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Pre-Study on Radiolabeling of Colistin with Lutetium-177 to Develop Theranostic Infection Agent

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ABSTRACT: Infection is one of the important burdens on the health care system, not only due to leading morbidity and mortality but also because of the development of antibiotic resistance. Although the infection can be diagnosed by imaging techniques, more effective agents including radiopharmaceuticals may be required to image deep-seated infections. Imaging also plays a critical role in the choosing of optimum treatment options and following treatment. Theranostic agents offer many advantages such as monitoring the biodistribution and targeting of therapeutic agents, as well as rapid diagnosis and treatment. Colistin, a cationic peptide, leads to bacterial death through interaction with lipopolysaccharides in the cell wall of bacteria. In our study, ^{177}Lu as radionuclide part and colistimethate sodium (a prodrug of colistin, CMS) as pharmaceutic part were chosen to prepare a radiopharmaceutical for imaging and treatment of infections. ^{177}Lu-CMS complex was formed under room condition, and radiolabeling efficiency was determined by paper and high-performance liquid chromatography. The effect of the filtration process on radiolabeling was evaluated among the labeling efficiencies of filtered and un-filtered complexes. The different incubation times (5, 30, and 60 min) effect on the radiolabeling process was also evaluated. Moreover, in vitro stability of ^{177}Lu-CMS complex in saline solution was assessed during 7 days. According to the results, desired radiolabeling efficiency was not obtained under tested conditions and stability studies. Therefore, various modifications such as the addition of chelating agents or stabilizers in the radiolabeling procedure should be made to increase the radiolabeling stability. Further studies regarding radiolabeling are surely needed, and our studies are continuing.

KEYWORDS: Lutetium-177; colistimethate sodium; infection; theranostic; radiolabeling.

1. INTRODUCTION

Infection is one of the important burdens on the health care system due to leading morbidity and mortality [1]. In addition, the use of over-dose or sub-optimal dose antibiotics in the treatment leads to the development of antibiotic resistance, side effects, or progression of infection [2]. Therefore, effective and early detection of infection foci in the body plays an important role for choosing the correct treatment option, obtaining a complete treatment response in acute stages, and preventing the disease progression.

Although biochemical tests or symptomatic evaluation of patients can be used in the diagnosis of infection, these are fall short of when it comes to early and accurate diagnosis of deep-seated infection. Imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), single-photon emission tomography (SPECT), and positron emission tomography (PET) are used in clinics as noninvasive techniques. Among these modalities, nuclear imaging techniques (SPECT, PET, and gamma camera) are advantageous owing to their providing physiological and functional three-dimensional whole-body images [3]. Although morphological and functional imaging techniques are used in radiology and nuclear medicine clinics, more sensitive diagnostic agents are required to obtain more accurate images and to distinguish infection cases from other pathological conditions such as inflammation or cancer.

The ideal radiopharmaceutical for infection imaging should be cheap, accessible, non-immunogenic, sensitive, and specific for infection foci [4]. The radioactivity ratio of the infected tissues to the blood, and the amount of radiopharmaceutical in the target area should be high. Moreover, radiopharmaceuticals should

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remain until obtaining the images. It should differentiate the infected tissue from non-microbial inflammation lesions [5]. The radiolabeling procedure should be easy, and the radiation exposure of patients should be low to minimize the ionizing effect of radiation on healthy cells [6]. Therefore, many studies in the literature regarding radiolabeled peptides, antibodies, antibiotics with different gamma or positron emitter radioisotopes were performed to develop an ideal radiopharmaceutical for infection imaging. To diagnosis of infection by SPECT/CT or gamma camera imaging, many radiopharmaceuticals such as ^{67}Ga-citrate, ^{111}In-oxine- or ^{99m}Tc-hexamethyl propylene amine oxime (HMPAO)- labeled leukocytes, ^{99m}Tc-besilesomab, and ^{99m}Tc-labeled ciprofloxacin were used [7-9]. In addition, the infection imaging ability of PET radiopharmaceuticals including ^{18}F-fluorodeoxyglucose (^{18}F-FDG), ^{68}Ga-citrate, and ^{68}Ga-apo-transferrin were evaluated in the literature [10-12].

