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Labeling peptides with rhenium-188

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Abstract

A direct labeling technique via EHDP for the preparation of ^{188}Re-somatostatin analogue peptide β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–amide complex was developed. The influence of reaction conditions such as pH, temperature, weak ligand concentration and stannous chloride concentration were investigated. Methods of analysis were also established permitting identification of radiochemical impurities which may be present in the radiopharmaceutical solution. Results showed that under the procedure reported herein ^{188}Re–peptide complex can be prepared with a radiochemical purity of 90% and a specific activity up to 1.8 GBq mg^{-1} without radiolytic degradation of the product.

Keywords: Radiochemical impurities; Stannous chloride; Weak ligand

1. Introduction

Somatostatin is a peptide hormone consisting of 14 amino acids. It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract and the pancreas. Various tumors contain high numbers of somatostatin receptors, which enable in vivo localization of the primary tumor and its metastases by scintigraphy with radiolabeled somatostatin analogue peptides (Krenning et al., 1993; Olsen et al., 1995).

Over the past years, techniques have been developed to label peptides with indium-111 (Krenning et al., 1993; Olsen et al., 1995), iodine-123 (Krenning et al., 1993) and more recently technetium-99m (Thakur et al., 1997; Hnatowich et al., 1998). However, an increased effort has been made to label these biomolecules with β-emitters such as ytrium-90 (Jong de et al., 1997; Otte et al., 1997) and rhenium-188 (Zamora et al., 1996, 1997) for radionuclide therapy. Zamora et al. (1996) labeled the somatostatin analogue peptide

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RC-160 with ^{188}Re by the stannous tartrate direct labeling method, and reported therapeutic effectiveness of ^{188}Re–RC-160 in athymic nude mice bearing experimental human prostate cancer PC-3.

Whereas, the β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–amide peptide has been previously reported by Taylor et al. (1988) as a somatostatin analog with cytostatic effect on small lung cancer cells.

In our laboratory, monoclonal antibodies (MoAb) have been labeled with Re-188 in high radiochemical yields based on the direct labeling method ^{188}Re–EHDP–MoAb (Ferro et al., 1997, 1999). This method comprises reduction of intrinsic disulphide bridges within the biomolecule by

