Original Article

Radiosynthesis of ^{191}Os-2-acetylpyridine thiosemicarbazone complex, as an *in vivo* therapeutic radionuclide generator

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ABSTRACT

Introduction: Due to the anti-proliferative properties of platinum group-thiosemicarbazone complexes, the production of ^{191}Os-labeled 2-acetyl pyridine 4-N-methylthiosemicarbazone (^{191}Os-APMTS) was investigated.

Methods: [^{191}Osmium ($T_{½}$= 15.4d) was produced via the ^{190}Os(n,γ)^{191}Os nuclear reaction using enriched target irradiated with thermal neutrons. Reaction of in-house synthesized 2-acetylpyridine thiosemicarbazone (APMTS) with ^{191}Os yielded [^{191}Os]APMTS checked by ITLC followed by stability, partition co-efficient and biodistribution determination.

Results: Following synthesis and spectroscopic determination of the ligand (>99% chemical purity), the complex was prepared with a radiochemical purity of more than 95% (RTLC) and specific activity of 21.5 GB/mM and was stable in the formulation and presence of human serum at 37°C for up to 48h. The partition coefficient was determined (log P. 1.23). The biodistribution study up to 4 days demonstrated significant tissue uptake differences in the bone, blood, heart and thyroid.

Conclusion: This is the first Os-191 labeled thiosemicarbazone designed as an in-vivo therapeutic radionuclide generator. Further investigation is ongoing on the evaluation of the complex in tumor bearing animals.

Key words: Thiosemicarbazone; ^{191}Os; Radiolabeling; Biodistribution

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INTRODUCTION

Thiosemicarbazone complexes have shown interesting anti-proliferative activity *in vitro* and *in vivo*. Pyridine-based compounds are mostly studied [1, 2], possibly due to their resemblance to pyridoxal metabolites that attach to co-enzyme B_6-dependant molecules leading to enzyme inhibition [3]. For example, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone, Triapine, has shown ribonucleotide reductase inhibitor activity and has already entered phase II clinical trials [4]. Reaction of acetyl thiosemicarbazones has been carried out with platimun metal group including Ru and Os, in which the acetyl thiosemicarbazone ligand coordinates the metal as a bidentate N, S-donor forming a five-membered chelate ring [5]. Various osmium aryl complexes have been reported for ultimate antineoblastic activity in the literature. An Osmium (VI)-salicylidene-2-aminophenol complex has demonstrated prominent *in vitro* and *in vivo* anti-cancer properties [6]. Also, osmium (IV) azole complexes have demonstrated cytotoxicity in various cancer cell lines [7]. Also for Os (II) complexes significant in vitro anticancer activity of arene complexes have been repeatedly reported [8-10]. Labeling of thiosemicarbazones with metallic radioisotopes including ^{67}Ga [11, 12], ^{64}Cu [13], ^{103}Pd [14], ^{61}Cu [15] led to the development of various diagnostic and/or therapeutic compound demonstrating significant tumor targeting properties in many reports.

Due to the importance of pyridine thiosemicarbazones in anti-neoblastic activity and the necessity of metal complexation in most of these compounds for their activity, the idea of developing a possible therapeutic/diagnostic agent using a suitable beta emitting osmium isotope, ^{191}Os decaying to a gamma emitter radionuclide ^{191}Ir, an in vivo therapeutic/diagnostic generator has interestingly potential for research and clinical applications.

^{191}Os can be produced in reasonable amounts using (n, gamma) reactions. ^{191}Os ($E_{\beta\text{- max}}$ = 313keV, $T_{1/2}$ = 15.4 day) (Figure 1), is one of the potential radionuclides for targeted therapy modalities. An ^{191}Os\\$^{191m}$Ir generator suitable for first-pass radionuclide angio-cardiography has been developed recently. This generator system allows repeated elutions of ^{191m}Ir 4.96-s from its 15.4 day ^{191}Os parent [16]. We have recently reported the development and production of ^{191}Os/^{191m}Ir generator for research purposes [17].

In this research we developed a possible *in vivo* tumor-targeting radionuclide generator by labeling 2-acetyl pyridine 4-N-methylthiosemicarbazone (APMTS) with ^{191}Os radionuclide (in ^{191}Os-k_2OsCl_6 form) considering the beta emission (313 kev) and its suitable physical half-life (Figure 2). Theoretically the complex can be used probably for both therapy and diagnosis based on the different characteristics of the parent and daughter radionuclides.

