ^{99m}Tc and high-molecular fraction. The next analyses (ELISA tests to check the immunoreactivity — ELISA VIDITEST anti-monoclonal towards neurotubulin, IgG1, SDS-electrophoresis to control biological stability. Checking the chemical stability and purity was ensured by paper chromatography) were made with the reaction mix.

Direct labelling — with 2-mercaptoethanol

2-mercaptoethanol was use to reduced the disulphide bonds in proteins of the monoclonal antibody and reduction ^{99m}Tc was ensured by SnCl2.2H2O which is contained in the 6-MDP kit. 2-mercaptoethanol and monoclonal antibody were mixed, and the reaction volume was complemented to 1 ml. Reaction time was 10 minutes. The mixture was purified by gel filtration (Sephadex G 25) and separated to 6 fractions. High-molecular fraction was detected by spectrophotometry (280 nm). 6-MDP kit was dissolved in physiological solution, and 10 μl was mixed with ^{99m}Tc and high-molecular fraction. The mixture was purified by gel filtration (Sephadex G 25) and separated to 6 fractions in order to gain all labelled monoclonal antibodies with ^{99m}Tc without free ^{99m}Tc and the detected high-molecular fraction.

The next analyses (ELISA, SDS-electrophoresis, paper chromatography) were made with this high-molecular fraction.

Direct labelling — electrolytic

Electrolytic methods are used for direct labelling of the monoclonal antibody with radionuclide. Anionic forms of technecium ($^{99m}TcO4$-) are reduced to lower oxidation grades to more reactive forms. In our case, we used for electrolysis mixture of monoclonal antibody-technetium or single eluate. Conditions of electrolysis were controlled by laboratory source and reduction was made by tin electrode. 1 ml of reaction volume was prepared (60 MBq of ^{99m}Tc (eluate) was coupled with NaCl with or without monoclonal antibody). Electrolytic time was 30 seconds and reaction time was 15 minutes. The mixture was purified by gel filtration (Sephadex G 25) and separated to 6 fractions to disjoin free ^{99m}Tc from the labelled monoclonal antibody. High-molecular fraction was detected by spectrophotometry (280 nm). Next analyses (ELISA, SDS--electrophoresis, paper chromatography) were made with this high--molecular fraction.

Results

Indirect labelling

Experiments were made in reaction conditions which were chosen according to reactivity, purity and stability of reaction mixture. Photometry of fractions of conjugate TU-20 — HYNIC produced by gel filtration are shown in Table 1. 3.5 ml, 5 ml, 7.5 ml and 9 ml volumes of Sephadex G 25 were used. Paper chromatography of reaction mixture of radiolabelled antibody is shown in Figure 2. There was no need for the reaction mixture to be purified — vide Figure 2.

Table 1. Concentration TU-20 of fractions obtained by purification of conjugate TU-20-HYNIC. Entering concentration TU-20 750 μg/ml

Gel volume	3.5 ml	5 ml	7.5 ml	9 ml
Fraction	Concentration TU-20 [μg/ml]	Concentration TU-20 [μg/ml]	Concentration TU-20 [μg/ml]	Concentration TU-20 [μg/ml]
1 (TU-20-Hy)	37	0	0	0
2 (TU-20-Hy)	44	97	0	0
3 (TU-20-Hy)	310	328	148	0
4 (TU-20-Hy)	147	119	451	42
5 (TU-20-Hy)	101	111	46	640
6 (TU-20-Hy)	66	63	53	22

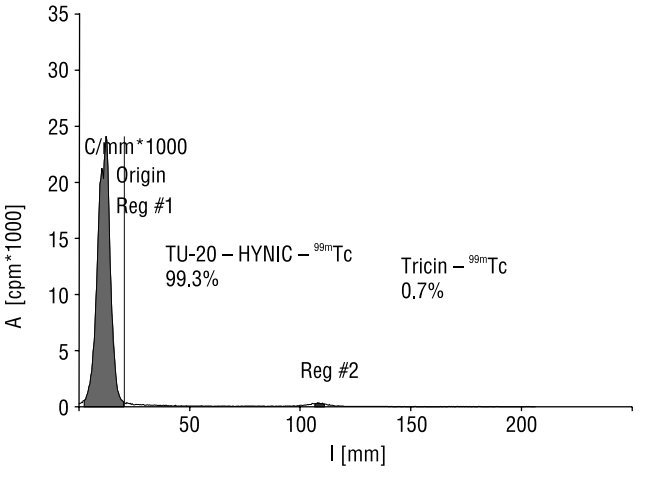

Figure 2. Paper chromatography of reaction mixture TU-20-HYNIC-^{99m}Tc.

