

Combining dose and injection volume for good performance of a specific radiopharmaceutical for sentinel node detection^{☆,☆☆}

Eutimio Gustavo Fernández Núñez^{a,*}, Erica Aparecida de Oliveira^a,
Nataaniel Gomes da Silva^a, Renato Santos de Oliveira Filho^b

^aRadiopharmacy Center, Institute of Energetic and Nuclear Research, Sao Paulo, SP 05508-000, Brazil

^bFaculty of Medicine, Federal University of Sao Paulo, SP 04020-041, Brazil

Received 29 April 2011; received in revised form 9 July 2011; accepted 12 July 2011

Abstract

Introduction: The aim of this work was to quantify the effects of injection volume at different technetium-99m specific radiotracer doses on its lymphatic movement in animal model.

Procedures: Effects of injection volume (50, 100 μ l) at different doses (0.05, 0.135, 0.22 nmol) on popliteal node (PN) detection were studied in rats. The radiotracer under study was ^{99m}Tc-technetium-cysteine-mannose-dextran conjugate (30 kDa).

Results: At 0.05 nmol dose, higher PN uptake was observed at 50 μ l injection volume (2.6 fold increase). Conversely, at 0.135 nmol dose, an increase of radiotracer retention in PN was achieved at 100 μ l volume, 78% higher than 50 μ l. However, at 0.22 nmol dose, the injection volume changes did not influence on the PN uptake. Considering as suitable radiotracer performance: high PN uptake and extraction, better combinations were 0.05 nmol/50 μ l, 0.135 nmol/100 μ l, 0.22/50 μ l.

Conclusion: Suitable performances could be reached by proper combinations of dose, injection volume and concentration for a specific radiotracer used in sentinel lymph node detection.

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Keywords: Sentinel lymph node detection; Technetium-99m; Tissue-specific radiopharmaceutical; Dosage; Injected volume

1. Introduction

Since 1992, sentinel lymph node biopsy have been extensively used in patients with breast cancer and cutaneous malignant melanoma [1]. Nowadays, the application of this technique for lymphatic metastasis diagnostic is expanding in other kinds of cancer like prostate, gynecological, gastrointestinal and oral/oropharyngeal squamous cell carcinomas [1–4].

Sentinel lymph node detection is basically performed by use of technetium-99m radiocolloids, noncarcinogenic inert dyes and [F-18] 2-deoxy-2-fluoro-d-glucose (FDG) [5,6]. The first two classes of compounds can be used separately, but better results are obtained when they are used together [7]. The nodal staging with technetium-99m radiopharmaceuticals and dyes leads to an invasive approach for histological studies. Conversely, positron emission tomography with FDG is not invasive but it has a poor sensitivity for detecting millimetric metastasis in lymph nodes [8]. Therefore lymph node biopsy is still the technique of choice for defining whether or not cancer cells have entered lymphatic system.

A new concept of technetium-99m radiopharmaceuticals for sentinel lymph node identification emerged with Lymphoseek. This radiotracer in clinical trials is the first tissue specific radiopharmaceutical for this purpose. This dextran-based product has long-lasting selectivity for mannose receptors expressed on macrophages and

[☆] The corresponding author gratefully acknowledges Philosophic Doctors, Bluma L. Faintuch, Adriano Duatti, Roberto Pasqualini, Ioannis Pirmettis and his collaborators at NCSR Demokritos, Greece, for supporting this study and his wife, Relma Tavares de Oliveira Fernández for the inspiration to write this paper.

^{☆☆} Conflict of interest: The authors declare that they have no conflict of interest.

* Corresponding author. Tel.: +55 11 31339558.

E-mail address: eutimiocu@yahoo.com (E.G.F. Núñez).

dendritic cells enriched within lymph node [9]. Lympho-seek meets three important properties for a suitable sentinel lymph node agent: fast clearance from injection site, rapid accumulation with prolonged retention in sentinel lymph node and low uptake in other nodes of lymphatic chain [10].

Nevertheless, the satisfactory performance of radiotracer for sentinel node detection not only depends on its own properties. In that sense injection site, injection activity, injection volume and utilization of imaging should be also considered [11,12]. Specifically, the injection volume can influence in a negative or positive way the lymphatic performance of radiotracers due to the fragile balance between internal and external pressures in a lymphatic channel [13].

Preclinical and clinical studies about the combined impact of injection dose or activity and volume in sentinel node agent performance have not been widely considered.

The aim of this paper was to evaluate the effects of injection volume at different technetium-99m specific radiotracer doses on its biological performance in a rat model.