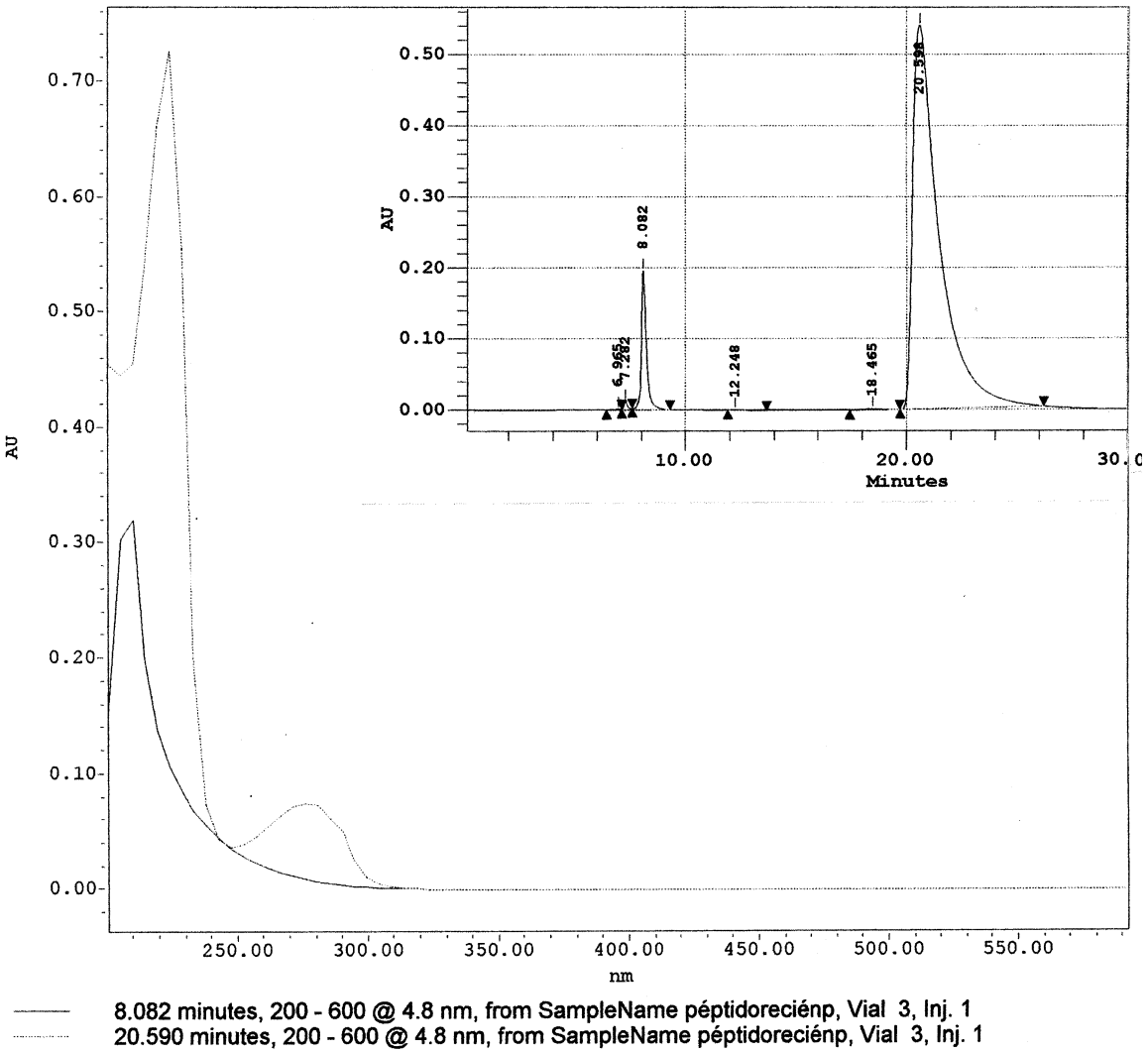

Fig. 1. Purification of reduced peptide on a ProteinPak 125 HPLC size exclusion column. Phosphate buffer (0.1 M, pH 7.4): flow rate 1.5 ml min^{-1}. $T_R = 8.082$ for 2-ME and $T_R = 20.598$ for peptide. The UV spectrum of each compound was obtained with a HPLC photodiode array detector.

Table 1
Factorial experimental design utilized during the labeling of peptides with ^{188}Re[a]

Class	Levels	Values
[EHDP] (mg ml^{-1})	3	10.5, 25, 40
[$SnCl_2$] (mg ml^{-1})	2	1.5, 2.2
Temperature (°C)	3	22, 37, 92
Incubation time (h)	6	0.5, 1, 1.5, 3, 5, 24

[a] Dependent variable: radiochemical purity

the use of the reductant 2-mercaptoethanol. Following a subsequent purification, the resulted reduced molecule is formulated with gentisic acid, stannous chloride and EHDP as a sterile solution to produce the specific radiobiomolecule after reaction with ^{188}Re-perrhenate. Therefore the mechanism that is carried out into the vial is: Sn^{2+}-reduction of perrhenate in the presence of the weak competing ligand ethane-1-hydroxy-1,1-diphosphonic acid (EHDP, chelating agent) promoting the mechanism of ligand exchange in order to obtain labeled molecules as stable complexes in high radiochemical yields.

In this report, we developed a radiopharmaceutical formulation via EHDP to obtain the β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–amide peptide labeled with Re-

Table 2
Systems employed during the determination of ^{188}Re–peptide radiochemical purity by ITLC-SG analysis (1 X 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

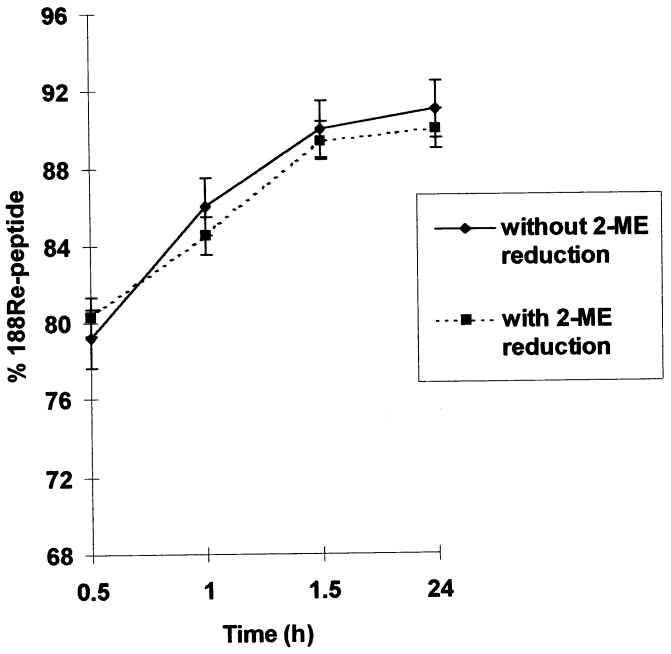

Fig. 2. Preparation of ^{188}Re–peptide complex employing 2.2 mg ml^{-1} of $SnCl_2$, 25 mg ml^{-1} of EHDP, pH 3 and incubation at 92°C during 1.5 h with and without previous reduction with 2-mercaptoethanol.

