In house development of ^{99m}Tc-Rhenium sulfide colloidal nanoparticles for sentinel lymph node detection

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Abstract: In this study, rhenium sulfide colloidal nanoparticles were developed as radiopharmaceutical for sentinel lymph node detection. We directly used rhenium sulfide as a starting material for the preparation of colloidal nanoparticles. UV-visible spectrophotometry was used for characterization of in house developed colloidal particles. The size distribution of radioactive particles was studied by using membrane filtration method. The percentage of radiolabeled colloidal nanoparticles was determined by paper chromatography (PC). The study also includes *in vitro* stability, protein binding in human blood and bioevaluation in a rabbit model. The results indicate that 77.27 ± 3.26 % particles of size less than 20nm (suitable for lymphoscintigraphy) were radiolabeled. ^{99m}Tc labeled rhenium sulfide labeling efficacy with the radiometal is 98.5 ± 0.5%, which remains considerably stable beyond 5h at room temperature. Furthermore, it was observed that 70.2 ± 1.3 % radiolabeled colloid complex showed binding with the blood protein. Bioevaluation results show the remarkable achievement of our radiopharmaceutical. The in house prepared ^{99m}Tc labeled rhenium sulfide colloidal nanoparticles reached the sentinel node within 15 min of post injection. These results indicate that ^{99m}Tc labeled rhenium sulfide colloid nanoparticles kit produced by a novel procedure seems of significant potential as a feasible candidate for further development to be used in clinical practice.

Keywords: Rhenium sulfide, colloids, nanoparticles, radiolabeling, biodistribution, sentinel node.

INTRODUCTION

In 1997, Cabanas introduced the concept of Sentinel Lymph Node is the first node that receives lymphatic drainage from the tumor (Cabanas, 1992). Biopsy of sentinel lymph node is gradually replacing dissection of lymph node in breast cancer patients (Krag *et al*., 1998). Radioisotopic detection of the sentinel lymph node and absence of its metastasis invasion should theoretically be predictive of total drainage of the tumor (Benateau *et al*., 2005). Several radiocolloidal nanoparticles such as ^{99m}Tc-sulphur colloids, ^{99m}Tc-antimonysulfide, and ^{99m}Tc-dextran have been evaluated for sentinel node detection (Yokoyama *et al*., 1990). Colloidal nanoparticles are significantly important as drug carriers (Yokoyama *et al*., 1990; Kreuter *et al*., 1991), as carrier for gamma emitters for scintigraphic imaging of phagocytic cell distribution (in spleen, liver, bone marrow and abscesses), as MRI contrast agents (Bulte *et al*., 1996; Babes et al., 1999), and as lymphoscintigraphic agent for sentinel lymph node detection and for radiotherapeutic applications (Tiefenauer *et al*., 1993; Pouliquen *et al*., 1993; Pouliquen *et al*., 1992; Chouly *et al*., 1996; Denizot *et al*., 1999).

Venkatesan and his co-workers (Tu *et al*., 2007) synthesized rhenium sulfide colloidal nanoparticles having size distribution of range 1-10 μm and applied them in radiation synovectomy, but it could not be applied as a carrier of rhenium activity as intravenous injection itself because such application needs the particle size less than 50 nm. Wang et al introduced the concept of solvent stabilization. Solvent stabilization is an efficient way for development of colloidal nanoparticles, by providing stability to "bare" surface newly synthesized small sized colloidal nanoparticles (Wang et al., 2000). The bare surface helps in further modification of nanoparticle's surface. The concept of solvent stabilization method was further developed by Weixia Tu *et al*. (2007), they synthesized rhenium sulfide nanoparticles by using rhenium precursors as starting material and ethylene glycol as stabilizer and solvent. They have reported the successful synthesis of rhenium sulfide nanoparticles of average diameter 5.5 nm but did not report any development of radiopharmaceutical kit for diagnosis of malignant tumors.