2. Materials and methods

2.1. Materials

$\text{Na}[^{99\text{m}}\text{TcO}_4]$ was eluted from $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator (Institute of Energetic and Nuclear Research, IPEN/CNEN-SP, Brazil), using 0.9% saline. The $[^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ was prepared from Isolink kit (Covidien, Petten, The Netherlands) and Vital dye for combined technique (sodium patent blue V, 2.5%) was donated from Guerbet (Roissy, France).

The modified dextran, 2-propylene-cysteine-mannose-dextran [Fig. 1], with chemical purity higher than 95% were provided without cost by National Center for Scientific Research “Demokritos” Athens, Greece. Its molecular weight is 30 KDa with 9 and 36 units of cysteine and mannose respectively.

Forty-eight young female Wistar EPM-1 rats were provided by the Animal Facility of IPEN-CNEN, weight ranging from 150 g to 200 g. Animal studies were performed at the Radiopharmacy Center, IPEN/CNEN, and the protocol was accepted by the animal Welfare Ethical Committee.

2.2. Preparation of the precursor $[^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$

$[^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ was prepared from Isolink kit, adding 1 ml of $\text{Na}[^{99\text{m}}\text{TcO}_4]$ (1000 MBq) as manufacturer’s instructions. The pH was neutralized with 320 μL of 1M phosphate buffer/HCl 1N (1:2) at the end of this radiochemical reaction.

2.3. Radiolabeling of dextran conjugate

Twenty microliters of 2-propylene-cysteine-mannose-dextran aqueous solution (1 $\mu\text{g}/\mu\text{l}$) were mixed with 100 μl precursor $[^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ (74 MBq) during 60 min at 100°C. Each radiolabeling was carried out in triplicate.

2.4. Radiolabeling quality control

The radiochemical purity of the $^{99\text{m}}\text{Tc}$ -modified dextran was determined by means of instant thin layer and paper chromatographies. Thin layer chromatography was carried out silica gel impregnated glass fiber sheets (ITLCTM-SG, Pall Corporation, New York, NY, USA) with Methanol/HCl (99:1) as mobile phase. Retention factors (R_f) in this chromatography system for $^{99\text{m}}\text{TcO}_4$,

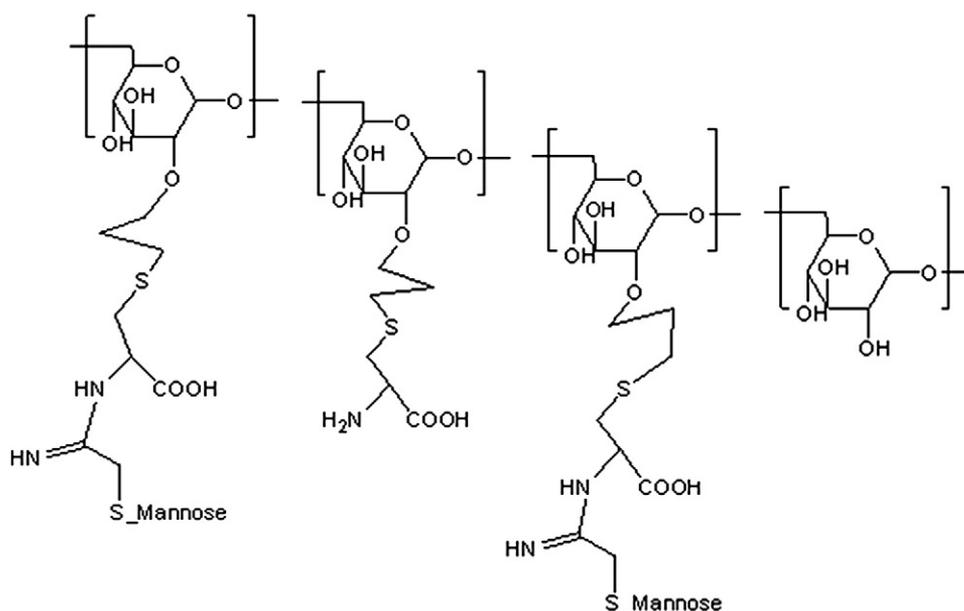


Fig. 1. Chemical structure of 2-propylene-cysteine-mannose-dextran.

$[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ and radiolabeled dextran were 1, 1 and 0 respectively. Paper chromatography was performed on Whatman 1 (RJM Sales Inc., New York, NY, USA) with acetone. $^{99\text{m}}\text{TcO}_4$ only migrated ($R_f=1$) from the sample application point.

The radioactive profile throughout the ITLC-SG and paper strips was measured in AR-2000 radio-Thin-Chromatography Imaging Scanner (Bioscan Inc., Washington, DC, USA).

The specific activity of radiotracers was calculated by means of activity per dextran unit weight and radiochemical purity [14].