The theranostic term can be defined as the combination of therapeutic and diagnostic approaches. This approach is attractive for personalized medicine owing to its ability to screen the pharmacokinetic behavior, biodistribution, targeting, and treatment efficiency of the therapeutic agent [13]. Lutetium-177 (^{177}Lu) is an important theranostic radionuclide due to its gamma photons with 208 keV and 113 keV (for diagnosis) and beta particulate with 497 keV (for therapy) emissions. The advantages of ^{177}Lu including its production in large quantities, and long physical half-lives ($T_{1/2}$=6.71 days) provide its use in distant sites from the reactor [14]. Therefore, the use of ^{177}Lu has been increasing day by day to develop new radiopharmaceuticals for imaging and/or therapy of different diseases including infection. Thanks to the +3 oxidation state of ^{177}Lu, different types of molecules including peptides, antibodies, calcium minerals were radiolabeled with ^{177}Lu for bone pain palliation therapy, tumor treatment, and synovectomy of inflamed joints [15, 16]. In addition, different types of antibiotics such as benzylpenicillin, kanamycin, and sulfadiazine were radiolabeled with ^{177}Lu to treat and image bacterial infections [17-19].

Colistimethate sodium (CMS), a prodrug of colistin, is activated to colistin by hydrolysis. CMS, a polypeptide cationic antibiotic, increases the bacterial membrane permeability and leds to bacterial death by the interaction with anionic lipopolysaccharides on the outer membrane of the gram-negative bacterial cells [20]. It is approved for many types of gram-negative bacteria infections such as skin infection and endocarditis. CMS showed a considerably toxic profile in multi-drug resistance gram-negative bacteria infections in the literature [21].

Taking these facts into consideration, in this study, the radiolabeling procedure of CMS with ^{177}Lu was assessed to develop theranostic radiopharmaceuticals for imaging and treatment of gram-negative bacterial infections. This study is significant in that it's the first study in the literature regarding radiolabeling, the evaluation of radiolabeling efficiency, and the stability of ^{177}Lu-CMS.

2. RESULTS

2.1. FTIR spectra of CMS

The broad hydrogen bonds of CMS were observed between 4000 and 2000 cm^{-1} of spectra given in Figure 1. In addition, the vibration of the methane sulfonate group in CMS, and its bond to colistin was determined in 1026 and 1175 cm^{-1} of spectra, respectively.

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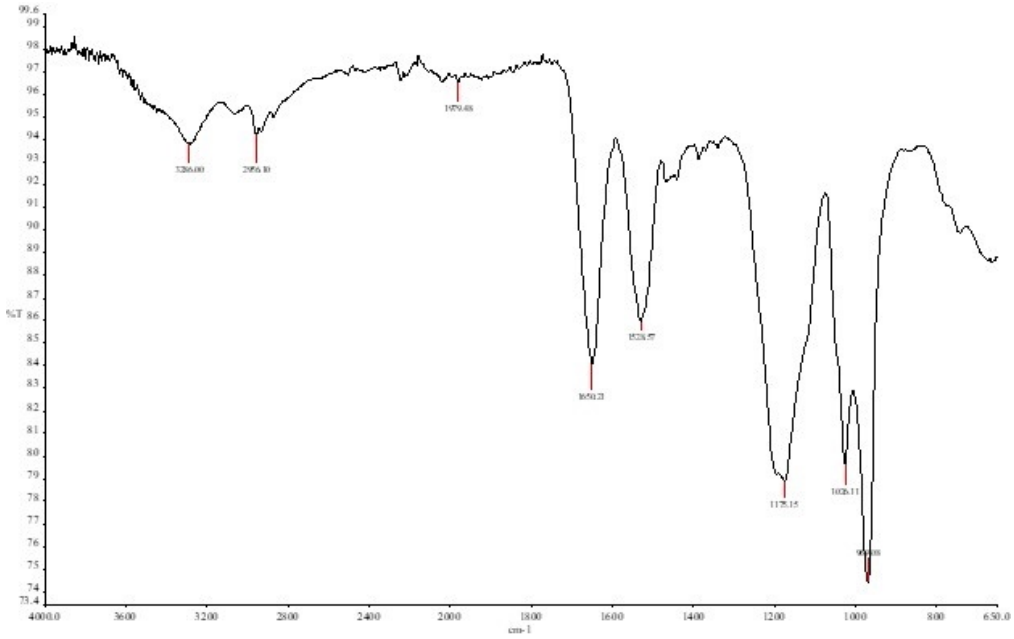

Figure 1. FTIR spectra of CMS.