In this work, we have described the synthesis of ^{99m}Tc labeled rhenium sulfide colloidal nanoparticles by using Re_2S_7, SnF_2 and ethylene glycol. The rhenium sulfide based nanoparticles were used to develop a radiopharmaceutical kit and tested in a rabbit model for sentinel lymph node imaging. During animal study, the newly-produced colloidal nanoparticles reached the target within 15 min after intradermal injection in left rear foot

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pad which could be easily detected in static views till 5h p.i.

MATERIALS AND METHODS

Materials

Rhenium sulfide and SnF_2 were purchased from Aldrich, USA. Acetone was purchased from Fisher Scientific, UK. $Na^{99m}TcO_4$ generator was taken from Pakistan Institute of Nuclear Science and Technology (PINSTECH), Islamabad, Pakistan. Saline was purchased from Ostuka, Pakistan. UV-visible spectra were developed on UVD-3500 UV-Visible double beam Spectrophotometer Labomed, Inc. Spectra were recorded through dual head SPECT gamma camera. ICON8.5 Macintosh System interfaced with the camera was used for data processing.

Methodology

Preparation of rhenium sulfide colloidal nanoparticles

20 mg L-cysteine hydrochloride monohydrate (L-cysteine.HCl.H_2O) and 10 mg rhenium sulfide was dissolved in 1 ml solution of 10N NaOH with stirring followed by addition of 18 ml of ethylene glycol (solvent) under vigorous agitation at room temperature. The solution was stirred continuously for 45 min. The pH of the freshly prepared colloids was adjusted at 7.4 by using 5N, 1N and 0.1N HCl. The resultant solution was passed through 0.22 µm membrane filter and 1 ml/ kit of this filtrate was dispensed in sterilized serum vials.

Preparation of pyrophosphate kit

200 mg anhydrous sodium pyrophosphate was dissolved in 19 ml of double distilled water. Solution was slightly warmed followed by addition of 80 mg of SnF_2 and 400 mg of D-manitol. The pH was adjusted at 6.0 - 6.2. The resultant solution was dispensed (1 ml/ vial) in sterilized serum vials by passing through 0.22 µm membrane filter.

UV-visible Spectrometry

UV-visible spectrometry is an effective and simple technique to study the metal species in the preparation of colloidal nanoparticles (Tu *et al*., 2007). The reaction for the preparation of rhenium sulfide colloidal nanoparticles was studied at various time intervals by using UV-visible spectrophotometry.

Membrane filtration

The filters of various pore sizes were pre-equilibrated in ethylene glycol for 15 min. 1 ml radiolabeled rhenium sulfide colloidal nanoparticles were withdrawn from the reaction vial and filtered through 220 nm Millipore filter having polyvinylidene difluoride composition. The filter was rinsed and both filter and filtrate were counted for radioactivity. The radiocolloid sample drawn from previous filtrate was again passed through 100 nm whatman filter with aluminium oxide composition. Filter and filtrate were counted for radioactivity. This procedure was repeated for 50 nm (Nucleopore polycarbonate) and 20 nm (whatman aluminium oxide) filters. All values were corrected for background activity.

Membrane adsorption study

The sterile filters of various membrane composition and size e.g., 220 nm (Millipore, polyvinylidene difluoride), 100 nm (whatman, aluminium oxide), 50 nm (Nucleopore, polycarbonate) and 20 nm (whatman, aluminium oxide) were used for membrane filtration. Each filter was broken in two equal pieces and membrane inside it was removed carefully, in order to avoid any contamination during handling. The membrane was cut into small pieces and separately placed in ^{99m}Tc-rhenium sulfide colloidal particles for 1h. The liquid was removed by syringe and membranes were rinsed with ethylene glycol. All rinses were combined with respective radioactive liquids. Both membranes and radioactive liquids were separately counted for radioactivity.

