

Preparation of ^{188}Re -HEDP lyophilized kit for instant bone metastases therapy*YANG Zhi (杨志),^{1,†} ZHU Hua (朱华),¹ LI Nan (李囡),¹ MA Yun-Xia (马云霞),¹ and ZHANG Yan (张岩)¹¹Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education),

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Hydroxyethylidene diphosphonate (HEDP) was prepared and labeled with rhenium-188. Its lyophilized kit was developed for instant preparing of ^{188}Re -HEDP. ^{188}Re -HEDP showed high foci uptake in normal mice ($> 30\%$ ID/g at carrier co-injection) in BABLC/SPF mice. High quality single photon emission computed tomography (SPECT) image of New Zealand rabbit was obtained at 4 h after intravenous injection of 74 MBq radiotracer. The lyophilized HEDP kit affords the new opportunity for routine clinical application in bone metastases therapy.

Keywords: Rhenium-188, Hydroxyethylidene diphosphonate, Lyophilized kit, Bone tumor therapy

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I. INTRODUCTION

Chronic pain syndrome is an important complications of bone metastases. It is well acknowledged that the first radio-nuclide therapy of bone metastases was done in 1960 by administration of ^{32}P -phosphate [1]. Since then, a variety of β -radioisotopes have been used to treat bone Metastases [2, 3]. Among them, ^{89}Sr (Metastron) is used the most frequently worldwide, despite the inconvenience in obtaining it, hence the expensive price. It is only recently available in Australia. In mid 1990s, it was found that ^{186}Re hydroxyethylidene diphosphonate (HEDP) is effective for pain palliation in patients with osseous metastases from prostate cancer [4–7]. Radiochromatographic method was developed later for quality control and stability test of [^{186}Re]-HEDP [8, 9]. However, the ideal agent for bone pain palliation has not yet been identified. ^{188}Re , emitting electrons in maximum energy of 2.1 MeV and 155 keV γ photons (15%) with $T_{1/2} = 16.9$ h, is an attractive candidate for bone tumor therapy. It can be obtained in no-carrier-added form of $^{188}\text{W}/^{188}\text{Re}$ generator. Palmedo *et al.* found in 2003 that repeated ^{188}Re -HEDP therapy was good for patients with prostate cancer patients with bone metastases [10]. Recently, Biersack *et al.* found that repeated administrations of ^{188}Re -HEDP reduced the pain and improved survival rates of prostate cancer patients with bone metastases [11]. The ^{188}Re -HEDP for bone pain palliation has become an effective method for treating bone metastasis pain, and has been a radiopharmaceutical used clinically. The preparation of ^{188}Re -HEDP has been reported by many researchers [12, 13]. However, the synthesis process is still complicated, and is not conducive to rapid production.

In this paper, we report a system labeling study of ^{188}Re -HEDP lyophilized kit with ^{188}Re obtained from $^{188}\text{W}/^{188}\text{Re}$ generator using radiometric methods. The ^{188}Re -HEDP's obtained by lyophilized kit is of high radiochemical purity (R-

CP) and radiochemical yields (RY). The relationship is investigated, too, between the bone uptake and precise rhenium mass levels. The biodistribution of mice and SPECT imaging of rabbit are performed. All these efforts are made towards routine clinical application in bone tumor therapy with the ^{188}Re -HEDP lyophilized kit.

II. EXPERIMENTAL

A. General

Hydroxyethylidene diphosphonate (HEDP), sodium acetate (NaOAc), phosphate-buffered saline (PBS), sodium bicarbonate (NaHCO_3), glacial acetic acid, phosphorus trichloride (PCl_3), 2,5-dihydroxybenzoic acid (DHB), and acetyl chloride were purchased from Sigma-Aldrich (St. Louis, MO). $^{188}\text{ReO}_4^-$ was eluted from $^{188}\text{W}/^{188}\text{Re}$ generator (Shanghai Institute of Applied Physics, CAS) using ^{188}W produced in the High Flux Isotope Reactor. Other commercial chemicals were purchased from VWR International (San Diego, CA). BABLC/SPF mice (about 30 g) and New Zealand rabbit were kept under pathogen-free conditions and were handled according to Institutional Animal Care and Use Committee guidelines. Thighbone and foci uptakes were analyzed using two-tailed, unpaired Student *t*-tests, with $p < 0.05$ being considered as statistically significant. All statistical computations were performed using Excel.