2.2. Radiolabeling Study

The radiolabeling of CMS with ^{177}Lu was realized. The percentages of radiochemical purity (RCP %) of ^{177}Lu-CMS complexes were calculated by using different mobile and stationary phases (Table 1).

Table 1. The mobile and stationary phases, and the locations of free ^{177}Lu and ^{177}Lu-CMS.

Mobile phases	Stationary phases	Free ^{177}Lu	^{177}Lu-CMS
Ammonium hydroxide: methanol: water solution (1:20:20 w:v:v)	Whatman 3MM	Origin	Front (Rf= 0.8)
Ammonium acetate: methanol solution (1:1 v:v)	Whatman 3MM	Origin	Front (Rf= 0.8)
Diethylenetriamine-pentaacetic acid (DTPA) solution (10 mM)	ITLC-SG	Front (Rf = 0.8)	Origin
Ethylenediamine-tetraacetic acid (EDTA) solution (50 mM)	ITLC-SG	Front (Rf = 1.0)	Origin
Mobile phases	Stationary phases	Free ^{177}Lu	^{177}Lu-CMS
Ammonium hydroxide: methanol: water solution (1:20:20 v:v)	Whatman 3MM	Origin	Front (Rf= 0.8)
Ammonium acetate: methanol solution (1:1 v:v)	Whatman 3MM	Origin	Front (Rf= 0.8)

The amounts of radioactive impurities were separated and quantified by using two different solvent systems. The RCP of the complex was examined at 5, 30, and 60 min to evaluate the effect of incubation time on the radiolabeling process at the same time. The radioactivity percentage of origin and front parts were given in Table 2 for all phases and different incubation times.

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Table 2. The radioactivity percentages (mean ± standard deviations) of origin and front parts for ^{177}Lu-CMS complex in different phases and incubation times.

Mobile phases	Ammonium hydroxide: methanol: water solution (1:20:20 w:v:v)			Ammonium acetate: methanol solution (1:1 v:v)			DTPA solution (10 mM)			EDTA solution (50 mM)		
Incubation time (min)	5	30	60	5	30	60	5	30	60	5	30	60
Origin	0.18 ± 0.11	0.93 ± 0.39	31.40 ± 1.10	0.22 ± 0.14	4.50 ± 1.60	16.3 ± 7.10	0.15 ± 0.07	5.10 ± 0.85	17.80 ± 7.90	5.00 ± 0.70	4.50 ± 1.50	11.6 ± 1.90
Front	33.90 ± 2.01	37.30 ± 4.30	11.00 ± 1.60	38.10 ± 4.32	24.30 ± 1.30	11.50 ± 1.30	8.80 ± 0.35	12.80 ± 2.30	5.90 ± 0.57	5.80 ± 0.99	12.60 ± 2.30	11.40 ± 1.10

The percentages of RCP for ^{177}Lu-CMS complex were given in Table 3. According to results, free ^{177}Lu migrated forward with DTPA and EDTA solutions as solvents, while ^{177}Lu-CMS remained at the spotting point in ammonium hydroxide: methanol: water (1:20:20 w:v:v) and ammonium acetate: methanol solutions (1:1 v:v) as the other solvents. Stationary phases were chosen as Whatman 3MM and Instant thin-layer chromatography silica gel (ITLC-SG) for mobile phases. Furthermore, RCP of ^{177}Lu-CMS was changed between sampling time intervals ($p < 0.05$), and the highest RCP was obtained after 60 min of incubation time. As a result, the incubation time was selected as 60 min for the radiolabeling process, however unfortunately, over 90% RCP was not achieved for ^{177}Lu-CMS (Table 3).

Table 3. The percentages of RCP of ^{177}Lu-CMS complex in different phases and incubation times.

