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潜在肿瘤治疗剂 ^{188}Re-Lanreotide 的研制

PREPARATION OF ^{188}Re-LANREOTIDE AS A POTENTIAL TUMOR THERAPEUTIC AGENT

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白红升　金小海　樊红强　贾 兵　王豫庆　陆巍巍

（中国原子能科学研究院， 北京，102413）

摘　要

放射性核素标记多肽在肿瘤诊断和治疗中具有巨大的潜在应用前景。用 ^{188}Re 以柠檬酸和酒石酸的混合物为转换络合剂直接标记 Lanreotide，研究了 ^{188}Re-Lanreotide 的标记条件、体外稳定性及动物实验，建立了 ^{188}Re-Lanreotide 的质量控制方法。 实验结果表明，在 pH 2～3 和 60 ℃反应 40 min，标记率为 88%～94%，经 Sep-Pak C_{18} 反相萃取柱纯化后放射化学纯度大于 95%。^{188}Re-Lanreotide 血清除快，经肝胆排出体外，肠和肺的摄取量较高。

Preparation of ^{188}Re-Lanreotide as a Potential Tumor Therapeutic Agent

BAI Hongsheng JIN Xiaohai FAN Hongqiang JIA Bing
WANG Yuqing LU Weiwei
(China Institute of Atomic Energy, Beijing, 102413)

ABSTRACT

Radiolabeled peptides hold unlimited potential in diagnostic applications and therapy of malignant tumor. Somatostatin analogue peptide (Lanreotide) is labeled directly with ^{188}Re via the mixture of citrate and tartate. The influences of reaction conditions such as pH, temperature, amount of stannous chloride, Lanreotide quantity, reaction time on labeling yield are investigated in detail. At the same time, the stability in vitro, quality control and animal test are evaluated. The experimental results show that Lanreotide reacts with ^{188}Re for 40 min at pH 2～3 and 60 ℃, the labeling yield is at range of 88%～94%. After purification of ^{188}Re-Lanreotide with Sep-Pak C_{18} reverse phase extraction cartridge, the radiochemical purity (RP) is more than 95%. ^{188}Re-Lanreotide is eliminated rapidly from the blood and is excreted through liver, the uptake of lung and intestine is high.

INTRODUCTION

Receptor specific peptides play an important role not only in the diagnostic and therapeutic applications of neoplastic diseases, but also in the pathogenesis of other diseases. The radiolabeled receptor specific peptides are an exciting subject in the field of nuclear medicine. Except for receptor specificity like monoclonal antibody, the receptor peptides have several advantages over monoclonal antibody. Most peptides are endogenous and bioactive analogue that have rapid blood clearance and high concentration in the target tissue; peptides can also withstand harsher chemical conditions of pH and temperature, making radiolabeling parameters more flexible and less damaging to the biological activity of peptides. Peptides are relatively less expensive and easily synthesized[1].

Somatostatin is a cyclic disulfide-containing peptide hormone of 14 amino acid. It exists in the hypothalamus, the cerebral cortex, the brain stem, gastrointestinal tract and pancreas, and exerts an inhibitory effect on several cell functions such as secretion of peptide hormones and growth factors. The clinic value of Somatostatin is limited due to its very short half-life in vivo [2]. In recent years, much attention are drawn to the development of radiolabeled somatostatin analogues (such as Octreotide, RC-160 and Lanreotide) with radionuclides (such as ^{188}Re, $^{131/125/123}$I, ^{99}Tc^{m}, ^{111}In, ^{90}Y etc.) for a variety of diagnostic applications as well as for therapy of malignant tumor[3~9].