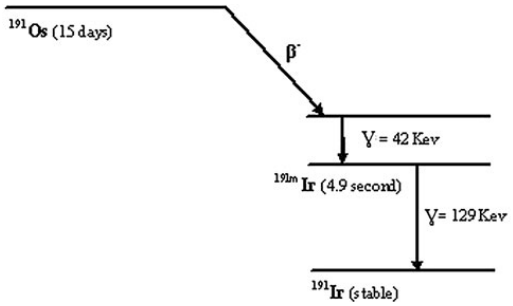

Fig 1. Salient characteristic of ^{191}Os decay.

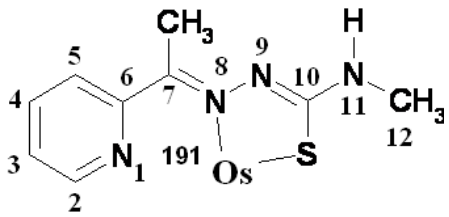

Fig 2. Possible chemical structure of the thiosemicarbazone complex [5].

METHODS

Isotopically Isotopically enriched granulated metallic osmium with purity of >90% was obtained from commercial vendors. Chromatography paper, Whatman No. 2 was obtained from Whatman (Maidstone, UK). Radio-chromatography was performed by using a bioscan AR-2000 radio TLC scanner instrument (Bioscan, Washington, DC, USA). A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for counting distributed activity in rat organs. All chemical reagents were purchased from Merck (Darmstadt, Germany). All values were expressed as mean ± standard deviation (Mean± SD) and the data were compared using student T-test. Statistical significance was defined as $P<0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. All of rats were purchased from Pasteur Institute

of Iran, weighing 180-220 g (n=5) and were kept at routine day/night light program and were kept under common rodent diet pellets.

^{191}Os production in form of K_2OsCl_6

^{191}Os was prepared by neutron irradiation of 10 mg amount of isotopically enriched granulated metallic osmium in the Research Reactor of Tehran for 15 days by $4\times10^{13}cm^{-2}s^{-1}$ average neutron flux with subsequent chemical fusion in a mixture of KOH–KNO_3. The optimal temperature for fusion is 500°C. Additional product of fusion reaction is volatile because evaporation of osmium tetroxide occurs at 120 °C. Fusion was performed in bath of 51.3 g KNO_3 and 48.7 g $NaNO_3$ that was connected to a circulator and KOH trap for collecting the evaporated gases. After fusion, the irradiated target is dissolved in water to give an 0.4 N KOH solution of ^{191}Os-$K_2[OsO_4(OH)_4]$, which is mixed with two volumes of ethanol to reduce the Os (VIII) to Os (VI). After 10 min, five volumes of concentrated hydrochloric acid are added quickly and the solution is heated in a boiling water bath for 30 min. The solution is then evaporated to dryness and the brickred precipitate of ^{191}Os-K_2OsCl_6 dissolved in 0.9% NaCl–0.01 N HCl [16].

Preparation of 2-Acetyl pyridine 4-N-methylthiosemicarbazone

The thiosemicarbazone was prepared with slight modifications according to the conventional methods [18]. (60%) m.p. 181 °C. ^{1}H NMR (D_6-DMSO) δ (ppm) 10.32 (s, 1H, NH_9 or NH_{10}), 8.61(bs, 1H, NH_9 or NH_{10}), 8.57-8.56 (d, J=4.5 Hz, 1H, H_2), 8.41-8.4 (d, J=8.06 Hz, 1H, H_5), 7.81-7.78 (t, J=7.1, 1H, H_4), 7.38-7.35 (t, J=5.6 Hz, 1H, H_3). 3.06 and 3.07 (2 single peaks for 2 diastereomer, 3H, N-CH_3), 2.38 (s, 3H, CH_3-7). IR (KBr) λ max 3288 (N-H), 3237 (N-H), 2989 (C-H), 2932 (C-H), 1538 (C=N), 1231 (C=S), 1470 (C=N), 1160 (C=S). Mass (electrospray) 208.1 (14%), 172(4), 157.1(76), 130(65), Elemental analysis for $C_9H_{12}N_4S$, calcd C, 51.9, H, 5.81, N, 26.9; found C, 52, H, 5.79, N, 27.0.