Mobile phases	Ammonium hydroxide: methanol: water solution (1:20:20 v:v) and DTPA solution (10 mM)			Ammonium acetate: methanol solution (1:1 v:v) and EDTA solution (50 mM)		
Incubation times (min)	5	30	60	5	30	60
RCP % of ^{177}Lu-CMS complex	57.63 ± 14.40	55.58 ± 3.62	72.72 ± 2.58	49.90 ± 16.46	71.62 ± 0.56	73.27 ± 6.29

The RCP % of ^{177}Lu-CMS was found statistically higher in ammonium acetate: methanol (1:1) and EDTA solutions (50 mM) eluting system compared to ammonium hydroxide: methanol: water (1:20:20) and DTPA solution (10 mM) at 30 and 60 min of incubation times ($p < 0.05$). The reason for this difference may be the higher affinity of ^{177}Lu to different eluting systems than that of CMS. Therefore, ammonium acetate: methanol (1:1) and EDTA (50 mM) solutions were chosen as the mobile phase for further studies.

The effect of filtration on the radiolabeling process was evaluated with filtered and un-filtered complexes for 7 days. The RCP % of ^{177}Lu-CMS was shown in Figure 2. According to the results, a statistically significant difference was observed among the labeling efficiencies of filtered and un-filtered complexes ($p < 0.05$) during 7 days. Although un-filtered ^{177}Lu-CMS complexes exhibited higher RCP % than filtered ^{177}Lu-CMS for 1, 4, and 7 days, the radiolabeling stability decreased at these times when compared to beginning values. The RCP % of un-filtered and filtered ^{177}Lu-CMS was found lower than 90 %. These results have been revealed that

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desired radiolabeling efficiency was not achieved for ^{177}Lu-CMS. The pH values of the filtered and un-filtered radiolabeled complex were appraised with litmus papers, and the pH values of both solutions were found to be 4.

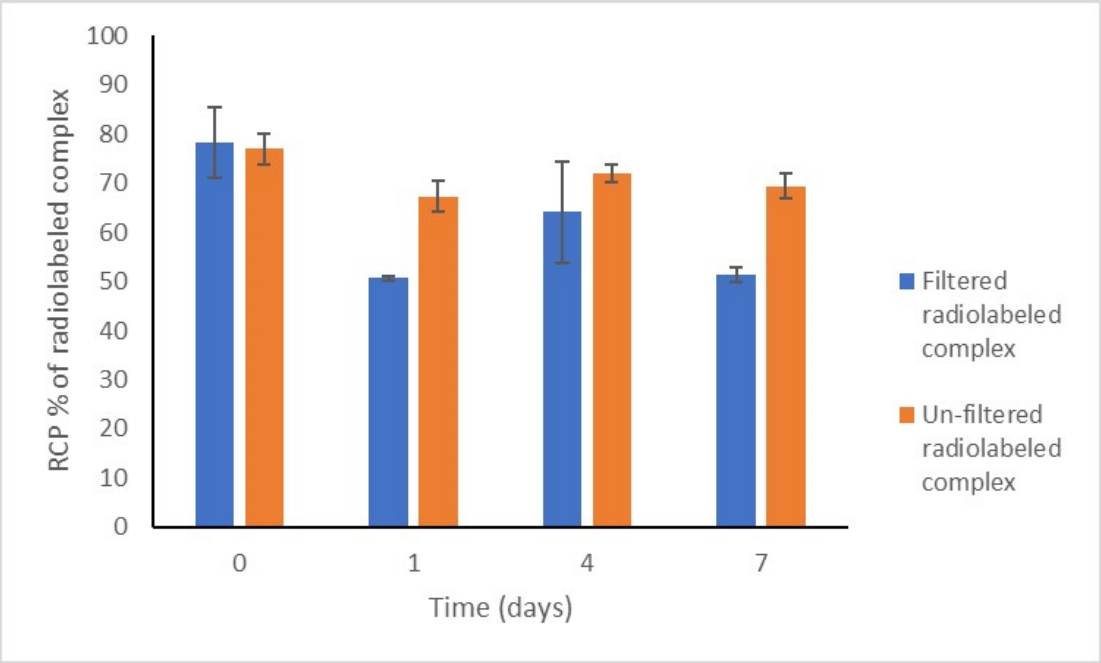

Figure 2. RCP % of filtered and un-filtered radiolabeled complex.

2.3. In vitro radiolabeling stability of ^{177}Lu-CMS

The stability of the radiolabeled complex in saline after storage at room temperature was assessed by measuring radioactivity in the dose calibrator and HPLC system for 7 days. Obtaining in vitro stability results of ^{177}Lu-CMS by using dose calibrator are shown in Figure 3. According to these measurements, ^{177}Lu-CMS had less than 90% of RCP after 7 days, confirming that metal didn't remain enough bound to CMS. A statistical difference was found between in vitro stability of the radiolabeled complex in saline solution for 0, 1 and, 7 days of storage at room temperature ($p<0.05$).