B. Analytical methods

^1H NMR spectra were recorded on a Varian XL-300 spectrometer (Varian, Inc., Palo Alto, CA) operating at 300 MHz with tetramethyl silane (TMS) as internal standard. The samples were analyzed by a radioactivity thin-layer scanner (Bioscan, IAR-2000, USA) and an automated gamma scintillation counter (Perkin Elmer, 1470-002, USA). High performance liquid chromatography (HPLC) analysis was performed using a Dionex P680 system equipped with a tunable

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absorption detector and a PDA-100 photodiode-array detector, using a Hypersil BDS C-18 reversed phase column (5 μm , 250 mm $\times$ 4.6 mm). The HPLC solvents were 0.1% TFA in H_2O (solvent A) and 0.1% TFA in acetonitrile (solvent B). Conditions: Gradient B: 0%–10%, 0–5 min; 10%–50%, 5–8 min; 50–80%, 8–10 min; 80%–10%, 10–12 min.

C. Radio-synthesis of ^{188}Re -HEDP by conventional methods

The synthesis and radiolabeling of ^{188}Re -HEDP is shown in Fig. 1. The precursor HEDP was synthesized by a previous procedure with some modifications [14]. Briefly, 30 g glacial acetic acid was dissolved in 15 mL water under N_2 atmosphere in a three neck bottle. PCl_3 was added dropwise in 60 min at 50 $^\circ\text{C}$. The mixtures were stirred for four more hours at 150 $^\circ\text{C}$. The acetyl diphosphonate mixtures were obtained. Then, the mixtures were further distilled at 150 $^\circ\text{C}$ till the acetic acid evaporated (about 4 h). The HEDP was obtained by crystallization from glacial acetic acid/ H_2O /ethanol (1/3/1) as white crystal in 90% total yield. Formation of HEDP was analyzed by electrospray ionization mass spectrometry using the Agilent LC/MSD TOF mass spectrometer (Santa Clara, CA, USA) equipped with a Vydac C-18 column (4.6 mm $\times$ 250 mm, 7 μm particle sizes, 30 nm pore size, Anaheim, CA, USA).

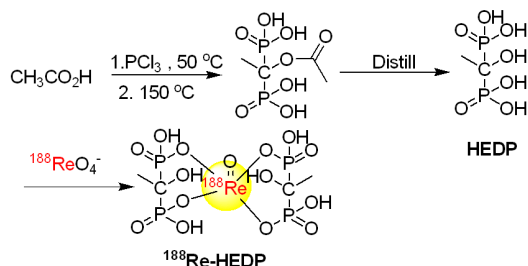


Fig. 1. (Color online) Synthesis and radiolabeling of ^{188}Re -HEDP.

^{188}Re was obtained from 370 MBq (10 mCi) alumina-based $^{188}\text{W}/^{188}\text{Re}$ generator. The specific volume of above eluates was 10 mL (saline), and then was concentrated to 1.5 mL. For preparation of the ^{188}Re -HEDP, 5.8 μL (0.22 μmol) of NH_4ReO_4 (10 mg/mL) in saline was added to the concentrated ^{188}Re solution. This carrier-added $^{188}\text{ReO}_4^-$ was used for the labeling reaction through a 0.22-mm sterile filter to a kit vial containing 8.3 mg (0.04 mmol) of HEDP, 3.0 mg (0.02 mmol) of DHB, and 3.9 mg (0.02 mmol) of SnCl_2 . The vials were kept for 20 min at 90 $^\circ\text{C}$, and the reaction was monitored by radio-TLC (eluted A: Acetone; eluted B: 5% saline buffer). Quality control of carrier-added ^{188}Re -HEDP was performed using instant Xin-Hua 1 test paper [11].

D. Kit formulation and labeling with ^{188}Re

Effects of reductant amount (SnCl_2) and carrier content (NH_4ReO_4) in labeling of ^{188}Re -HEDP were investigated.

The radio-labeling yields were determined with Radio-TLC. Each set of reaction conditions was run 2–3 times.