^{188}Re($T_{1/2}$=16.9 h) decays by emission (E_{max}=2.11 MeV) followed by emission of 155 keV gamma photons, and thus has attractive energy characteristics for therapy and evaluation of targeted tissue uptake and dosimetry. Carrier-free ^{188}Re, as sodium perrhenate, can be obtained from ^{188}W/^{188}Re generator [10, 11]. Rhenium has similar chemical property with Technetium, and chemical reduction of perrhenate to lower oxidation state by reduced agents (such as stannous chloride, ascorbic acid, $NaBH_4$ etc.) allows attachment of therapeutic agents [12, 13]. Perrhenate does not concentrate in the bone marrow and is rapidly cleared through kidney [14]. Lanreotide (D-β-Nal-Cys-Try-D-Trp-Lys-Val-Cys-Thr • NH_2) is a new somatostatin analogue. It can bind to human somatostatin receptor (hSSTR) subtype 2 through 5 with high affinity and to hSSTR subtype 1 with low affinity. Virgolini I., et al., investigated biodistribution, safety and radiation absorbed dose of ^{111}In-DOTA-Lanreotide in 1998 and their experimental results were promising[3]. In this paper, we will introduce labeling condition, quality control, stability in vitro and animal test in detail.

1 Reagent and Instrument for Experiments

1. 1 Reagent

Water used for the experiment is redistilled, deionized and degassed. Lanreotide is provided by IAEA (HPLC grade). Stannous chloride (HPLC grade), acetate, sodium acetate, concentrated hydroxyl chloride, ethanol (analysis grade, Beijing Chemical Reagent Co.) and 0.9% sodium chloride solution (The fourth Pharmacy Factory, Shijiazhuang) are purchased from market. $^{188}ReO_4^-$ solution is obtained by elution for commercially available $^{188}W/^{188}Re$ generator (China Institute of Atomic Energy, Beijing) with 0.9% aqueous sodium chloride solution.

1. 2 Instrument

Model RM—905 Radioisotope active calibrator (China Institute of Dose) and Model FH—408 calibrator (Beijing Nuclear Instrument Factory) are used for radioactive measurement. MM—1 mixer is used for mixture of reactants (Nantong Xingyun Medical Electronic Instrument Factory). MD—110—2 electric balance (Shanghai Balance Factory) and thin layer chromatography analyzer (Berthold LB285, USA) are used for weighing and radiochemical purity analysis respectively. Model JHK—4 temperature controller (Hebei Huanghua Instrument Factory) is used for heating of reaction and incubation of stability investigation.

2 Method

2. 1 Preparation of ^{188}Re-Lanreotide

Lanreotide is labeled with ^{188}Re by two steps. Step one, $^{188}ReO_4^-$ solution reacts with transchelator using $SnCl_2$ as reduced agent to form ^{188}Re-transchelator. Step two, ^{188}Re-transchelator reacts with reduced Lanreotide to prepare ^{188}Re-lanreotide.

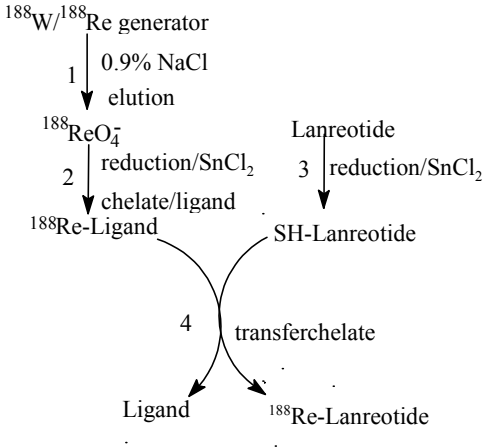

Fig. 1 Scheme of labeling Lanreotide with ^{188}Re

2. 1. 1 Preparation of ^{188}Re- citrate /tartate (^{188}Re-CT/TT)

1 ml of tartate solution (0.15 mol/L) and 1 ml of citrate solution (0.015 mol/L) are added to 10 ml of vial, mixed with 0.2 ml $SnCl_2$ solution (10 mg/ml), added 1 ml of $^{188}ReO_4^-$ solution (20 mCi/ml) (1 Ci = 3.7×10^{10} Bq). The mixture reacts for 45 min at 60 ℃ and pH 2～3. The pH value of mixture is adjusted to 5～7 with 0.5 mol/L sodium acetate solution. The labeling efficiency is determined by paper chromatography using acetone and 0.9% sodium chloride solution as mobile phase respectively. The labeling yield is more than 97%.

2. 1. 2 Preparation of ^{188}Re-Lanreotide

0.02 ml of Lanreotide solution (0.4 mg/ml) and 0.05 ml stannous chloride (1 mg/ml) are added to the vial together. The mixture incubates for 20 min at room temperature, then adds 0.2 ml of ^{188}Re-CT/TT (37 MBq), mixes and reacts for 40 min at 60 ℃. After cooling, pH value of the mixture is adjusted to 5～6 with 0.5 mol/L sodium acetate solution.