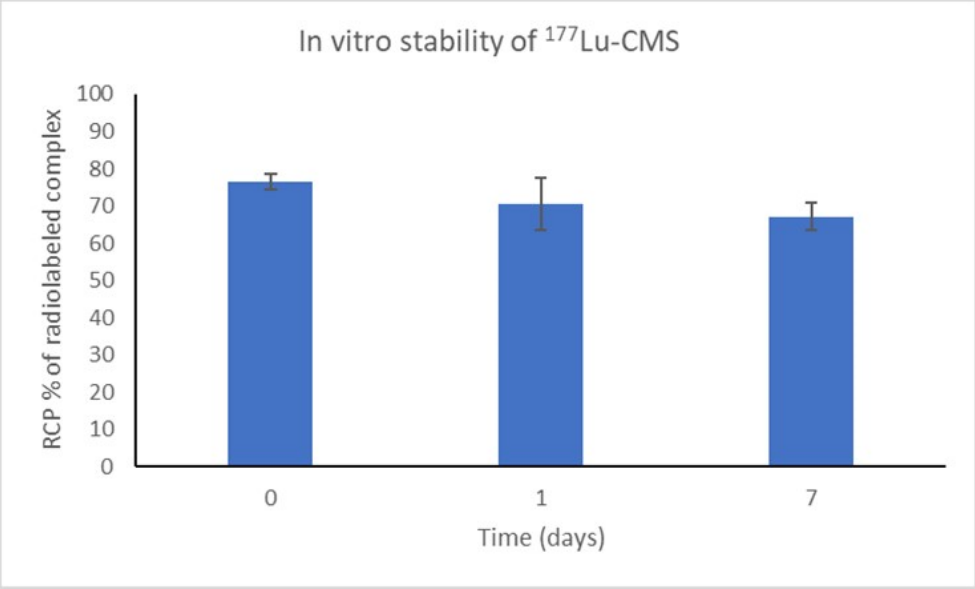

Figure 3. In vitro stability of 177Lu-CMS.

The HPLC chromatograms of free ^{177}Lu and ^{177}Lu-CMS were indicated in Figure 4. The first chromatogram obtained from free the ^{177}Lu sample (Figure 4A) displayed one peak, with 2.41 minutes of retention time. The second chromatogram obtained from a ^{177}Lu-CMS sample (Figure 4B) displayed more than one peaks, with 9.98 minutes of retention time. Furthermore, the calculated RCP % of ^{177}Lu-CMS from HPLC chromatogram was demonstrated in Table 4. ^{177}Lu-CMS also had less than 90% of RCP after 7 days, confirming that metal did not remain enough bound to CMS. The HPLC and dose calibrator measurements were found to be coherent with each other.