Radiolabeling and Quality control

1 ml from solution of rhenium sulfide colloidal nanoparticles was mixed with 250 µl pyrophosphate kit which was used as a reducing agent. The mixture was incubated at room temperature for 5 min. For radiolabeling, 10 mCi/ 0.5 ml $Na^{99m}TcO_4$ was added, heated the solution in boiling water bath for 30 min, cooled to room temperature and passed through 20 nm nucleopore polycarbonate membrane filter that was pre-equilibrated in ethylene glycol for 15 min.

Paper Chromatography (PC) was used to perform the quality control. To determine the amount of free pertechnetate in the reconstituted kit, acetone was used as a mobile phase on a chromatography paper (20 x 2 Whatman No. 3 MM). Small aliquots from the reconstituted kit were spotted on the respective strip. The strip, after elution, was cut in fractions of 1 cm and counted for radioactivity in a well type gamma scintillation counter.

Stability of ^{99m}Tc complex at room temperature

Stability of the in house developed ^{99m}Tc- rhenium sulfide kit was studied at time intervals of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5h at room temperature. Change in stability of the radiolabeled colloidal nanoparticles was analyzed at each time interval by paper chromatrography to detect any dissociation of the complex.

Protein binding

5 ml of fresh blood from a healthy volunteer was taken in a tube. A volume of 250 µl (3 mCi, 111 MBq) from kit was added in it and incubated at room temperature for 1h and then in water bath for 10 min, pre-set at 37^{o}C. The tube was centrifuged for 10 min at 3000 rpm, followed by separation of serum and blood cells in two different layers. The equal volume of 10% trichloroacetic acid

(TCA) was added into separated serum. TCA was used as a precipitating agent for the serum proteins. Tube containing serum and TCA solution was placed on the shaker for 10 min and then centrifuged at 3000 rpm for another 10 min. Residues were separated from supernatant and counted for radioactivity.

Bioevaluation

For evaluating potential of the newly-produced radiopharmaceutical as a feasible sentinel lymph node imaging agent, the acquisition study was carried out in healthy, New Zealand white rabbit models. The animals were originally obtained from a farm house and preserved in an animal house at INMOL Hospital, with a free access to green fodder and water. Ethical approval for the experiments was arranged according to Animal Ethics Guidelines of the Institute of Nuclear Medicine and Oncology (INMOL), Lahore. Images were taken at various time intervals up to 4h p.i. using a dual head SPECT gamma camera by applying a matrix size 256 x 256 and a zoom of 1. The imaging process was started by dynamic study of 15 min followed by static images at varying intervals of time. As an experimental procedure, 500 µCi/100 µl was injected intradermally to left rear foot pad (RFP) of the rabbit.

For evaluation of the uptake in nodes and its percentage of extraction, we performed dissection of rabbit. The dissection was performed under approved protocol by the ethical committee. Intramuscular injection of 200 µl ketamine and xylazine (2:1) was used as anesthesia. $^{99m}Tc(CO)_3$ rhenium sulfide colloidal nanoparticles (300 µl, 1 mCi) was injected intradermally in each rear foot pad. Both feet pads were massaged for 3 min after injection. 200 µl isosulfan blue dye was intradermally injected at 50 min p.i. in both feet pads, followed by dissection of animals at 10 min p.i. of blue dye. The nodes were carefully removed to measure the uptake of radioactivity and its extraction from the node. Each organ was separately counted for radioactivity. Fig. 1 shows the lymph node in dissected rabbit.