2. 2 Quality control

2. 2. 1 Thin layer chromatography

Thin layer chromatography (TLC) is used to monitor labeling yield. In the TLC studies, TLC-SG (Gelman Sciences Inc. Ann Arbor, Mich) chromatography paper is cut into 1.5×15 cm strip and activated by heating for 30 min at 110 ℃ according to manufacture's instructions. After heating, the strips are stored dry at room temperature until use. 0.005 ml portion of sample is spotted at 2 cm from lower end of the TLC paper, dries in air. The strips are developed in the 85% acidic ethanol solution (pH 3.5) and 0.9% sodium chloride solution respectively until the solvent reaches up to 12 cm of strip. After drying, the strip is cut into 1cm piece and its radioactivity is measured in a NaI(Tl) well detector.

2. 2. 2 Sep-Pak C_{18} cartridge method

Sep-Pak C_{18} Cartridge is used for labeling yield and purification of ^{188}Re-Lanreotide. Each cartridge is washed with 10 ml of 100% ethanol followed by 10 ml of 0.001 mol/L HCl solution. Aliquots of 0.1 ml sample is loaded onto the cartridge, unbound peptide ($^{188}ReO_4^-$ or ^{188}Re-CT/TT) is eluted with 0.001 mol/L HCl solution, ^{188}Re-Lanreotide is eluted with 80% aqueous ethanol solution, but radiocolliod is kept on the cartridge.

2. 3 Animal test

Bio-distribution of ^{188}Re-Lanreotide is performed in male Kunming white mice (weight: 20±2 g). 20 μCi of purified ^{188}Re-Lanreotide in 0.1 ml volume is injected

through tail vein and these mice are sacrificed at specific time intervals. The tissues and organs are excised, weighed and counted in a NaI(Tl) well detector. The uptake of activity in different organs is calculated as percent injected dose per gram organ.

3 Results and Discussions

3. 1 The TLC and Sep-Pak C_{18} cartridge elution pattern of ^{188}Re-Lanreotide

The determination of labeling yield and RP of ^{188}Re-Lanreotide is performed with two developing systems respectively. The TLC method is used widely for measurement of labeling yield of radiolabeled peptide, there is three components (unbound ^{188}Re, ^{188}Re-Lanreotide and radiocolliod) in the labeling mixture. The R_f value of those components in different developing system is listed in the Table 1. Fig. 2 and Fig. 3 are the TLC pattern of ^{188}Re-Lanreotide in 0.9% sodium chloride solution and 85% acidic ethanol solution (pH 3.5) respectively. The labeling yield is at range of 88%～94%.

Table 1 R_f value of component in two kinds of mobile phase

	$^{188}ReO_4^-$	$^{188}ReO_2$	^{188}Re-Lanreotide
85% acidic ethanol (pH 3.5)	0.8～1.0	0.0～0.1	0.8～1.0
0.9% sodium chloride	0.8～1.0	0.0～0.1	0.0～0.1

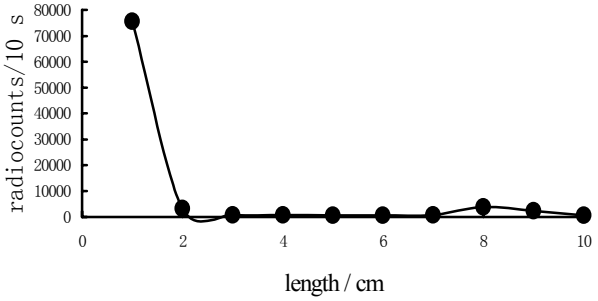

Fig. 2 TLC pattern of ^{188}Re-Lanreotide in 0.9% sodium chloride solution

Fig. 4 is the elution pattern of $^{188}ReO_4^-$ or ^{188}Re-tt/ct passing through a Sep-Pak C_{18} reverse phase extraction cartridge. After loading $^{188}ReO_4^-$ or ^{188}Re-CT/TT, the Sep-Pak C_{18} cartridge is firstly eluted with 10 ml of 80% aqueous ethanol solution, no radioactivity is found in the eluate. The $^{188}ReO_4^-$ or ^{188}Re-CT/TT is eluted with 0.001 mol/L HCl solution within 4 ml. Fig. 5 is the elution pattern of ^{188}Re-