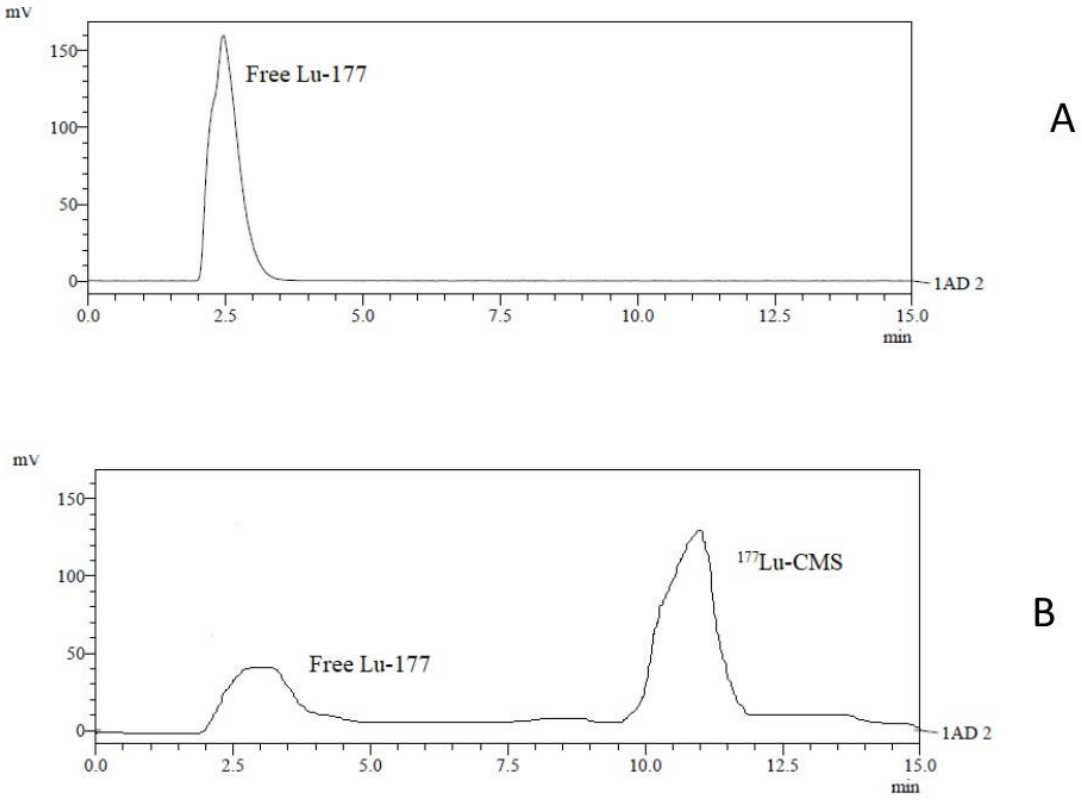

Figure 4. HPLC chromatograms of free ^{177}Lu (A) and ^{177}Lu-CMS (B).

Table 4. RCP % of ^{177}Lu-CMS from HPLC chromatogram

Time (days)	RCP % of ^{177}Lu-CMS±SD
0	75.25 ± 3.43
1	70.12 ± 4.02
7	68.78 ± 4.32

3. DISCUSSION

Infection can be an important burden for health systems if not diagnosed and treated on time. Although different methods including imaging techniques can be used in the diagnosis of infection, more effective imaging agents are required to image deep organs and obtain accurate images. Nuclear medicine techniques provide therapy and dose adjustment for infected organs in the body in addition to imaging. Although many ^{99m}Tc radiopharmaceuticals are commercially available in nuclear medicine clinics, studies using different radionuclides were made to develop new and disease-specific radiopharmaceuticals in the literature [22, 23].

Therefore, in our study ^{177}Lu as radionuclide part and CMS as pharmaceutic part was chosen to prepare a radiopharmaceutical for infection imaging and treatment. CMS was successfully identified by the FTIR analysis. The observed characteristic peaks of the methane sulfonate group in CMS were found in similar ranges of spectra with the previous study in the literature [24].

Radiopharmaceuticals should be sterile because of their parenteral use. The sterilization procedure of radiopharmaceuticals may be made by filtration before application [25]. The pH value of the reaction mixture directly affects the radiolabeling efficiency. In the literature, the optimal pH was reported as 4 for radiolabeling of different molecules with ^{177}Lu, and the pH of ^{177}Lu-CMS complexes was found to be 4 in our study [17, 26, 27]. In addition, no differences were detected between pH values of filtered and un-filtered ^{177}Lu-CMS complexes. As expected, no negative effect of filtration was observed for pH values.

In ammonium hydroxide: methanol: water (1:20:20), and ammonium acetate: methanol (1:1) eluting systems, ^{177}Lu-CMS complex eluted with the mobile phase (Rf =0,8), while free ^{177}Lu remained at spotting point (Rf = 0). In EDTA solution (50 mM) as eluting systems, free ^{177}Lu eluted with the mobile phase (Rf=1) due to the presence of complex formation between CMS and ^{177}Lu. In addition, this complexation led to obtaining low RCP values for the ^{177}Lu-CMS solution. According to the results obtained from a study performed by Yousefnia et al., a similar complex formation between $^{177}LuCl_3$ and DTPA solution was reported. They also reported that the Rf value of free $^{177}Lu^{+3}$ was determined as 0.7 in the DTPA solution elution system, while free $^{177}Lu^{+3}$ was remained at Rf 0 in ammonium acetate: methanol (1:1) [28]. In the studies using ammonium acetate: methanol as mobile phase in paper chromatography, the Rf value was determined as 0 for free $^{177}Lu^{+3}$ [18, 27]. EDTA solution was used as mobile phase to evaluate the radiolabeling efficiency of ^{177}Lu-sulfadiazine complex, and impurities moved with the solvent front [19]. All these findings are similar to our results.