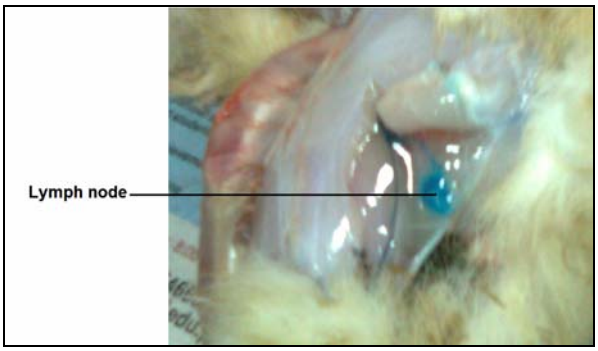

Fig. 1: Lymph node of dissected rabbit

The percentage (%ID) at site of injection, popliteal and iliac nodes has been calculated by the formula given below:

$\%ID = cpm_{tissue} \times 100 / (cpm_{standard} \times 10)$

Popliteal extraction (PE) was calculated as:

$PE = (\%ID_{popliteal} - \%ID_{iliac}) \times 100/(\%ID_{popliteal})$

Percentage ID/g of various organs was also calculated.

RESULTS

In house development of rhenium sulfide colloidal nanoparticles

Rhenium sulfide colloidal nanoparticles have been synthesized by direct use of rhenium sulfide and stabilizing them by ethylene glycol. As the finally formulated kit contains only 0.9ml ethylene glycol per 1.75ml kit so this tiny amount should not cause any side effect as reported lethal dose of ethylene glycol is 1.4ml/kg (Brent *et al.,* 2001). The surface of rhenium sulfide colloidal nanoparticles was modified by using thiol containing molecule. The surface active group directly chemisorbed on nanoparticle surface. L-cysteine hydrochloride monohydrate forms ester with ethylene glycol which peptized the rhenium sulfide colloidal nanoparticles. The L-cysteine ethyl ester modified nanoparticles provide a basis for further functionalization and surface modification with biocompatible bioactive molecules or layers (Nina *et al.,* 2007). By this procedure, we obtained stable colloidal solution of rhenium sulfide nanoparticles (fig. 2). Pyrophosphate kit was used as a reducing agent which reduced oxidation state of tehnitium-99m from +7 to +4 for radiolabeling of colloidal nanoparticles.

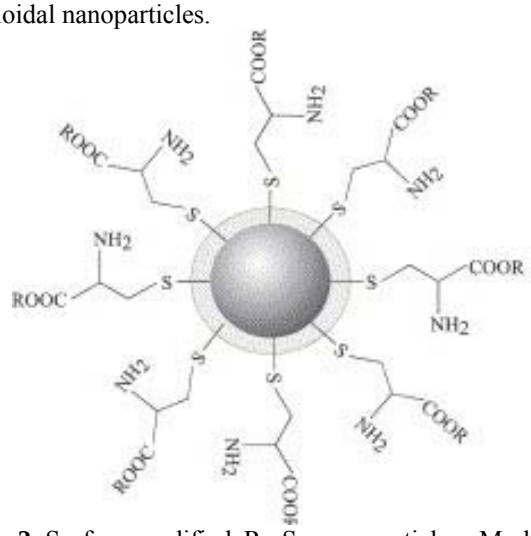

Fig. 2: Surface-modified Re_2S_7 nanoparticles: Modification of surface through surface active thiol group by using L-cysteine ethyl ester

UV-Visible spectrometry

Fig. 3 shows the absorption curve of the in house developed rhenium sulfide colloid nanoparticles at various time intervals of stirring. A uniform optical density of plasma absorbing colloid rhenium sulfide nanoparticles is seen in the curves that indicate the uniformity of size of the nanoparticles, which is the basic