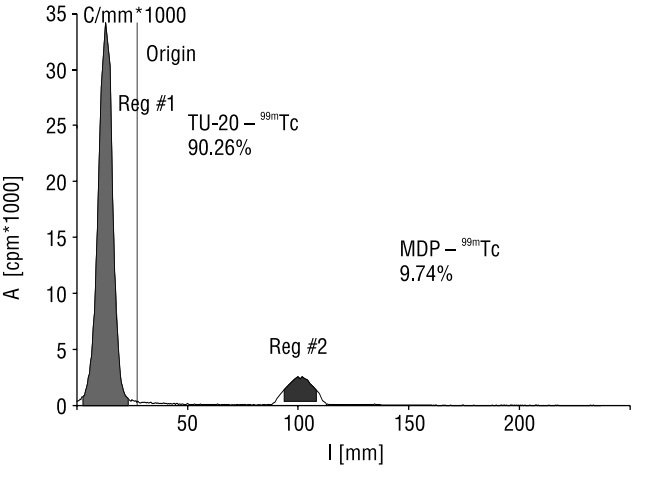

Figure 3. Paper chromatography of reaction mixture TU-20-^{99m}Tc.

Table 2. Concentration TU-20 of fractions obtained by purification of mixture TU-20- 2(ME). Entering concentration TU-20°750 μg/ml

Gel volume	7.5 ml	9 ml
Fraction	Concentration TU-20 [μg/ml]	Concentration TU-20 [μg/ml]
1 TU-20 (ME)	0	0
2 TU-20 (ME)	12	1
3 TU-20 (ME)	67	3
4 TU-20 (ME)	358	93
5 TU-20 (ME)	220	530
6 TU-20 (ME)	78	46

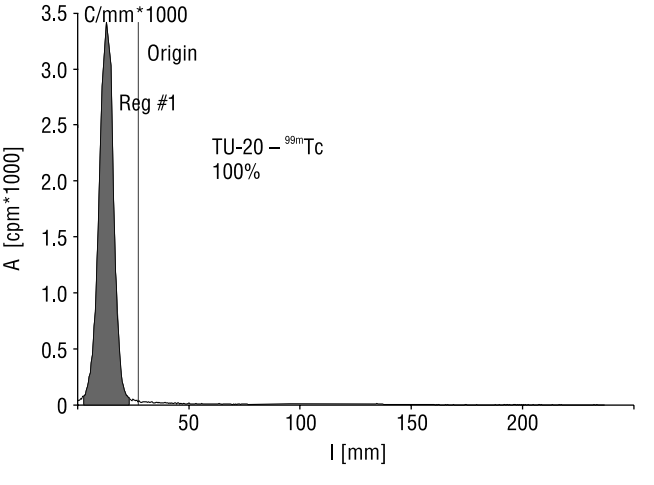

Figure 4. Paper chromatography of 5 fraction past purification of reaction mixture TU-20-^{99m}Tc.

Direct labelling — with 2-mercaptoethanol

In this case 5 ml, 7.5 ml and 9 ml cells of Sephadex G 25 were used for the separation of unneeded 2-mercaptoethanol from reduced TU-20, and the reaction mixture of radiolabelled antibody was purified from unnecessary MDP and free ^{99m}Tc. 7.5 ml cell was used. This means that there were large losses of monoclonal antibody. After first purification, there was 530 μg/ml TU-20 of 750 μg/ml entering monoclonal antibody. However, after the second purification of reaction mixture there was only 358 μg/ml (Table 2, Figures 3, 4).

Direct labelling — electrolytic

Monoclonal antibody was added to electrolysed eluate of generator. Electrolytic labelling was done with 7.5 ml cell of Sephadex G 25, and free ^{99m}Tc was separated from the radiolabelled monoclonal antibody. Photometry of fraction after sepa-

Table 3. Concentration TU-20 of fractions obtained by purification of mixture TU 20 — ^{99m}Tc. Entering concentration TU-20°750 μg/ml

Gel volume	7.5 ml
Fraction	Concentration TU-20 [μg/ml]
3 (TU-20-^{99m}Tc)	43
4 (TU-20-^{99m}Tc)	469
5 (TU-20-^{99m}Tc)	73
6 (TU-20-^{99m}Tc)	28
7 (TU-20-^{99m}Tc)	11
8 (TU-20-^{99m}Tc)	6

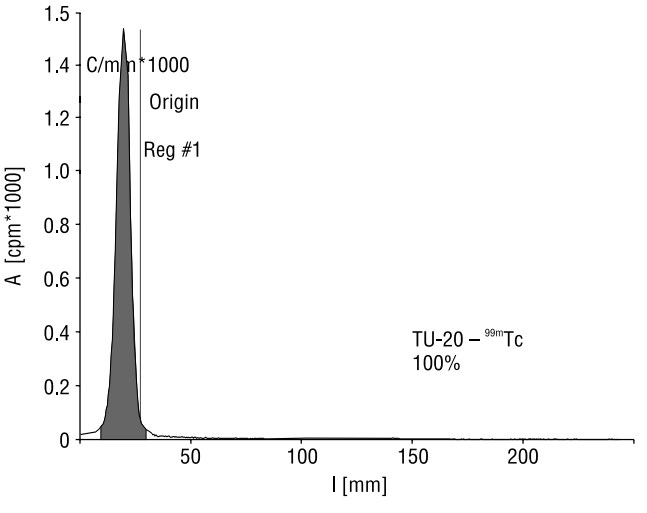

Figure 5. Paper chromatography of 4 fraction past purification of reaction mixture TU-20-^{99m}Tc.