Lanreotide on a Sep-Pak C_{18} reverse phase extraction cartridge. After loading the sample, the Sep-Pak C_{18} cartridge is eluted with 0.001 mol/L HCl solution, radioactivity in the eluate is less than 8% (this is unbound ^{188}Re). More than 88% radioactivity is eluted with 80% ethanol solution (this is ^{188}Re-Lanreotide) within 5 ml. Less than 2% radioactivity is found on the cartridge (this is radiocolliod).

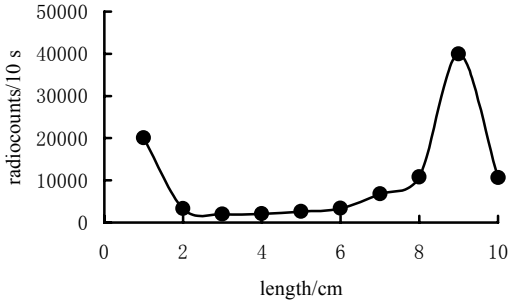

Fig. 3 TLC pattern of ^{188}Re-Lanreotide in 85% acidic ethanol solution (pH 3.5)

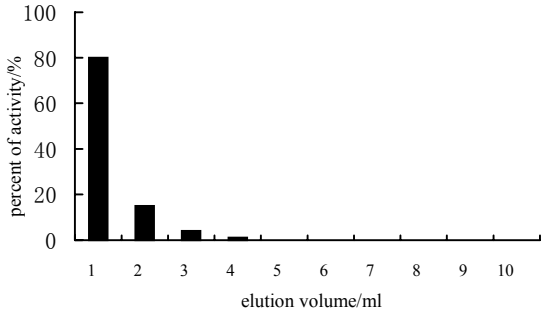

Fig. 4 Elution pattern of $^{188}ReO_4^-$ or ^{188}Re-CT/TT in 0.001 mol/L HCl solution

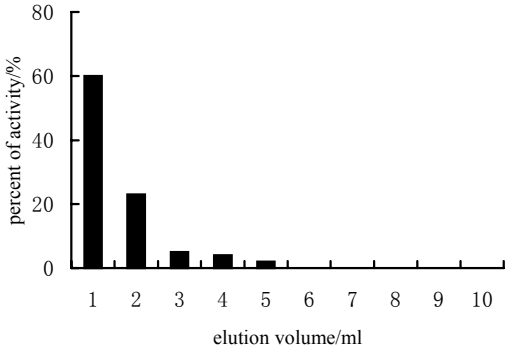

Fig. 5 Elution pattern of ^{188}Re-Lanreotide in 80% aqueous ethanol solution

3. 2 Optimal labeling condition

The effect of pH on labeling yield is studied by carrying out the reaction different pH value and the result is depicted in Fig. 6. It is shown that the labeling yield is maximum at pH 2～3, but labeling yield is lower at high pH value.

The effect of amount of stannous chloride on labeling yield is listed in Fig. 7. It is observed that labeling yield increases with amount of stannous chloride increasing. However, when amount of stannous chloride is more than 80 μg, labeling yield decreases with the increment of stannous chloride, the amount of radiocolliod increase.

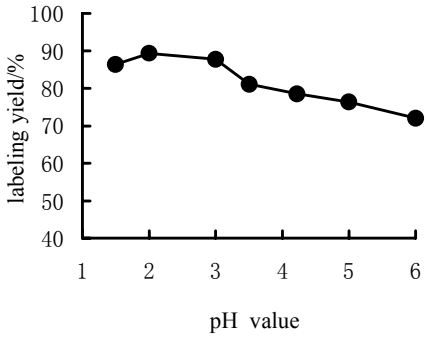

Fig. 6 Effect of pH value on labeling yield

The labeling yield of ^{188}Re-Lanreotide as function of Lanreotide quantity is given in Fig. 8. It is revealed that the labeling efficiency increases with the increment of Lanreotide quantity and reaches up to maximum at the amount of Lanreotide greater than 8 μg. Though Lanreotide is more expensive for us, higher amount of Lanreotide is needed in order to prepare high stable ^{188}Re-Lanroetide complex.

