STUDY ON THE PREPARATION AND STABILITY OF ^{188}Re BIOMOLECULES VIA EHDP

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Abstract. A direct labelling technique via ethane-1-hydroxy-1,1-diphosphonic acid (EHDP) as a weak competing ligand was developed for the preparation of several biomolecules: ^{188}Re-monoclonal antibody ior ceal against carcinoembryonic antigen (^{188}Re-MoAb), biotinylated ^{188}Re-MoAb (^{188}Re-MoAb-biotin), ^{188}Re-polyclonal IgG (^{188}Re-IgG), ^{188}Re-peptide (somatostatine analogue peptide b-(2-naphtyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-amide), ^{188}Re-MoAb fragments (^{188}Re-F(ab')$_2$) and biotinylated ^{188}Re-F(ab')$_2$ (^{188}Re-F(ab')$_2$-biotin). The reaction conditions such as pH, temperature, weak ligand concentration and stannous chloride concentration were optimized during the radiolabelling of each biomolecule. Before the labelling procedure, disulphide bridge groups of the biomolecules were reduced with 2-mercaptoethanol (2-ME). To obtain ^{188}Re labelled antibodies and peptides in high radiochemical yields (>90%) via EHDP, it was necessary to use acidic conditions and a high concentration of stannous chloride to allow the redox reaction $Re^{+7} \rightarrow Re^{+5}$:$Re^{+4}$. The labelling of MoAb and F(ab')$_2$ with ^{188}Re via EHDP was also evaluated employing a pretargeted technique by avidin-biotin strategy in normal mice, demonstrating that the ^{188}Re-labelled biotinylated antibodies are stable complexes *in vivo*. The ^{188}Re-peptide complex prepared by this method, was stable for 24 h and no radiolytic degradation was observed.

1. INTRODUCTION

Increased effort has been made to label monoclonal antibodies (MoAb) and peptides with rhenium-188 because of their potential role in the radioimmunotherapy of cancer and the availability of Re-188 from a W-188/Re-188 generator [1–3].

The β-(2-naphthyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-amide peptide is a somatostatin analog with cytostatic effect on small lung cancer cells [4].

Whereas the avidin-biotin system, has shown that target-to-nontarget radioactivity ratios and radioimaging scanning can be significantly improved by introducing a two-step or three-step system. One two-step approach is based on the administration of streptavidin conjugated to the antibody followed by a radioactive biotin derivative or biotinylated antibody is injected followed by radioactive streptavidin [5]. In the three-step system, biotinylated antibody is injected followed by an excess of cold avidin or streptavidin and, as a third step, radioactive biotin is administered. An avidin "chase" of biotinylated antibody has been also reported improving clearance of radiolabelled MoAb without decreased accumulation in the target tumour [6].

In this report, the MoAb murine anti-CEA IgG1 designated ior ceal (Havana, Cuba), its F(ab′)$_2$ fragments and the β-(2-naphthyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-amide peptide, were labelled with Re-188 with high radiochemical purity based on the direct labelling method ^{188}Re-EHDP-MoAb [7]. The labelling of biotinylated MoAb ior ceal and its F(ab′)$_2$ fragments with Re-188 from instant freeze dried kit formulations was also performed. The biodistribution and dosimetry of these radioimmunoconjugates were determined in Balb/c mice after injection of avidin as a "chase" under the hypothesis that if the ^{188}Re-labelled biotinylated MoAb ior ceal and its ^{188}Re-labelled biotinylated F(ab′)$_2$ fragments prepared by this method, are stable complexes *in vivo*, an increased blood clearance of the radiolabelled agent with reduction of radiation dose would be obtained.

2. MATERIALS AND METHODS

2.1 Preparation and Purification of the F(ab')$_2$ Fragments

F(ab')$_2$ fragments of ior ceal were prepared by digestion with pepsin following by a purification step using a Protein A-Agarose column and a ProteinPak 300SW (1 mL loop) HPLC size-exclusion column. Finally, F(ab')$_2$ fragments were concentrated by ultrafiltration (Ultrafree-PFL 30,000 NMWL, Millipore Co.) to obtain a concentration of approximately 10 mg/mL.

2.2 Biotinylation of Antibodies and Antibody Fragments

To prepare biotin labelled whole MoAb or F(ab')$_2$ fragment, 5.0 mg of succinimidyl-6-(biotinamido)hexanoate (ImmunoPure NHS-LC-Biotin, PIERCE Co.) were dissolved in 30 μL DMSO and 0.1M phosphate buffer was added to a final volume of 0.5 mL. Immediately, 40 μL of the NHS LC-Biotin, were added to 1.0 mL of the MoAb solution (≈10 mg/mL) and the mixture was incubated with gentle stirring for 30 minutes at 18°C. The biotinylated antibody was separated from the unreacted NHS-LC-Biotin employing a ProteinPak 300SW (1 mL loop, Waters) HPLC size-exclusion column run in 0.1 M phosphate buffer (pH7.4). Under these conditions, ratios of 4–5 moles of biotin per mole of whole antibody and 2–3 moles of biotin per mole of F(ab')$_2$ fragment were obtained.