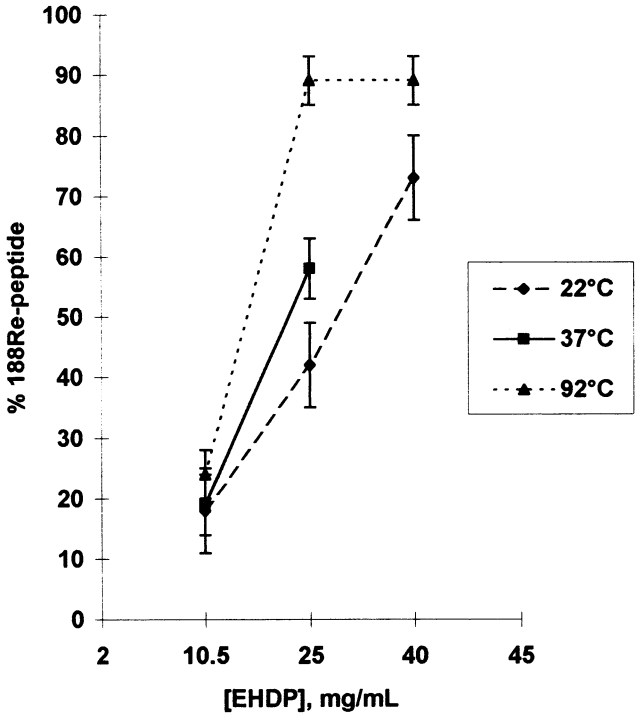

Fig. 3. Influence of EHDP concentration and the temperature on yield of ^{188}Re–peptide complex (2.2 mg ml^{-1} of $SnCl_2$, pH 3).

188. Methods of analysis were also established permitting identification of radiochemical impurities which may be present in the radiopharmaceutical solution.

2. Materials and methods

2.1. Peptide reduction

The peptide with amino acid sequence β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–NH_2 was supplied as a lyophilized powder (Sigma Chemical) into vials containing 1.0 mg. Reconstitution of the lyophilized powder was performed by adding 1.0 ml of sterile and apyrogenic water (peptide solution). To 0.5 ml of peptide 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) high performance liquid chromatography (HPLC) size-exclusion column, using 0.1 M phosphate buffer (pH 7.4) as mobile phase at a flow rate 1.5 ml min^{-1} This system produced retention times of 20–21 and 8–8.5 min for the peptide and 2-ME, respectively. The UV spectrum of each compound was obtained with a HPLC Photodiode Array Detector as is shown in Fig. 1.

2.2. Preparation of ^{188}Re–peptide complex

The general procedure for the preparation of ^{188}Re–peptide complexes was as follows: ethane-1-hydroxy-1,1-diphosphonic acid (EHDP) was synthesized in our laboratory by the reaction between acetic acid and phosphorous trichloride following a subsequent purification through steam distillation. This distillation was carried out at 120°C until the pH of the distilled was 5 indicating that the excess acetic acid was completely eliminated. The residual semisolid product was

the EHDP.EHDP and 0.5 mg (3 μmol) of gentisic acid (as antioxidant) were dissolved in 0.5 ml of stannous chloride solution ($SnCl_2$ in 0.06 M HCl), the pH was adjusted to 3.0 with 1 M NaOH and then 1.0 ml of reduced peptide (0.25 mg ml^{-1} in 0.1 M phosphate) or unreduced peptide (0.25 mg ml^{-1} in distilled water) was added. The W-188/Re-188 generator (Oak Ridge National Laboratory, USA) was eluted with 0.9% saline, and the radioactivity was assayed using a dose calibrator (Capintec Radioisotope Calibrator, Model CRC-7). Perrhenate solution(2 ml) ($\approx$ 225 MBq ml^{-1}), previously adjusted to pH 3.0 or 4.0, was added to a vial containing the peptide. The pH of the reaction mixture was measured and the solution was allowed to react at room temperature, at 37 or at 92°C. Radiochemical purity was determined at time periods from 30 min to 24 h after addition of perrhenate. The general factorial experimental design is shown in Table 1.