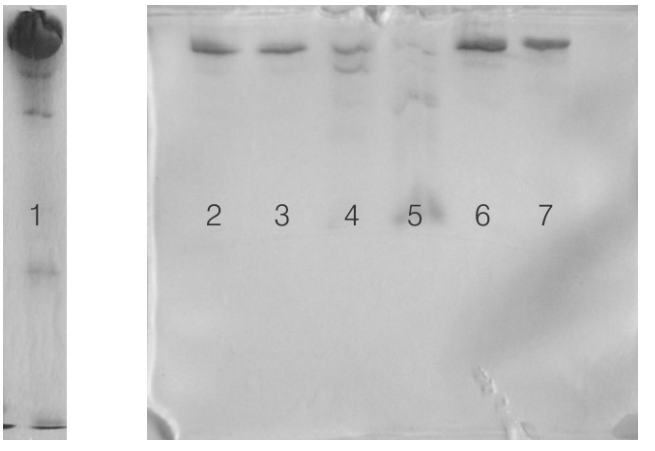

Figure 6. Immunoreactivity.

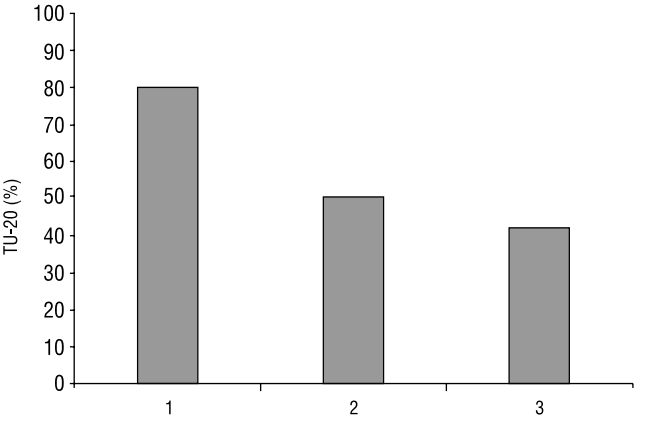

Figure 7. Percentage of immunoreactivity found in preparations prepared by different methods of labelling.

ration reaction mixture is shown in Table 3. The yield of monoclonal antibody is high, but in the paper chromatography shown in Figure 5 radiolabelled monoclonal antibody yield is shown. Activity of this preparation is low because there are not enough binding places for the radionuclide. The yield of the radiolabelled antibody is also low.

Samples:

1. standard TU-20,
2. TU-20-HYNIC- ^{99m}Tc,
3. TU-20-HYNIC- ^{99m}Tc,
4. TU-20 (ME)- ^{99m}Tc,
5. TU-20 (ME)- ^{99m}Tc,
6. TU-20- ^{99m}Tc (El),
7. TU-20- ^{99m}Tc (El).

Samples:

1. TU-20-HYNIC- ^{99m}Tc,
2. TU-20 (ME)- ^{99m}Tc,
3. TU-20- ^{99m}Tc (El).

Discussion

Chemical purity, biological stability and immunoreactivity

Immunoglobulin TU-20 is a macromolecule with large molecular weight; this is the reason for using gel filtration to separate simple molecules from the monoclonal antibody. In our case, mixtures HYNIC, 2-ME and free ^{99m}Tc containing from TU-20 — HYNIC and labelled TU-20 were separated. Chemical stability was ensured by paper chromatography. Biological stability of the structure of monoclonal antibody was checked by SDS-electrophoresis, and the radiolabelled structure was confronted with native structure of the monoclonal antibody. To follow the immunoreactivity of the radiolabelled monoclonal antibody the ELISA test was used (Figure 6). It can be claimed that the best preparation of our three tested methods is the method of indirect labelling of the monoclonal antibody over bifunctional chelator HYNIC. Our final preparation showed high biological and chemical stability, the specific bond on the antigen is safe, the yield of radiolabelled antibody is high and it is not necessary for the reaction mixture to be purified. High biological and chemical stability any more than immunoreactivity of the final preparation was proven

by direct electrolytic labelling (Figure 7). However, it is necessary to purify the reaction mixture to separate the labelled monoclonal antibody from free ^{99m}Tc, which is, in relation to the present radionuclide, a disadvantage of this method. The second problem of this method is the unreduced degree of monoclonal antibody which causes there to be very few binding places for the radionuclide on the monoclonal antibody, and the yield of the preparation is low, of course. As worst, seems to be direct labelling with 2-mercaptoethanol where this reduction reagent destroys the structure of the monoclonal antibody and biological and immunoreactivity is damaged.

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