2.3 Biomolecules reduction

To 0.5 mL of peptide or MoAb solution were added 25 μL of 2-mercaptoethanol (2-ME) previously diluted with distilled water 1 :10. After allowing the mixture to react at room temperature for 30 min with continuous rotation, the resulting solution was purified on a ProteinPak 125 (Waters) HPLC size-exclusion column, using 0.1 M phosphate buffer (pH7.4) as mobile phase at a flow rate 1.5 mL/min This system produced retention times of 20–21 min, 8–8.5 min and 4–4.5 for the peptide, 2-ME and MoAb respectively. The U.V. spectrum of each compound was obtained with a HPLC Photodiode Array Detector In the case of peptides, the radiolabelling was carried out using reduced and unreduced molecule.

2.4 Preparation of ^{188}Re-biomolecules

The general procedure for the preparation of ^{188}Re-biomolecules was as follows: EHDP and 5 mg of gentisic acid were dissolved in 0.5 mL of stannous chloride solution ($SnCl_2$ in 0.06 M HCl), and 1.0 mL of reduced or unreduced biomolecule was added followed by addition of 1.5 mL of ^{188}Re-perrhenate solution (Oak Ridge National Laboratory).

2.5 Radiochemical Purity

The radiochemical purity for MoAb, F(ab')$_2$ and IgG was determined by a combination of instant thin layer chromatography (ITLC) and HPLC as reported previously [7]. The evaluation of the radiochemical purity for peptides was determined by ITLC-SG analysis (Table I) and C-18 Sepak cartridges (Waters). The immunoreactivity of the labelled antibodies and its fragments was evaluated using affinity thin layer chromatography (ATLC) as Zamora et al reported [8].

2.6 Biodistribution and dosimetry

Female Balb-c mice (27–30 g) were used in Biodistribution studies. 50 μg of avidin (Pierce Co.) was injected 15 min after injection of the biotinylated radioimmunoconjugate.

TABLE I. SYSTEMS EMPLOYED DURING THE DETERMINATION OF ^{188}Re-PEPTIDE RADIOCHEMICAL PURITY BY ITLC-SG ANALYSIS (1 × 10 cm STRIPS)

Solvent:	0.9% NaCl	Acetone	Acidified ethanol (10% HCl 0.01N)
Rf $^{188}ReO_4^-$	1.0	1.0	1.0
Rf $^{188}ReO_2$	0.0	0.0	0.0
Rf ^{188}Re-peptide	0.0	0.7–1.0	1.0
Rf ^{188}Re-EHDP	1.0	0.0	1.0

Cumulative activities and residence times in all organs studied were calculated from the biological data obtained in the animals. Human absorbed dose calculations were performed according to the methods outlined by the MIRD committee using the computer program MIRDOSE3 developed at Oak Ridge Associated Universities.

3. RESULTS AND DISCUSSION

The reduction of intrinsic disulphide bridges within the antibody molecule by the use of the reductant 2-ME, was an essential step during the preparation of ^{188}Re-MoAb complexes. However, contrary to the labelling studies with MoAb's, the reduction of peptides is not necessary to obtain ^{188}Re-peptides complexes in high radiochemical yields. This result is expected as the Sn(II) ion works strongly in the acidic region reducing the rhenium to a reactive species, and reducing the peptide for subsequent chelation to the metal.