2.3. Radiochemical and chemical quality control

The radiochemical purity was determined by a combination of instant thin layer chromatography (ITLC), HPLC and reverse phase C-18 cartridges (Waters). The systems used for the analysis by ITLC-SG are shown in Table 2.

2.3.1. High performance liquid chromatography

Quality control of the labeled peptide was evaluated by size exclusion HPLC analysis employing a ProteinPak 125 gel filtration column (Waters), with photodiode array detector. 0.1 M phosphate pH 7.4 at a flow rate 1.5 ml min^{-1} was used as mobile phase. The radiochromatographic profile

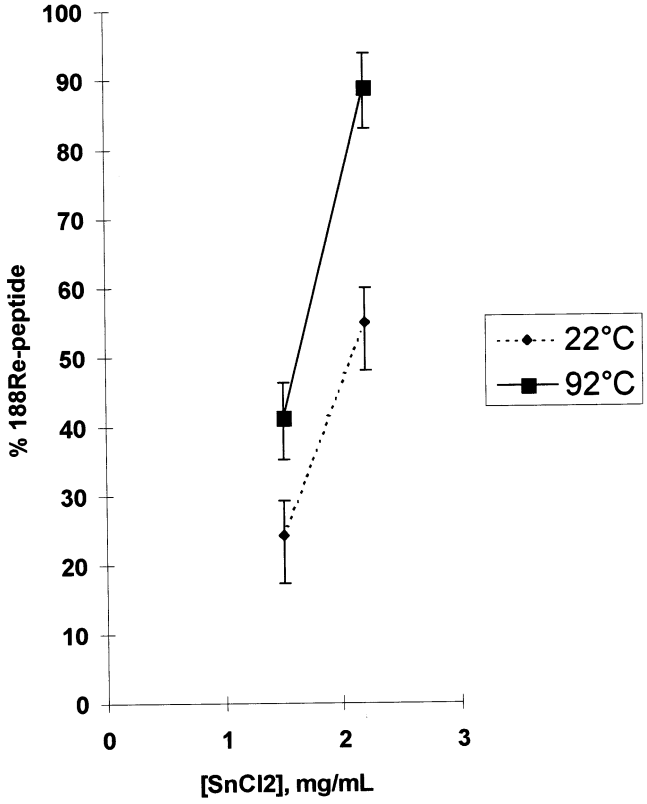

Fig. 4. Influence of $SnCl_2$ concentration and the temperature on labeling yield of ^{188}Re–peptide complex (25 mg ml^{-1} of EHDP, pH 3).

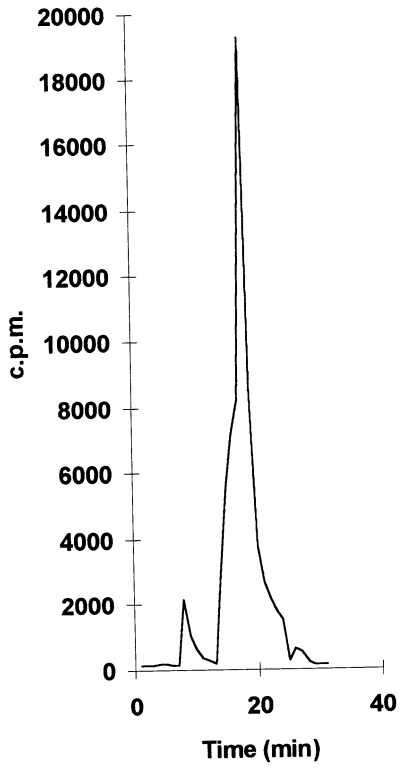

Fig. 5. Radio-HPLC chromatogram of ^{188}Re–peptide complex (2.2 mg ml^{-1} of $SnCl_2$, 25 mg ml^{-1} of EHDP, pH 3 and incubation at 92°C during 1.5 h). ProteinPak 125 HPLC size exclusion column. Phosphate buffer (0.1 M, pH 7.4), flow rate 1.5 ml min^{-1}.

was determined by collecting samples (Waters fraction collector) of uniform volume (0.5 ml) for counting in an external NaI (Tl) detector (NML, Laboratories).