Radiolabeling and Quality control of ^{191}Os-APMTS

^{191}Os-K_2OsCl_6 (0.7–2.2 mCi) solution in 2 mL vial with activity was adjusted to pH 1 by HCl 1 M. Vial solution was evaporated in a boiling water bath and heated to dryness using a flow of N_2 gas at 50-60°C. The thiosemicarbazone, dissolved in supra-pure ethanol (4.8 µmol, 1mg/ml) was added to the residue and vortexed at 25°C for 3-5 min. The mixture was then left at various temperatures (25, 50, 75 and 95°C) for 30 min up to 12 hours to optimize the reaction for best yield. The mixture (about 1 ml) was then cooled in an ice bath and mixed with saline (1 ml). A 5µL sample of the final mixture was spotted on Whatman No. 2 chromatography paper and developed in a mixture of 10% ammonium acetate: methanol (1:1) as mobile phase to discriminate free osmium from radiolabeled compound. After obtaining the desired radiochemical purity the solution was concentrated by a flow of N_2 gas at 50ºC and a 5% ethanol mixture was prepared by the addition of isotonic acetate buffer for biological tests.

Stability of ^{191}Os-APMTS complex in the final product

A sample of ^{191}Os-APMTS (18–180MBq) was kept at room temperature for 48 h while checked by RTLC every 2 h. A micropipet sample (5 µL) was taken from the shaking mixture and the ratio of free radio-osmium to ^{191}Os-APMTS was checked by ITLC in a mixture of 10% ammonium acetate:methanol (1:1) as mobile phase to discriminate free osmium from the radiolabeled compound.

Stability of ^{191}Os-labeled compounds in the final product

Stability tests were based on previous studies performed for other radiolabeled thiosemicarbazone [11]. A sample of ^{191}Os-APMTS (18–180MBq) was kept at room temperature for 48 hours while checked by RTLC every two hour. A micropipette sample (5 µl) was taken from the shaking mixture and the ratio of free radio-osmium to ^{191}Os-APMTS was checked by ITLC in a mixture of 10% ammonium acetate: methanol (1:1) as mobile phase.

Serum stability studies

To 36.1 MBq (976 µCi) of ^{191}Os-APMTS was added 500µL of freshly prepared human serum. The resulting mixture was incubated at 37°C for 5 h, and 5-µL aliquots were analyzed by radio-TLC up to 48h of incubation to determine complex stability.

Determination of partition coefficient

Partition coefficient of ^{191}Os-APMTS was measured according to reported methods [19]. Briefly, ^{191}Os-APMTS (300-500 µCi) was transferred to a 5 ml-vial containing 3M (4 ml) sodium following 1 min of vigorous vortex mixing of 1 mL of 1-octanol and 1 mL of isotonic buffered saline (pH=7) at 37°C. Following centrifugation at >1200*g* for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well counter. A 500 *µ*L sample of the octanol phase from this partitioning was repartitioned two to three times with fresh buffer to ensure that trace hydrophilic ^{191}Os impurities did not alter the calculated *P* values. The reported log *P*

values are the average of the second and third extractions from three to four independent measurements, log *P* values represent the mean (standard deviation) of five measurements.

Biodistribution

Biodistribution and in vivo stability of complex studies was evaluated in 20 wild-type mice of 25 g weight each. Ten mice received 50 µCi of ^{191}Os-APMTS complex intravenously via dorsal tail vein. Five groups were killed 4, 24, 48, 96h after injection of the radiolabeled compound. Samples of 12 organs including blood, liver, lung, heart, bone, spleen, stomach, kidney, thyroid, intestine and other organs were excised, weighed wet and counted by NaI(Tl) well counter. Extra care and caution was observed while performing the excision and the activity counts of the tissue samples. The absolute tissue concentrations expressed as a percentage of the administered dose per gram of the wet tissue. The above procedure was repeated on other ten mice by administration of ^{191}Os-K_2OsCl_6 as free cation for comparison.

RESULTS AND DISCUSSION

Selection of ligand

Although the 2-Acetylpyridine thiosemicarbazone ligand seems a non-selective ligand however the broad application of pyridine-based thiosemicarbazone metal complexes including Triapine in the literature for the detection and therapy of malignancies suggests the possible application of ^{191}Os-APMTS as an in-vivo therapeutic/diagnostic complex. The half life is also a drawback, however, various radionuclides with half lives at the day scales are still applicable including I-131 and Phosphorous-32, still none possess the potential as being used as an in-vivo therapeutic/diagnostic system.