The radiolabeling efficiency is the critical parameter in radiopharmaceutical development to determine the impurities and quantity of radiolabeled compounds. In ^{177}Lu radiopharmaceuticals, the radiolabeling efficiency is calculated by the evaluation of free $^{177}Lu^{+3}$ quantity because it is the main radiochemical impurity [29]. In the quality control study using ammonium acetate: methanol and EDTA as mobile phases, the radiolabeling efficiency for ^{177}Lu-CMS complexes was found to be 75-50 %, which is similar to the literature [30]. Yousefnia et al. was also reported this mobile phase and the RCP % was found lower due to possible complexation between free $^{177}Lu^{+3}$ ions and chelating agents by using DTPA and EDTA solutions [28]. Therefore, it can be concluded that ammonium acetate: methanol and EDTA solutions are more proper compared to ammonium hydroxide: methanol: water and DTPA solutions in the evaluation of ^{177}Lu-CMS radiolabeling efficiency.

The conjugation between radionuclide and pharmaceutical part should be stable until the reaching of the radiopharmaceutical to the targeted area and obtaining image or treatment response. Hence, radiolabeling stability is one of the critical issues in radiopharmaceutical development [31]. Unfortunately, the radiolabeling of ^{177}Lu-CMS complexes was found as unstable in our paper chromatography study performed after 30 min. Although on the 7th day RCP % values were found lower than 90% in both paper chromatography and HPLC, it is not an indicator for stabile radiolabeling. Therefore, some differences such as the addition of chelating agents in the radiolabeling procedure should be made to obtain stabile ^{177}Lu-CMS complex. In the literature, stabile radiolabeled complexes with ^{177}Lu were obtained by using various bifunctional chelating agents such as DTPA, 1,4,7,10-tetraazacyclododecan-N,N´,N´´,N´´´-tetraacetic acid (DOTA), 5p-C- NETA [32, 33].

The results of the study in which evaluated the effect of incubation time on radiolabeling efficiency were shown in Table 3. Statistically important increases were observed in the RCP % of the radiolabeled complex when the incubation time was adjusted to 60 min ($p < 0.05$). Hence, 60 min was chosen as the optimum incubation time for the preparation of the ^{177}Lu-CMS complex. This finding is in agreement with the incubation time of a ^{177}Lu labeling study in the literature [34].

The separation of the pharmaceutical part from the radionuclide part causes the failure of imaging or treatment of target tissue in radiopharmaceuticals. Hence, radiolabeling stability is critical for obtaining accurate imaging and effective treatment. The in vitro radiolabeling stabilities of complexes in saline are shown in Table 4. ^{177}Lu-CMS exhibited low stability in terms of their radiolabeling efficiencies, which were below 90 % up to 7 days. These results are in agreement with previous studies performed for radiolabeled anticancer drug-chelating agents [35, 36].

4. CONCLUSION

Infection is one of the critical diseases around the world not only due to infection-related deaths but also because of antibiotic resistance development. Although the diagnosis of infection can be performed by mini-invasive techniques such as biopsy or non-invasive techniques consisting of various imaging techniques, they are inadequate to obtain sensitive and specific images from deep-seated infected organs. Therefore, more specific, and effective imaging agents are required to diagnose infection at molecular and early stages. Theranostics offer various advantages including monitoring the biodistribution and targeting of therapeutic agents, detection of efficacy and pharmacokinetic behavior of the therapeutic agent, and rapid diagnosis and treatment due to consisting of the therapeutic and diagnostic agents at the same system.

In this study, a radiopharmaceutical was developed for infection imaging and treatment using ^{177}Lu as radionuclide part and CMS as pharmaceutic one. The effect of filtration and different incubation times on radiolabeling efficiency was evaluated by paper chromatography and ITLC-SG after the radiolabeling procedure. A significant difference was detected among RCP % values of filtered and un-filtered radiolabeled complexes. In the radiolabeling efficiency study, different mobile and stationary phases were evaluated to optimize the protocol. According to the results of paper chromatography using ammonium acetate: methanol solution (1:1 w:v:v) and EDTA (50 mM) solutions eluting system, better radiolabeling efficiencies for both filtered and un-filtered radiolabeled complexes were obtained. Moreover, results of the radiolabeling stability showed that the better RCP % values were obtained after 60 min of incubation time but desired RCP % values were not acquired under tested conditions.