2.3.2. C-18 Cartridges

C-18 SepPak cartridges were activated by eluting 10 ml of absolute ethanol through the column followed by 10 ml of 0.001 N HCl. The cartridge was drained by flushing it with 5 ml of air. A 0.1 ml sample of the labeled peptide was loaded onto the top of the cartridge, 10 ml of 0.001 N HCl was eluted through the column with the eluent collected into a test tube (free perrhenate). This was followed by eluting the column with acidified ethanol (10% HCl 0.01 N) also collected into a test tube (labeled peptide). The radioactivity of the first sample elution, the second sample elution and the cartridge (hydrolyzed-reduced rhenium) were counted in a NaI(Tl) detector (NML, Laboratories) in order to calculate the percent of ^{188}Re–peptide complex.

3. Results and discussion

The reduction of intrinsic disulphide bridges within the antibody molecule by the use of the reductant 2-ME, is an essential step during the preparation of ^{188}Re–MoAb complexes when a direct method is used (Ferro et al., 1997, 1999). However, contrary to the labeling studies with MoAb's, the reduction of peptides is not necessary to obtain ^{188}Re–peptides complexes in high radiochemical yields as is shown in Fig. 2. 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 (Zamora et al., 1997).

According to previous results employing proteins (Ferro et al., 1997, 1999), to obtain Re-188 labeled peptides in high yields, it was essential to use a high concentration of stannous chloride (Fig. 3) and therefore the reaction had to be carried out under acidic conditions (pH 3) to allow the readily reversible redox reaction of Re^{7+}/Re^{5+}:Re^{4+} (in comparison to the analogous Tc redox reaction). When the reaction was carried out at pH 4, the radiochemical purity decreased 30%.

Fig. 4 shows the influence of EHDP concentration and reaction temperature on yield of ^{188}Re–peptide. It can be seen that the radiopeptide yield was increased with EHDP concentration because more EHDP was available to stabilize the reduced perrhenate which can be easily reoxidized to $^{188}ReO_4^-$. The labeling yield of ^{188}Re–peptide was increased by heating the reaction mixture at 37 and 92°C with respect to an incubation at 22°C. Ideally, the radiolabeling of peptides should be carried out at 22°C, however, other investigators (Jong de et al., 1997; Thakur et al., 1997; Zamora et al., 1997) have labeled peptides at 92 and 100°C without any loss of biological properties, but the

process has to be validated for each biomolecule of interest.

The radiochromatographic profile, correlated with the UV-chromatogram, showed retention times for perrhenate, radiopeptide and radiopeptide fragments (hydrolyzed peptides) of 8.5–10, 18–19 and 26–27 min, respectively (Fig. 5). As can be observed, peptide fragmentation was low ($\approx 2\%$) despite the use of acidic conditions (pH 3). It was also noticeable that the retention time for radiopeptide diminished 2 min respect to the unlabeled peptide. The reason to explain this finding could be associated with the aggregation of the peptide due to the presence of EHDP as in the case of MoAb (Ferro et al., 1997) As was discussed, it is possible that at low pH the amine groups of the peptide are in the protonated state promoting some interactions with EHDP molecules which could result in cross-linking.

Zamora et al. (1997) detected radiolysis at 2.5 h post-labeling during the preparation of the ^{188}Re–RC-160 somatostatin analogue by the stannous tartrate direct labeling method. They stabilized the formulation against radiolysis by post-labeling addition of ascorbic acid. The ^{188}Re–peptide complex prepared by the direct labeling method via EHDP, was stable for 24 h and no radiolytic degradation was observed. This stability could also be associated with the presence of EHDP molecules as was discussed above. The structure of Re–RC-160 complex was studied by Varum et al. (1994) and Rhenium appears to be coordinated by Phe^1, Cys^2, Trp^8, and the C-terminal NH_2 group forming a cyclic complex. Then, a similar structure could be expected for the peptide studied herein. However, the difference between the RC-160 peptide and the β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–amide peptide is the presence of aromatic rings in the first that could be converted to free radicals producing the radiolytic effect into the molecule.

In summary, ^{188}Re-somatostatin analogue peptide β-(2-naphthyl)-D-Ala–Cys–Tyr-D-Trp–Lys–Val–Cys–Thr–amide complex was prepared with a radiochemical purity of 90% and a specific activity of 1.8 GBq mg-1 employing 2.2 mg ml^{-1} of $SnCl_2$, 25 mg ml^{-1} of EHDP, pH 3 and incubation at 92°C during 1.5 h. In order to increase the radiochemical purity, a desalting column could also be used. However, this method is limited to labeling with ^{188}Re only those peptides which contain cysteine bridges. The biological properties also have to be evaluated since reaction conditions are not an appropriate environment to keep the biological properties of biomolecules.

Acknowledgements

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