The effect of temperature on labeling yield can be seen in the Fig. 9. It is discovered that labeling yield reaches up to maximum when temperature higher than 60 ℃.

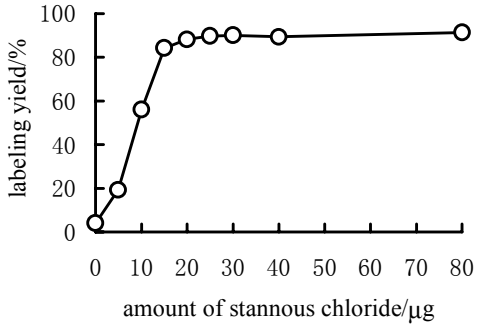

Fig. 7 Effect of amount of stannous chloride on labeling yield

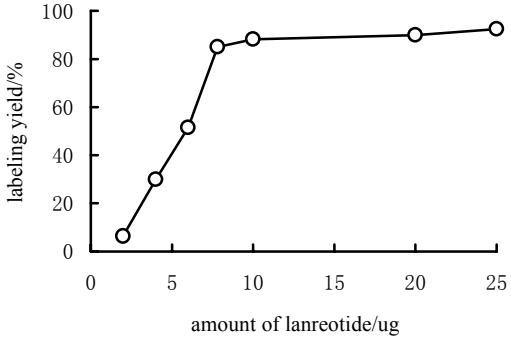

Fig. 8 Effect of amount of Lanreotide on labeling yield

The effect of reaction time on labeling yield is listed in Fig. 10. It is also observed that labeling yield arrives at the maximum when reaction time more than 40 min.

The effect of volume of ^{188}Re-CT/TT solution on labeling yield is given in Fig. 11, it is indicated that labeling yield decreases with the volume of ^{188}Re-CT/TT solution increasing.

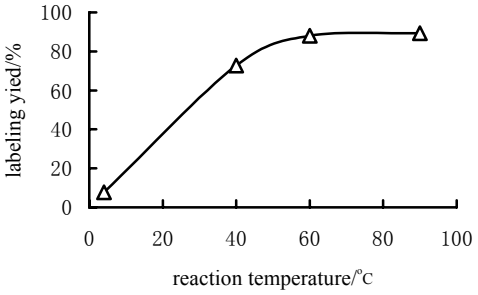

Fig. 9 Effect of reaction temperature on labeling yield

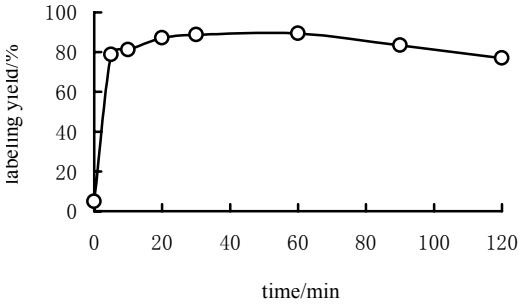

Fig. 10 Effect of reaction time on labeling yield

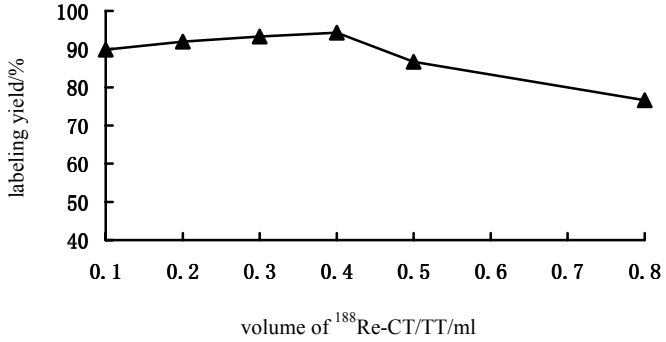

Fig. 11 Effect of the volume of ^{188}Re-CT/TT on labeling yield

In general, the optimal labeling condition is that 10 μg of Lanreotide reacts with 0.4 μl of ^{188}Re-CT/TT solution for 40 min at pH 2～3 and 60 ℃, the labeling yield is at the range of 88%～94%.