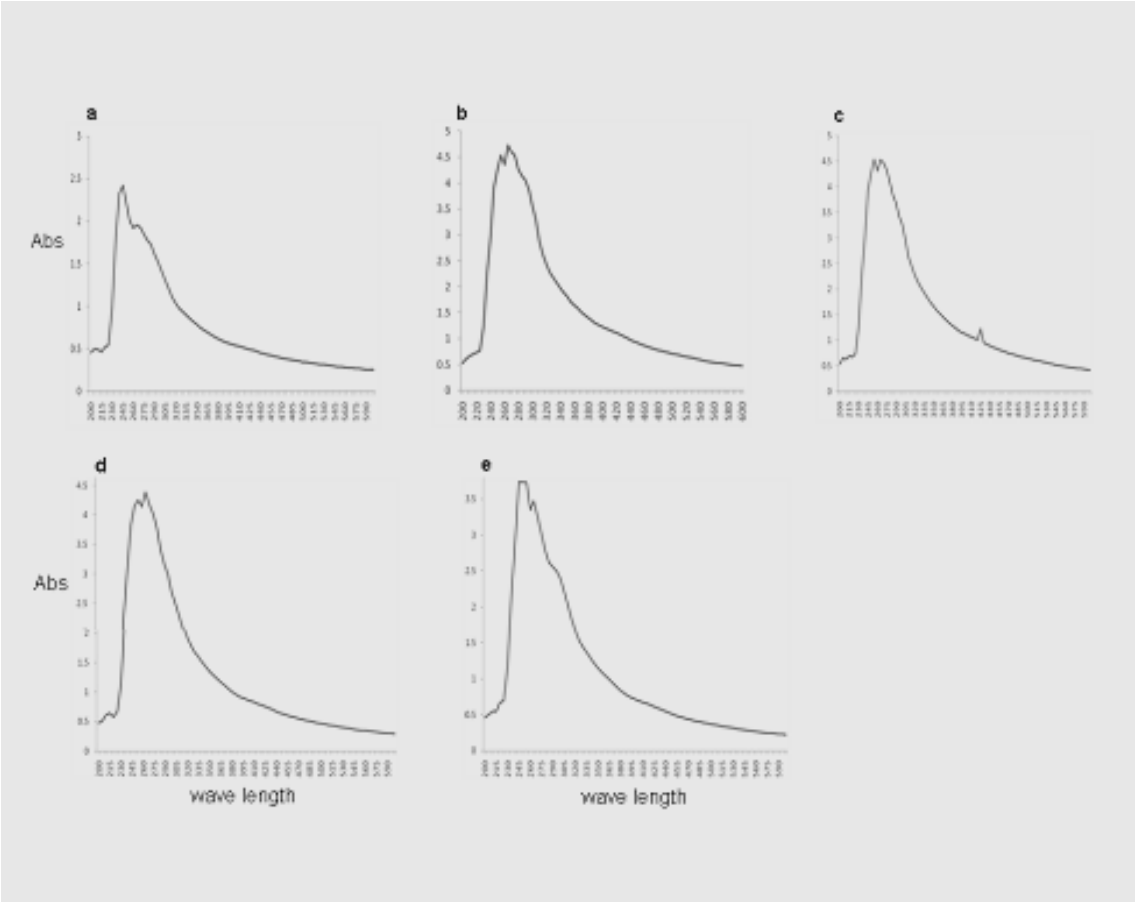

Fig. 3: UV-visible spectra of reactive solution during the formation of rhenium sulfide colloids at discrete intervals of stirring time, plotting between wavelength (X-axis) and absorption (Y-axis). The segments from 'a – e' show the absorbance of rhenium sulfide nanoparticles at 0, 15, 30, 45 and 60 min.

criterion to determine the uniformity of size of nanoparticles (Tu *et al*., 2007). The optical absorption appearing at 425 nm after 30 min stirring in ethylene glycol might be due to formation of some unknown intermediate product which disappears in 15 min. The curve at each time interval reveals that as the time is prolonged, optical density of rhenium sulfide colloid nanoparticles increases and absorption of metal species decreases. No change in optical density shows the uniform size of colloid nanoparticles (Tu *et al*., 2007).

Table 1: Radioactive particle size distribution

Particle size range (nm)	% Activity
>220	24.42 ± 3.46
220-100	29.98 ± 2.76
100-50	36.98 ± 4.65
50-20	48.57 ± 1.98
<20	77.27 ± 3.26

Membrane filtration and membrane adsorption study

The results in table 1 indicate percentage fraction of particles that were radiolabeled after passing through filters of various size ranges. The adsorption of ^{99m}Tc-rhenium sulfide on filter membranes of various compositions was studied to evaluate the fraction of non-adsorbed activity. Results are shown in table 2. None of the membranes significantly adsorbed ^{99m}Tc-rhenium sulfide colloidal particles.