Preparation and structure confirmation of the ligand

2-Acetylpyridine thiosemicarbazone (APMTS), not commercially available, was prepared according to the general procedure of thiosemicarbazones. The reaction was performed in 5% acetic acid solution containing N_4-methyl thiosemicarbazide. The ^{1}H-NMR spectrum demonstrated the formation of two different diastromers which was supported by previous reports. Figure 3 shows the route to prepare the ligand and labeled compound.

Labeling

The radiochemical yields were determined by RTLC. At optimized condition, total labeling and formulation of ^{191}Os-APMTS took about 12 h, with a yield of 95%.

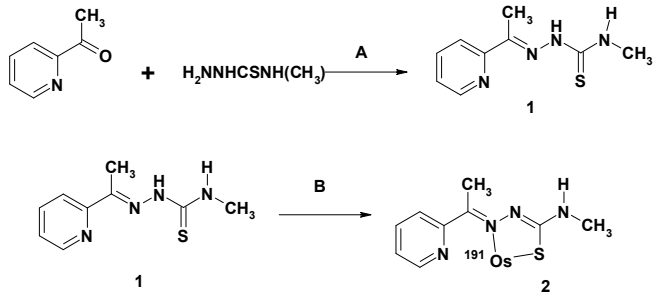

Fig 3. Production of ^{191}Os-APMTS; A: 5% AcOH, 50°C, B: ^{191}Os-K_2OsCl_6, EtOH, N2, 90°C.

The radiolabeled complex was stable in aqueous solution for at least 72 h and no significant amount of other radioactive species was observed by RTLC. Trace amount of ^{191}Os-K_2OsCl_6 (~5%) were detected by RTLC which showed that radiochemical purity of the ^{191}Os-APMTS was higher than 95%. Radiochemical impurities in the ^{191}Os-K_2OsCl_6 sample used in the radiolabeling step were checked by two solvent systems; A, a mixture of 10 mM DTPA solution as mobile phase on Whatman No. 2 paper (pH 3), the ^{191}Os-K_2OsCl_6 cation in $^{191}Os^{4++}$ form, was chelated with the polydentate eluting leading to the migration of the cation in ^{191}Os-DTPA form to higher R_f (R_f =0.9), any other ionic species would lead to the observation of new radiopeaks, especially at the origin (R_f 0.0–0.1) (data not shown). B, a mixture of 10% ammonium acetate:methanol (1:1) was used as another solvent system on the Whatman No 2 paper, free osmium cation remains at the origin using this system while other ionic species would migrate to higher R_f (Figure 4).

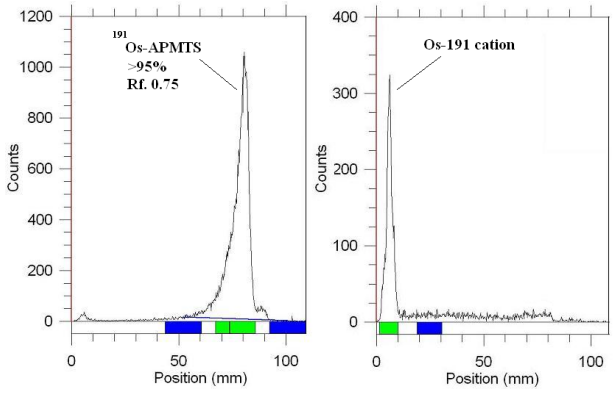

Fig 4. Radio-choromatogram of K_2OsCl_6 (right) and ^{191}Os-APMTS (left) developed in 10% ammonium acetate:methanol (1:1).

Optimization

No detectable complex was formed at room temperature. The best temperature was found to be 85-90°C. At this temperature, when prepared ^{191}Os-K_2OsCl_6 was used, all the radio-osmium was inserted into the complex. While heating the reaction mixture over 90°C or for more than 1 h, the radiochemical yield dropped. The final radiolabeled complex in alcoholic media was diluted in normal saline to a 5% solution.

The solution was stable at room temperature up to 2 days post-formulation, allowing completion of biological experiments. Before the experiments, the solution passed through a 0.22 microns filter (Millipore). At optimized conditions, (2-2.5 mCi Os-191, 90ºC and 5 µM of starting ligand), a specific activity of 21.5 GB/mM was obtained using calculation method.