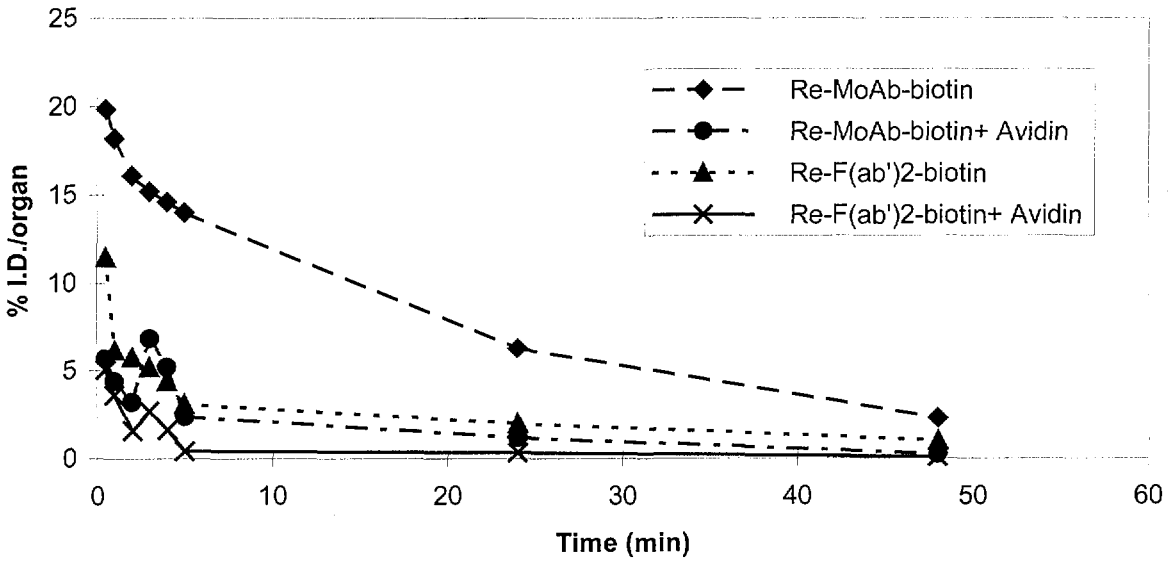

FIG. 1. Blood clearance in mice of ^{188}Re-MoAb-biotin and ^{188}Re-F(ab')2-biotin with or without avidin as a chase.

^{188}Re-MoAb, ^{188}Re-MoAb-biotin, ^{188}Re-F(ab')$_2$ and ^{188}Re-F(ab')$_2$-biotin preparations were produced for these studies with specific activities of 1.30 ± 0.18 GBq/mg (36 ± 5 mCi/mg). Radiolabelled F(ab')$_2$ fragment was eluted from the Protein Pak 125 HPLC column as a monomeric peak (retention time of 8.9 ± 0.2 min).

There were no significant differences ($p > 0.05$) between the biodistribution of biotinylated and unbiotinylated ^{188}Re-labelled immunoconjugates. When avidin was injected as a chase after injection of ^{188}Re-MoAb-biotin or ^{188}Re-F(ab')$_2$-biotin, the blood radioactivity level decreased approximately 50 70% (FIG. 1), the cumulated activity in blood decreased almost 75% (from 191.05 $\pm$ 26.92 to 40.54 $\pm$ 6 MBq·h) and the effective dose diminished 25% (from 0.173 to 0.130 mGy/MBq) respect to that of the radioimmunoconjugates where the "chase effect" was not used.

The ^{188}Re-peptide complex showed that under the procedure reported herein it can be prepared with a radiochemical purity of 90% and a specific activity up to 1.8 GBq/mg without radiolytic degradation of the product.

TABLE II. REACTION CONDITIONS TO LABEL DIFFERENT BIOMOLECULES VIA EHDP

Biomolecule	[$SnCl_2$] (mM)	[EHDP] (mM)	pH	Labeling time (h)	Temperature (°C)	Yield (%)
^{188}Re-IgG	0.88	30	3	18–22	22	98
^{188}Re-IgG	3.52	120	3	0.5	37	97
^{188}Re-IgG	7.04	120	4	2	37	97.5
^{188}Re-IgG	7.04	120	5	18–22	22	97
^{188}Re-MoAb	3.52	120	3	2	22	99
^{188}Re-MoAb	3.52	120	3	0.5	37	98
^{188}Re-MoAb	3.52	120	4	5	37	97
^{188}Re-MoAb-biotin	3.52	120	3	0.5	37	96
^{188}Re-F(ab')$_2$	3.52	120	3	2	37	96
^{188}Re-F(ab')$_2$-biotin	3.52	120	3	2	37	95
^{188}Re-peptide (reduced)	11.76	120	3	1.5	92	90
^{188}Re-peptide (unreduced)	11.76	120	3	1.5	92	90

4. CONCLUSIONS

To obtain ^{188}Re labelled antibodies and peptides in high radiochemical yields (>90%) via EHDP, it was necessary to use acidic conditions and a high concentration of stannous chloride to allow the redox reaction $Re^{+7} \rightarrow Re^{+5}$:$Re^{+4}$ (Table II).

Results showed that the immunoreactivity of the antibodies remains unaffected after the labelling procedure. However, MoAb and its fragments are unstable *in vitro* at neutral pHwhich is in agreement with the results obtained by other workers [1,3]. These investigators have also found that the ^{188}Re-MoAb complex is stable *in vivo*, maybe due to a protective effect of serum proteins against the processes of ^{188}Re reoxidation.

In this work the labelling of MoAb and F(ab')$_2$ with ^{188}Re via EHDP was also evaluated employing a pretargeted technique by avidin-biotin strategy in normal mice, demonstrating that the ^{188}Re-labelled biotinylated antibodies are stable complexes *in vivo*.

The ^{188}Re-peptide complex prepared by this method, was stable for 24 h and no radiolytic degradation was observed. In order to increase the radiochemical purity, a desalting column could also be used. However, this method is limited to labelling with ^{188}Re only those peptides which contain cysteine bridges. The biological properties of the radiopeptides have to be evaluated since reaction condition are not an appropriate environment for their integrity.

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