The kit formulation was prepared under aseptic conditions, with Vial A containing 1 mL 5% NaHCO_3 buffer and 5.0 mg DHB in final pH 6.0, while Vial B containing HEDP and SnCl_2 . Each vial was transferred to the freeze-dryer and the process continued. The vials were closed under dry sterile nitrogen gas and stored at 2–8 $^\circ\text{C}$.

The freeze-dried Vial B was added with 2 mL of generator-eluted $^{188}\text{ReO}_4^-$ (370 MBq), extracted with injector, added to Vial A and incubated for a while to form ^{188}Re -HEDP.

E. Radiochemical analysis of ^{188}Re -HEDP lyophilized kit

Dependence of the ^{188}Re -HEDP labeling yields upon the reaction temperature and times were investigated. Radio-labeling yields were determined with Radio-TLC. Each set of reaction conditions was run 2–3 times.

F. Quality control of radioactive ^{188}Re -HEDP lyophilized kit

The *in vitro* stability of ^{188}Re -HEDP in saline and with vitamin C solution was evaluated by measuring RCP with Radio-TLC at each time point after incubation at 37 $^\circ\text{C}$. Radiolabeling yield of the kits was tested for three months.

G. Biodistribution study in normal mice

Radiolabeling yield of ^{188}Re -HEDP kit was over 95%. It was used without further purification in the following biodistribution and SPECT imaging studies.

Relationship between bone uptake and precise rhenium mass levels was studied. Each amount of NH_4ReO_4 was added to $^{188}\text{ReO}_4^-$ with final concentration of 0.05 to 0.3 mg/5 mg HEDP. About 0.37 MBq (100 μL) of ^{188}Re -HEDP with each amount of NH_4ReO_4 was injected through the tail vein of mice ($n = 4$). Three hours after injection, the mice were sacrificed, and the tissues and organs of interest were collected, wet weighed and counted in a γ -counter. The percentage of injected dose per gram (%ID/g) for each sample was calculated by comparing its activity with appropriate standards of the injected dose (ID), and the values are expressed as mean $\pm$ SD. The contrast study was performed to acquire the relation between bone uptake and precise rhenium mass levels. The results were expressed as %ID/g. Averages and standard deviations were calculated. The T/NT and F/NT (thighbone or foci to normal tissues) values were calculated.

Biodistribution of optimal NH_4ReO_4 added ^{188}Re -HEDP were investigated. The mice were injected intra-venously with ^{188}Re -HEDP (0.37 MBq), and sacrificed at 0.5, 1, 4 and 6 h after injection.

H. SPECT imaging of ^{188}Re -HEDP

About 74 MBq of ^{188}Re -HEDP (contain 0.15 mg NH_4ReO_4 /5 mg HEDP) in 0.2 mL of PBS solution was injected in marginal ear vein of a New Zealand rabbit. It was placed near the center of the field of view (FOV) of a Siemens SPECT scanner, where the highest image resolution and sensitivity are available. The rabbit was deeply anesthetized by intravenous injection of a mixture of ketamine (25 mg/kg) and diazepam (1.1 mg/kg). Anesthesia was supplemented as needed. Computer acquisition of the gamma camera data was initiated after 4 hours administration of the ^{188}Re -HEDP.

III. RESULTS AND DISCUSSION

A. Chemistry and radiolabeling produce

The reaction procedure is described in Fig. 1. HEDP was prepared by recrystallization from three phase system as white crystal in 90% total yield. It was identified by elemental analysis, mass spectrum, and ^1H -NMR, and the results agree well with the expected chemical structures. HEDP contains phosphoric acid and alcohol hydroxyl groups, we performed electrospray ionization mass spectrometry. ESI-MS $m/z = 205.0082$ for $[\text{M-H}]^-$, calculated for $\text{C}_2\text{H}_7\text{O}_7\text{P}_2$: 204.9667. ^1H NMR (D_2O , 300 MHz) δ : 2.0–2.2 (s, 5H, -OH), 1.1 (s, 3H, -CH₃). Anal. calcd for $\text{C}_2\text{H}_8\text{O}_7\text{P}_2$ (%): C, 11.66; H, 3.91; Found (%): C, 11.58; H, 4.13.