Serum stability studies

Incubation of ^{191}Os-APMTS in freshly prepared human serum for 24 h at 37°C showed no significant loss of ^{191}Os from the complex during the course of the studies after RTLC study of the cut-off filter flow-through, and the radiochemical purity of complex remained at 95% for 24 h under physiologic conditions.

Partition co-efficient of ^{191}Os-APMTS

As expected from the RTLC behavior, the lipophilicity of the ^{191}Os-APMTS compound was high as determined by the octanol/water partition coefficient (P) for the 191Os-complex and was found to depend on the pH of the preparation. At a pH of 7 (final formulation) the lipophilicity was 1.23±0.05. The water solubility of the tracer slightly changed when the pH is out of 5.5-7 range.

Biodistribution

In order to investigate biodistribution of ^{191}Os-APMTS in wild-type mice we had to obtain the biodistribution data for free osmium cation. After injection of the ^{191}Os-k_2OsCl_6 pre-formulated by the normal saline (pH = 6.5–7), through the tail vein of mice. The biodistribution of the cation was checked in various vital organs. The animals were sacrificed by CO_2 asphyxiation at selected times after injection. Dissection began by drawing blood from the aorta, followed by collecting heart, spleen, thyroid, bone, kidneys, liver, intestine, stomach, lung and other organs.

The average of percent dose per unit weight of selected tissues from ^{191}Os-K_2OsCl_6 is demonstrated in Figure 5.

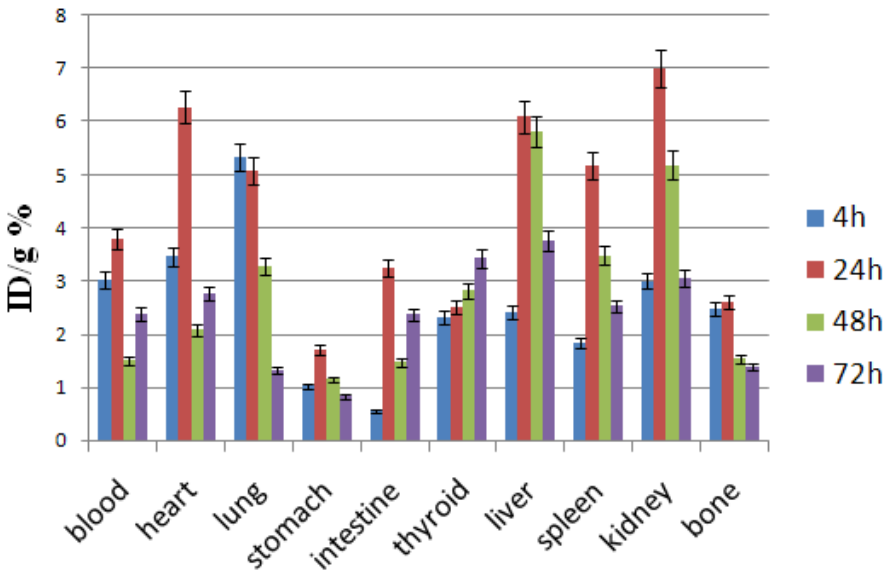

Fig 5. Percentage of injected dose per gram of ^{191}Os-K_2OsCl_6 in mice tissues at 4, 24, 48 and 72 h post injection.

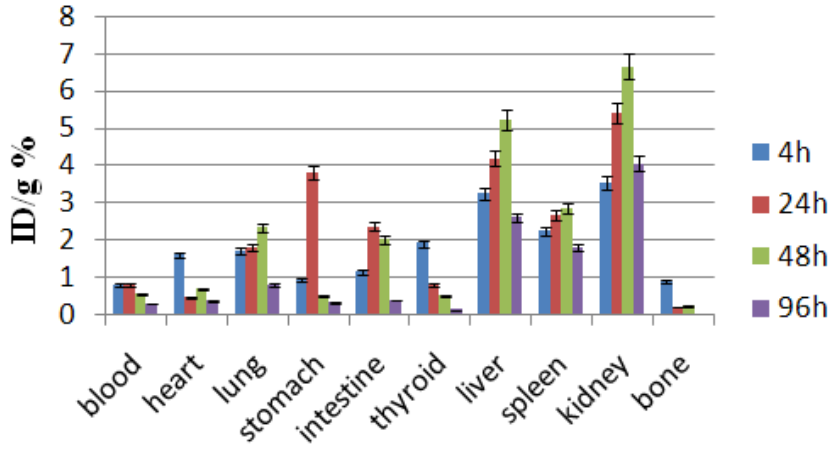

Fig 6. Percentage of injected dose per gram of ^{191}Os-APMTS in mice tissues at 4, 24, 48 and 72 h post injection.