3. 3 Stability study

Though labeling yield is 88%～94%, ^{188}Re-Lanreotide must be purified before stability study in order to get correct evaluation about stability in vitro. 0.1 ml of purified ^{188}Re-Lanreotide is added respectively to one vial containing 1 ml of normal saline solution and other vial containing 1 ml mixed solution of normal saline and 1 mg ascorbic acid. Then two vials are incubated for 24 h at 37 ℃ and radiochemical purity (R.P.) is tested with TLC at specific time intervals. The results are seen in Fig. 12. It is demonstrated that the R.P. of ^{188}Re-Lanreotide is greater than 95% within 2.5 h without ascorbic acid, but R.P. keeps no change for 6 h with ascorbic acid. This shows that ^{188}Re-Lanreotide is unstable in vitro and ascorbic acid promotes stability of ^{188}Re-Lanreotide.

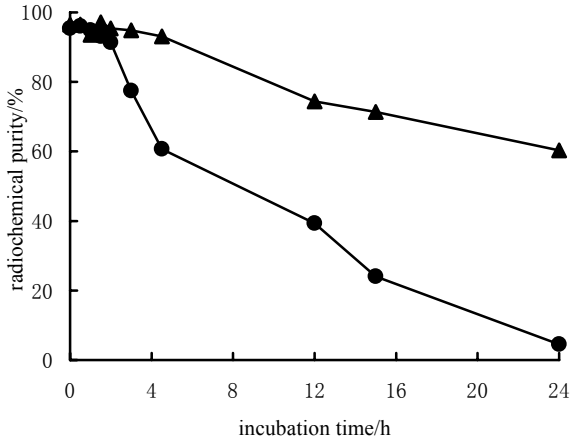

Fig. 12 Stability of ^{188}Re-Lanreotide without ascorbic acid and with ascorbic acid

—●— without ascorbic acid ; —▲— with ascorbic acid.

3. 4 Biodistribution

The biological behavior of ^{188}Re-Lanreotide is given in the Table 2. Biodistribution studies with ^{188}Re-Lanreotide shows about 2% I.D./g in the blood at 3 h post-injection, ^{188}Re-Lanreotide is eliminated rapidly from the blood and concentrates in the lung and intestine. The uptake of thyroid increases with time of post-injection increasing. This indicates that dissociation of ^{188}Re-Lanreotide occurs. Uptake of adrenal gland is 3.05% I.D./g at 3 h post-injection and uptake of muscle is very low.

Table 2 Biodistribution in vivo of ^{188}Re-Lanreotide in rat（*n* = 3，%I.D./g）

Tissue	Time/h				
	0.5	1	3	6	24
Blood	3.52	2.11	1.65	0.86	0.28
Liver	10.12	6.76	3.48	1.58	0.98
Spleen	3.18	4.50	2.15	1.25	0.56
Lung	2.18	3.58	1.91	1.03	0.37
Kidney	1.54	2.52	1.19	0.86	0.46
Heart	1.45	1.09	0.86	0.53	0.13
Muscle	0.64	0.76	0.38	0.15	0.07
Intestine	2.42	4.58	5.69	2.89	1.32
Thyroid	0.87	1.52	4.95	2.45	0.65
Adrenal Gland	0.68	2.87	3.02	2.53	1.87

4 CONCLUSION

Preparation of ^{188}Re-Lanerotide carries out by two steps using stannous chloride as reducing agent. The reaction of ^{188}Re with mixture of citrate and tartate solution forms ^{188}Re-ligand, then ^{188}Re-ligand reacts with reduced Lanerotide at optimal condition, the labeling yield is at range of 88%～94%. The radiochemical purity of purified ^{188}Re-Lanerotide on Sep-Pak C_{18} cartridge is more than 95%. ^{188}Re-Lanerotide is unstable in vitro without ascorbic acid, but its stability is improved after adding ascorbic acid. ^{188}Re-Lanerotide is cleared rapidly from blood and excreted through liver, concentrates highly in the lung and intestine. ^{188}Re-Lanreotide will be a promising peptide-pharmaceutical for tumor therapy.

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