HEDP was labeled with ^{188}Re obtained from $^{188}\text{W}/^{188}\text{Re}$ generator by reduction with SnCl_2 in the conventional method. For Radio-TLC analysis, with the 5% saline system, the R_f values of ^{188}Re -HEDP, and free $^{188}\text{ReO}_4^-$ were about front (R_f 0.9–1.0), while ^{188}Re -colloidal impurities remain at original. With the acetone system, the ^{188}Re -colloidal impurities and ^{188}Re -HEDP remains at the origin and free $^{188}\text{ReO}_4^-$ moves with the front (R_f 0.9–1.0). For each radiolabeled complex, the single peak in the HPLC-chromatogram clearly showed the formation of only one complex and excluded the possibility of residual $^{188}\text{ReO}_4^-$ or other component. This means that the chelation of rhenium with the bisphosphonates moiety is complete.

B. Kit formulation and radiolabeling procedure

The composition of the kit was similar to conventional methods (ligand-HEDP, antioxidant-DHB, reducing agent- SnCl_2 , and carrier- NH_4ReO_4). It was found that over 95% yields of complex were at pH 4–6 and the labeling yield did not depend on the DHB concentration. We concerned with the influence of reductant amount (SnCl_2), and carrier content (NH_4ReO_4) obtained the optimal condition. The labeling yield of HEDP increased with the SnCl_2 content ($[\text{HEDP}] = 10 \text{ mg/mL}$, Fig. 2(a)). However, as shown in Fig. 3(a), the absence of carrier decreased the labeling yield to 90%. At a concentration of 0.01–1 mg NH_4ReO_4 carrier/5 mg HEDP

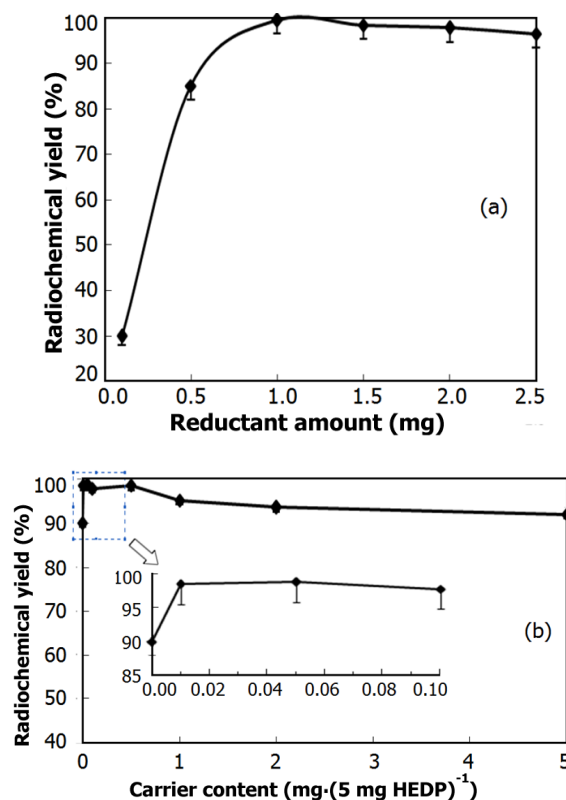


Fig. 2. (Color online) Influence of reductant amount (a) and carrier content (b) on labeling efficiency, for ^{188}Re -HEDP reaction of 20 min at 100 °C.

the radiolabing yields were > 95%.

A lyophilized kit was prepared under optimal conditions: ^{188}Re -labeled HEDP in 5% NaHCO_3 buffer with the final pH 6.0. Dispensed cold kits were tested according to Chinese Pharmacopoeia to be both sterile and pyrogen free.

Figure 3 shows the labeling yield as a function of temperature and reaction time. The temperature effect was investigated in the synthesis of ^{188}Re -HEDP (Fig. 3(a)), and the desired compound was prepared in good yield at 90 °C. As shown in Fig. 3(b), 15 min is suitable for this reaction. At 90 °C, the reaction was rapid and efficient. After 15 min, the highest radiochemical yield was $96 \pm 2\%$ ($n = 3$) with ^{188}Re -HEDP kit.

So, to the above freeze-dried vial, 2 mL of generator-eluted $^{188}\text{ReO}_4^-$ (370 MBq) was added to Vial B, which was extracted with injector and added to Vial A and incubated at 90 °C for 15 min to give ^{188}Re -HEDP.