For free ^{191}Os the radioactivity was mainly located in the heart, lung, liver, spleen and kidney as previously shown by other researchers [20].

Iridium-191m has been used effectively for the evaluation of intracardiac shunts in children and for the determination of left ventricular ejection fraction in adults and is mostly a perfusion agent, however medium heart uptake in this study is related to the Ir-191 myocardial uptake as the daughter radionuclide. Due to the resemblance of the Os cations especially at higher oxidation states to iodine anion thyroid uptake was observed as shown in the figure as already shown for pertechnetate anion due to the charge/size resemblance. Also as a free metallic cation, osmium is carried through the circulation in protein-bound form and finally is accumulated in liver. On the other hand, the water solubility as well as negatively charge complex cation is excreted through kidneys too.

A few hours post-injection of ^{191}Os-thiosemicarbazone, the radioactivity content increased in the kidneys and liver and this pattern remained constant up to 24 hours. Major part uptake of radioactivity accumulated was observed in the reticulloendothelial system including liver and spleen. Intestines exhibited a significant uptake which could be attributed to liver excretion of the tracer or metabolites (Figure 6). Lung uptake also showed increased during 24h and remained constant.

Comparison of vital organs uptake for ^{191}Os-K_2OsCl_6 and ^{191}Os-APMTS demonstrate kinetic pattern difference for both species. For blood, free ^{191}Os is significantly higher at 24 h, subsequently; it decreased up to 48 h. For ^{191}Os-APMTS the blood content is low and no significant amount is observed in 96h (Figure 5). In the case of heart, free ^{191}Os is significantly higher at 24 h, it decreased afterward up to 72 h. For ^{191}Os-APMTS the uptake is not significant and remained constant up to 96 h. In case of liver, both compounds have similar uptakes possibly due to different mechanisms. In the case of kidney, both compounds are mainly excreted through this organ. Despite the lipophilic nature of the complex as obtained by log P determination (1.23), it is highly possible that the kidney excretion can be the result of the second metabolite formation leading to more water soluble species. Osmium cation is filtered through the glumeroles as a small cation, while in case of the complex the water solubility is a major cause and the possibility of formation of ionic complex is possible as shown in Figure 2.

Thyroid uptake is mainly different in both species, the osmium in the form of $^{191}OsCl_6^-$ or possible hydrolyzed forms is taken up in thyroid possibly due to the resemblance to TcO_4^- anion, as described above, however, the thyroid uptake is totally insignificant at all time intervals.

As an anion, $OsCl_6$ or the hydrolyzed products , the bone uptake at the hydroxyl apatite structure is observed which is over 1% even after 72 h, however, as a possible cationic water soluble complex, ^{191}Os-APMTS, shows no significant bone uptake.

In contrast with other radiolabeled APMTS complexes, [^{67}Ga]-APMTS has been prepared and reported as a possible tumor imaging agent as described earlier, this complex also demonstrates the same behavior in the excretion modes as well as biodistribution. Like ^{191}Os-APMTS, [^{67}Ga]-APMTS demosnstrates either kidney as well as liver uptake which is possibly due to the dual excretion route (2h; liver uptake; 25% and kidney uptake; 21%) [11].

CONCLUSION

Total labeling and formulation of ^{191}Os-APMTS took about 12h, with a radiochemical purity of higher than 95%. The radiolabeled complex was stable in

aqueous solutions for at least 2 days. Trace amounts of ^{191}Os cation were detected by ITLC indicating that radiochemical purity of the ^{191}Os-APMTS was higher than 95%. The biodistribution of the tracer in wild-type rats demonstrated that the major route of excretion is a urinary tract. Comparison of vital organs uptake for ^{191}Os-K_2OsCl_6 and ^{191}Os-APMTS demonstrate kinetic pattern difference for both species. ^{191}Os-APMTS can be a potential in vivo generator for therapy because of beta emission (313 keV), and also, for localization and dosimetry study in relevant organs by gamma emission of the daughter radionuclide. Our experiments have shown satisfactory quality, and stability suitable for future studies. Further investigations concerning tumor models and trapping mechanisms are required.

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