Quality control

The ^{99m}Tc-labeled rhenium sulfide colloidal nanoparticles remained settled at the bottom (A) and free pertechnetate (B) moved with the solvent front. The percentage binding (%) of radiolabeled nanoparticles was then calculated as (A x 100)/(A + B)%. It was observed that 98.5 ± 0.5 % of the in house developed nanoparticles were radiolabeled.

Stability of radiolabeled nanoparticles at room temperature

Preliminary labeling of the in house developed nanoparticles with the radiometal was sufficiently stable under physiological conditions. The rhenium sulfide colloidal nanoparticles remained intact with the radiometal in colloidal form and no significant change was observed in the percent binding till 5h. It was observed that the labeling efficacy of newly-produced

Table 2: Membrane adsorption of ^{99m}Tc rhenium sulfide colloids

Filters			% Non-adsorbed Activity
Size (nm)	Type	Membrane Composition	^{99m}Tc-Rhenium Sulfide (%)
220	Millipore	Polyvinylidene difluoride	99.66 ± 0.22
100	Whatman	Aluminium oxide	98.21 ± 0.17
50	Nucleopore	polycarbonate	99.78 ± 0.15
20	Whatman	Aluminium oxide	98.98 ± 0.35

nanoparticles after 5h incubation at room temperature was 97.7 ± 0.5%. The high labeling efficacy of the radiopharmaceutical shows the validity of labeling technique with the radiometal. Data are shown in fig. 4.

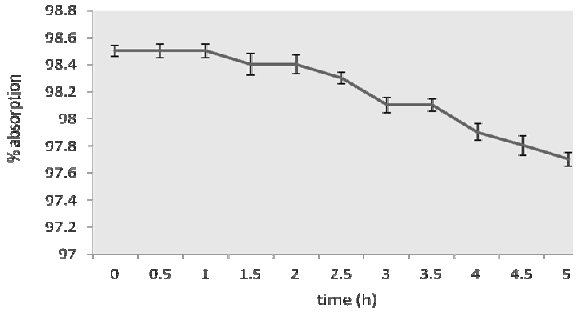

Fig. 4: Stability curve of newly-produced ^{99m}Tc-rhenium sulfide colloid nanoparticles.

Bioevaluation

The newly-produced ^{99m}Tc-labeled nanoparticles showed some retention at injection site, reached the sentinel lymph node within 15 min, retained there for more than 4h and then started clearing from the node (fig. 5).

Table 3: Biodistribution of ^{99m}Tc-labeled rhenium sulfide nanoparticles in various body organs

Organs	ID/g
Liver	2.80 ± 0.03
Heart	0.43 ± 0.01
muscle	0.36 ± 0.10
Spleen	0.11 ± 0.01
Lungs	0.22 ± 0.05
Bladder	11.15 ± 1.12
Kidneys	28.63 ± 1.21
Foot pad 1 (injection site)	33.21 ± 1.52
Foot pad 2 (injection site)	34.06 ± 2.12

The most probable route of excretion observed for this radiopharmaceutical was through kidney and bladder with no significant evidence of hepatobiliary excretion. These results indicate that ^{99m}Tc-nanoparticles kit seems of significant potential as feasible candidate for further development to be used in clinical practice. The rabbit were dissected and counted for percentage of injected dose (% I.D) of nodes and popliteal extraction. Table 3 shows the distribution of activity in various organs and table 4 show data of injected dose in popliteal and iliac node along with popliteal extraction.