In vitro stability of the complex was evaluated by measuring the RCP with Radio-TLC at different hours after preparation (Fig. 4(a)). The RCP were still over 95% at 5 h, indicating that ^{188}Re -HEDP was stable *in vitro* at least for 4 h at room temperature. The stability was improved by adding 5 mg/mL Vitamin c (Vc) under the same condition. As shown in Fig. 4(b), long term deposit of kit did not affect the radiolabeling yields in three months, and the kit stored at 2–8 °C for three months had more than 95% radiolabing yield in optimal conditions.

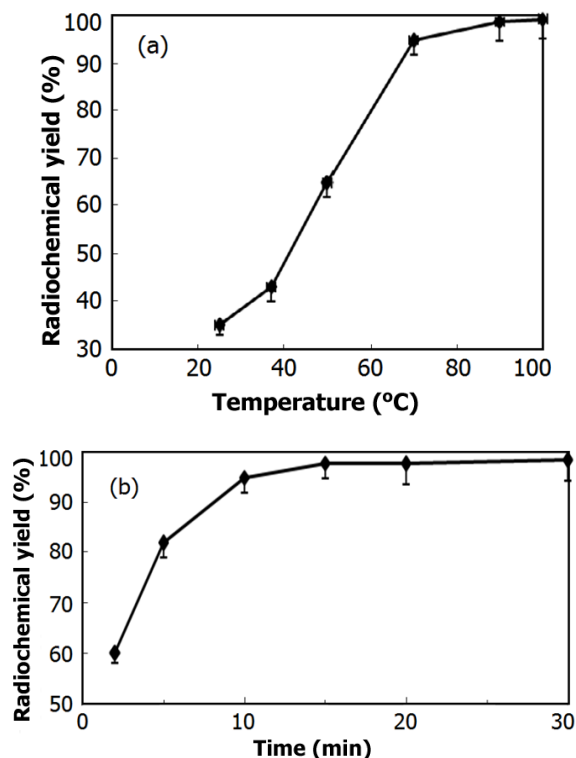


Fig. 3. Effects of (a) temperature (20 min of reaction time) and (b) reaction time (at 90 °C) on labeling efficiency.

C. Biodistribution

Many authors focus on ^{188}Re radiolabeling of phosphonate compounds for bone metastases therapy [15–17], but evidence shows that coordination compound [^{188}Re] Re-HEDP is a successful alternative for palliation of metastatic bone pain. This stimulates studies on ^{188}Re -HEDP kit formulation and its effect on biological activity and therapeutic use.

Tissue distribution of ^{188}Re -HEDP in BABLC/SPF mice was studied. The biodistributions of 0.37 MBq ^{188}Re -HEDP co-injected with 0.05–0.3 mg NH_4ReO_4 /5 mg HEDP at 3 h after injection were given in Table 1.

It is well known that the presence of macroscopic amounts of stable rhenium in Re-HEDP preparations is decisive with respect to the form of phosphonate chemical species [18]. In Re carrier-added preparations, these species consist of rhenium-rhenium bonds that cannot be formed using carrier-free ^{188}Re . As shown in Fig. 3(a), the carrier-added HEDP showed higher RY.

From Table 1, the biodistribution and bone uptake of each amount of carrier-added ^{188}Re -HEDP differ from each other. The radioactivity in the blood was 0.13 ± 0.03 , 0.23 ± 0.09 , 0.19 ± 0.06 , 0.11 ± 0.01 and 0.11 ± 0.02 %ID/g for uptake of 0.05, 0.10, 0.15, 0.20 and 0.30 mg Re per 5 mg HEDP, respectively, indicating that the ^{188}Re -HEDP can be eliminated quickly from the blood. The uptakes of ^{188}Re -HEDP in liver (< 0.18 %ID/g) and spleen ($(0.07\text{--}0.09)$ %ID/g) were especially low. No significant uptake was seen in any other organ