In conclusion, although the radiolabeling of CMS was tried with ^{177}Lu, further studies regarding the modification in the radiolabeling procedure are surely needed to obtain stable radiolabeling.

5. MATERIALS AND METHODS

5.1. Materials

CMS was a generous gift of Polifarma (Turkey). $^{177}LuCl_3$ was taken from Eczacibasi-Monrol Nuclear Products. DTPA, EDTA, ammonium hydroxide, and ammonium acetate were obtained from Sigma-Aldrich. Acetonitrile and chromatography papers were purchased from Merck and Agilent, respectively. All other reagents were of analytical grade.

5.2. Fourier transform infrared (FTIR) analysis of CMS

FTIR spectrum of CMS was collected using a potassium bromide tablet in the 4000 to 650 cm^{-1} range by a spectrometer (Perkin Elmer Spectrum 100 FT-IR).

5.3. Radiolabeling of CMS with ^{177}Lu

In the radiolabeling procedure of CMS with ^{177}Lu, various parameters such as incubation time and filtration were tested to obtain a high percentage of radiochemical purity/radiolabeling efficiency (RCP %).

In the radiolabeling study, 4 mL of the CMS physiological saline solution with 2.5 $mg.mL^{-1}$ concentration was radiolabeled by using 1 mL of $^{177}LuCl_3$ solution with 10 mCi radioactivity. The mixture was incubated for 5, 30, and 60 min at room temperature. After the end of incubation periods, RCP % was calculated by using paper chromatography and dose calibrator.

To evaluate the filtration effect on radiolabeling efficiency of the ^{177}Lu-CMS complex, the radiolabeled complex was prepared and separated into two samples. One of them was filtered through a cellulose nitrate membrane filter having 0.22-µm pore size. After this process, RCP % was calculated by using paper chromatography and dose calibrator for 7 days.

5.4. Evaluation of radiolabeling efficiency of ^{177}Lu-CMS

To determine the radiolabeling efficiency of ^{177}Lu-CMS, RCP % was calculated by dose calibrator (Biodex Atomlab). Different stationary and mobile phases were tested. DTPA and EDTA solutions were used to detect the radioactivity of free ^{177}Lu, while the radioactivity of ^{177}Lu-CMS complex was measured by using ammonium hydroxide: methanol: water, and ammonium acetate: methanol solutions. After the applying of ^{177}Lu-CMS complexes at the origin of stationary phases, the RCP of ^{177}Lu-CMS complex was calculated by using the following equation:

$$RCP(\%) = \frac{Lu-177-CMS\ radioactivity}{(Lu-177-CMS\ radioacitivty)+(Free\ Lu-177\ radioactivity)} \times 100$$

Furthermore, the RCP % of ^{177}Lu-CMS complexes was assessed by Radio-HPLC for in vitro stability studies. ^{177}Lu-CMS complexes were injected into the HPLC system equipped with a C18 (Inerstsil ODS-3 GL Science 150 × 3 mm) column, the photodiode array (PDA), and NaI Gamma detectors, and LC Solution software data analyzer (Shimadzu SCL-10A). The injection volume was 10 µL. Elution was obtained by using the following gradient steps of solvents A (ultrapure water) and B (acetonitrile): 90:10 (A:B) for 5 min, then 80:20 for 5 min, then 95:5 for 5 min, and then 90:10 for 5 min at a flow rate of 50 µL.min^{-1}. Analyses were carried out at room temperature.

5.5. In vitro radiolabeling stability of ^{177}Lu-CMS

The radiolabeling stability of ^{177}Lu-CMS was evaluated at room temperature. For this purpose, the un-filtered radiolabeled formulation was prepared. 200 µL of the radiolabeled formulations were kept in 800 µL saline for 7 days, and RCP % of formulations were examined at 0, 1, and 7 days by paper chromatography and Radio-HPLC analyses. Therefore, the RCP % values were compared with the findings in paper chromatography and Radio-HPLC analyses.

5.6. Statistical Analysis

All experiments were performed in triplicates, and the differences in the same group were represented by standard deviation (SD). Statistical differences among groups were determined by Student's t test, and regarded as statistically significant when $p<0.05$ (*).

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