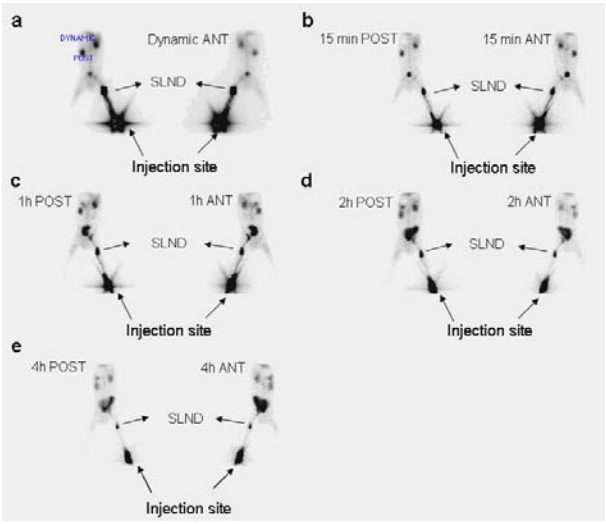

Fig. 5: Bioevaluation in rabbit by using newly-produced ^{99m}Tc-nanoparticles kit. 'a' shows the summation of 15 frame projections (posterior and anterior views, 1 min for each frame) of dynamic study performed in 15 min. The segments 'b - e' show the static images at 15 min, 1h, 2h and 4h p.i., respectively.

Table 4: Biodistribution of ^{99m}Tc-labeled rhenium sulfide nanoparticles in lymph nodes

Lymph nodes		Popliteal extraction (%)
Popliteal	Iliac	
5.8 ± 1	0.78 ± 0.12	86.55 ± 2

DISCUSSION

Sentinel lymph node biopsy has considerably improved diagnosis of metastases of melanoma and breast cancer. Lymph node radiodetection is the technique of choice, and most radiopharmaceuticals use technetium-99m as the radionuclide. In this research work, we have reported development of a rhenium sulfide based radiopharmaceutical kit by synthesizing rhenium sulfide nanoparticles involving rhenium precursors as starting material and ethylene glycol as stabilizer. The idea is

based on the concept of solvent stabilization method developed by Weixia Tu (Tu *et al*., 2007). Surface chemistry and conjugation method affect the particle stability, biocompatibility and biological functionalities of prepared materials. The uniformity of size of rhenium sulfide nanoparticles was confirmed by UV visible spectrometry, due to presence of uniform optical density of plasma absorption seen in the curves, which is the basic criterion to determine the uniformity of size of nanoparticles (Tu *et al*., 2007).

Drugs show binding to plasma proteins or many other biological materials like glycoprotein, lipoprotein, albumin, erythrocytes, and α, β-, and γ-globulins. Radiolabeled drug and plasma protein interactions affect the pharmacokinetic parameters such as metabolism, volume of distribution, and excretion of the drug, thus accordingly its dosage. Radiolabeled drug binding with plasma proteins is an essential parameter for measuring the effectiveness of the chelating moiety to coordinate the radiometal. The transchelation is involved in the process in which radiometal in labeled drug transchelates to blood proteins, particularly, albumin. So it is important to study the *in vitro* blood protein binding with radiolabeled drug before it is applied to any organism. The binding of drug with blood protein decreases the concentration of drug in plasma. The free or unbound drug is responsible for the side effects and pharmacological activities in the body. Plasma proteins provide a depot for drugs by maintaining buffered free drug levels and assist its distribution. The radiolabeled rhenium sulfide nanoparticles showed 70.2 ± 1.3% binding with blood proteins.

CONCLUSION

The rhenium sulfide colloidal nanoparticles have been synthesized in a novel procedure by using stable isotope of rhenium precursor and ethylene glycol as stabilizer. The radiopharmaceutical kit of nanoparticles was formulated by using pyrophosphate kit as a reducing agent. The great advantage of this radiopharmaceutical is its quick uptake in the sentinel node. The successful development of stable rhenium sulfide colloidal nanoparticles provides the basis for future research in targeted molecular modification and thereby, the promising application of rhenium nanoparticles in tracing diagnosis of sentinel lymph node imaging.

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