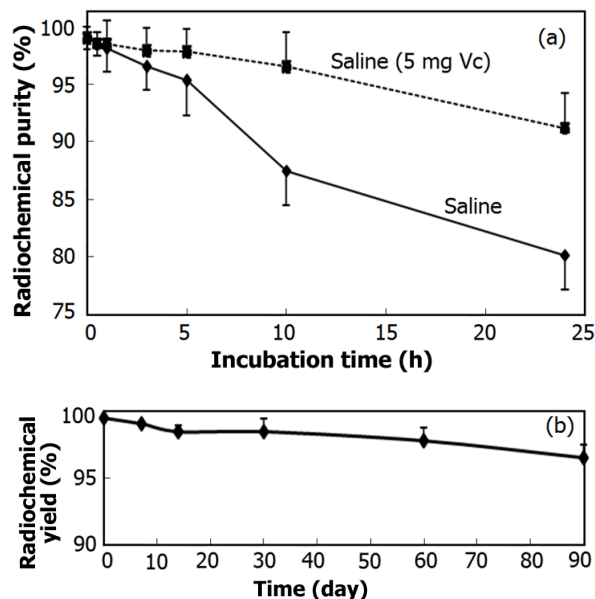


Fig. 4. *In vitro* (Saline, 5 mg/mL Vc Saline) stability of ^{188}Re -HEDP conjugate (a) and radiolabeling yield of the kit deposit in three months (b).

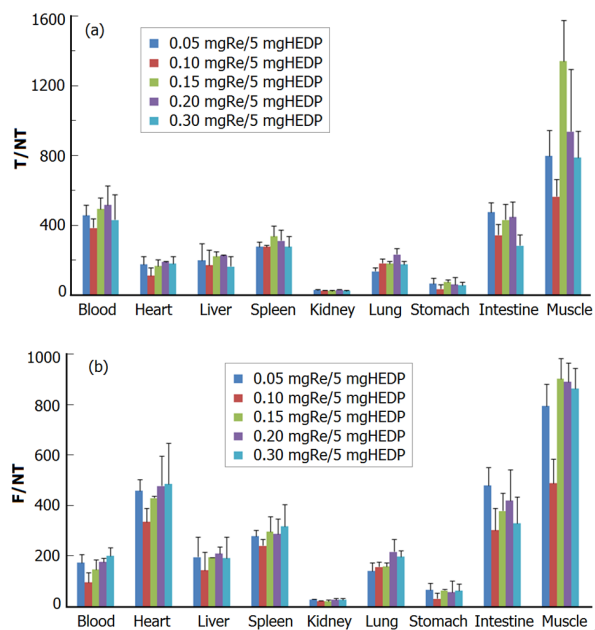


Fig. 5. (Color online) Thighbone (a) and focal (b) to normal tissues (T/NT , F/NT) uptake ratios for ^{188}Re -HEDP in BABLC/SPF mice 3 h after injection.

except bone. The biodistribution also showed that skeletal uptake of 0.15 mg Re were the highest ($26.66 \pm 1.02\%$ and $30.55 \pm 5.02\%$ in thighbone and focal, respectively). There was no statistical significance in bone uptake between thighbone and focal ($P = 0.16$, unpaired two tail test).

The T/NT and F/NT values were calculated, as shown in Fig. 5. Due to high radioactivity level in bones, the uptakes in

TABLE 1. Biodistribution (in %ID/g) of ^{188}Re -HEDP in BABLC/SPF mice

Organ ^a	0.05 mg Re	0.10 mg Re	0.15 mg Re	0.20 mg Re	0.30 mg Re
Blood	0.13 ± 0.03	0.23 ± 0.09	0.19 ± 0.06	0.11 ± 0.01	0.11 ± 0.02
Heart	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.0	0.04 ± 0.01	0.05 ± 0.01
Liver	0.13 ± 0.07	0.18 ± 0.12	0.13 ± 0.01	0.10 ± 0.01	0.12 ± 0.06
Spleen	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
Kidney	0.68 ± 0.08	0.83 ± 0.03	1.13 ± 0.26	0.66 ± 0.08	0.69 ± 0.10
Lung	0.15 ± 0.03	0.13 ± 0.02	0.17 ± 0.02	0.09 ± 0.02	0.11 ± 0.02
Stomach	0.35 ± 0.16	0.58 ± 0.21	0.40 ± 0.03	0.61 ± 0.61	0.36 ± 0.15
Intestine	0.04 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	0.07 ± 0.01
Muscle	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
Thigh	21.16 ± 1.09	20.58 ± 0.57	26.66 ± 1.02	20.04 ± 3.06	21.06 ± 2.88
Focile	21.06 ± 1.86	23.82 ± 2.47	30.55 ± 5.02	21.62 ± 1.29	18.58 ± 1.79

^a Each datum is expressed as mean ± SD of five animals.TABLE 2. Biodistribution of ^{188}Re -HEDP in BABLC/SPF mice at different hours after injection

Organ	0.5 h	1.0 h	4.0 h	6.0 h
Blood	1.74 ± 0.06	0.43 ± 0.06	0.27 ± 0.10	0.17 ± 0.07
Heart	0.85 ± 0.03	0.16 ± 0.03	0.08 ± 0.02	0.04 ± 0.01
Liver	0.54 ± 0.05	0.23 ± 0.02	0.27 ± 0.02	0.21 ± 0.04
Spleen	0.30 ± 0.005	0.14 ± 0.03	0.16 ± 0.06	0.11 ± 0.01
Kidney	2.08 ± 0.42	1.72 ± 0.41	1.33 ± 0.26	1.45 ± 0.18
Lung	0.79 ± 0.06	0.24 ± 0.03	0.22 ± 0.08	0.15 ± 0.04
Stomach	0.62 ± 0.04	0.44 ± 0.19	0.50 ± 0.10	0.66 ± 0.22
Intestine	0.32 ± 0.06	0.18 ± 0.08	0.26 ± 0.18	0.15 ± 0.07
Muscle	0.27 ± 0.03	0.21 ± 0.08	0.11 ± 0.04	0.06 ± 0.02
Thighbone	24.97 ± 0.70	25.96 ± 3.11	26.50 ± 1.72	27.72 ± 1.40
Focile	24.42 ± 4.23	28.42 ± 10.14	30.03 ± 11.01	26.94 ± 1.22
Blood	1.74 ± 0.06	0.43 ± 0.06	0.27 ± 0.10	0.17 ± 0.07

Fig. 6. SPECT imaging of New Zealand rabbit at 4 h after injection of 74 MBq ^{188}Re -HEDP.

non-target organs decreased, such as muscle, blood, spleen and liver ($T/NT > 200$). 0.10 mg Re/5 mg HEDP carrier added ^{188}Re -HEDP showed the lowest T/NT value. F/NT showed same tendency compared with T/NT . For all this, both thighbone and focile showed excellent uptake of ^{188}Re -

HEDP. Among then, 0.15 mg Re/5 mg HEDP carrier added tracer showed the highest T/NT and F/NT value.

Biodistributions of 0.15 mg NH_4ReO_4 per 5 mg HEDP in BABLC/SPF mice are given in Table 2. No sign of toxicity through the study period was observed. This is consistent with the general observation that Re-HEDP has low toxicity and can be used therapeutically at a high dose. ^{188}Re -HEDP cleared from the blood rapidly, being 1.74 ± 0.06 , 0.43 ± 0.06 , 0.268 ± 0.100 and 0.17 ± 0.07 %ID/g at 0.5, 1.0, 4.0 and 6.0 h post injection. ^{188}Re -HEDP showed predominant kidney uptake. As described above, the re-uptake of radio-metabolites may be the main reason for high kidney uptake.

These results show that ^{188}Re -HEDP lyophilized kit has high selective uptake in the skeletal system and low background uptake in soft tissues, and is of great potential for instant clinical assessment of bone tumor therapy.

D. SPECT imaging

A typical SPECT image is shown in Fig. 6. High bone activity accumulation was observed at 4 h after injection. From this image, it can be seen that ^{188}Re -HEDP complexes has highly selective skeletal uptake in the rabbit after administration of intravenous injection. The SPECT results agree well with the biodistribution studies in mice, indicating that ^{188}Re -

HEDP kit possesses excellent characteristics for instant clinical assessment of bone tumor therapy.

IV. CONCLUSION

In this paper, we focus on the development of ^{188}Re -HEDP kit for instant clinical bone tumor therapy. The kit preparation process was simple and fast. The sterile and pyrogen-

free HEDP kit was developed to produce instant preparation of ^{188}Re -HEDP suitable for clinical bone tumor therapy. The amounts of stable rhenium in ^{188}Re -HEDP preparations and biodistribution are determined. Both the biodistribution experiments and SPECT imaging studies demonstrate bone targeting. The development of the lyophilized HEDP kit affords the new opportunity for routine clinical assessment of bone tumor therapy.

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