The dextran conjugate was also characterized by reverse-phase high performance liquid chromatography (RP-HPLC), including $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ precursor. Analysis was performed on LC-10 AT VP Liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with in-line flow scintillation analyzer (Shell Jr. 1000/2000, VA, USA). HPLC solvents consisted of H_2O containing 0.1% trifluoroacetic acid (Solvent A) and methanol containing 0.1% trifluoroacetic acid (Solvent B). A C-18 column (5.0 μm , 100 Å, 4.6×250 mm, Waters, Milford, MA, USA) was employed with volumetric flow rate of 1 ml/min. HPLC gradient began with a solvent composition of 95% A and was followed by a linear gradient to 30%A: 70%B from 1 to 25 min, and 30%A:70%B to 5%A:95%B from 25 to 28 min.

2.5. Cysteine and histidine challenge

Stability of the labeled compound was assessed using plasma concentration of cysteine (264 μM) [15] and histidine (94 μM) [16] in PBS 0.1 M pH=7.4. Radioconjugate was incubated with an excess amount of both aminoacids (1000-fold molar excess compared to the dextran conjugate) at 37°C for 90 minutes. For transchelation studies the technetium-99m conjugate was not purified. A sample without radiotracer was used as a control. When statistical differences were detected between control and radioconjugate samples, the transchelation percentage was calculated. The amino acids transchelation was quantified by thin layer and paper chromatography described previously (section 2.4). Technetium-cysteine and technetium-histidine have $R_f=1$ in thin layer chromatography system (ITLC-SG/Methanol/HCl (99:1)).

2.6. Lymphoscintigraphy and ex-vivo uptake study in rats. Effects of injection volume and dose

The rats were anesthetized using 25 mg/kg tiletamine hydrochloride associated with 25 mg/kg zolazepam hydrochloride administered intraperitoneally. Afterwards 0.05 or 0.1 ml with different doses (0.05; 0.135; 0.22 nmol) of radioconjugate preparation was subcutaneously injected in the footpad central point of the left posterior limb. Each combination of injection volume and dose was performed in triplicate.

Images at 30 minutes post injection were performed in a Mediso Imaging System, Budapest, Hungria, employing a low-energy high-resolution collimator using a 256×256×16 matrix size with 20% energy window set at 140 keV.

A second injection with 0.05 ml vital dye was administered in the footpad in similar area of the radiotracer injection, five minutes before sacrifice time (30 minutes).

Blood samples were taken by cardiac puncture. Then, after sacrifice the popliteal region was incised permitting access and removal of the popliteal lymph node. Laparotomy with removal of inguinal lymph node, kidneys and liver was done at the same time. Muscle and bone tissues and injection site were also analyzed. The radioactivity of organs and tissues were determined by γ -counting using as standard the injected dose of radiotracer. Results were expressed as percentages of injected dose per organ (%ID), injected dose per gram (% ID/g) or (nmol/g, nmol/ml).

Popliteal extraction was also calculated as the following equation [17]:

$$\text{Popliteal extraction} = \frac{\text{Uptake}_{\text{Popliteal LN}}[\%ID] - \text{Uptake}_{\text{inguinal LN}}[\%ID]}{\text{Uptake}_{\text{Popliteal LN}}[\%ID]} \times 100$$

2.7. Ex-vivo uptake study in rats. Massage effect on radiotracer biodistribution over the course of 24 hours post-injection

The procedure was almost similar with the described one in section 2.4. The modifications were a massage for 1 min after radioconjugate injection, the injection volume and dose were 0.05 ml and 0.05 nmol respectively, the biodistribution studies were carried out at 15, 30, 60, 90, 1080 and 1440 min post injection.

2.8. Influence of radiochemical purity on radiotracer biodistribution

The radioconjugate was previously purified (99.5%) by means of size exclusion chromatography, in PD-10 column. Injected volume and dose were 50 μl and 50 nmol respectively. A gentle massage was performed for 1 min after injection. The biodistribution was assessed 30 min p.i., five animals were included in this study.

2.9. Statistical analysis

Comparisons of two means were performed by t-student test for independent samples at 0.05 level of significance ($\alpha=0.05$). One way ANOVA was carried out to determine differences among three or more means ($\alpha=0.05$). Turkey's test was used to define which means are significantly different. The software used for this purpose was Statgraphics Plus 5.0 (Statistical Graphics Corp., Fairfax, VA, U.S.A.). All the bar graphics were generated using Origin 8 SR2 (OriginLab Corporation, Northampton, MA, U.S.A.).

3. Results

3.1. Preparation of the precursor $[^{99m}\text{Tc}(\text{OH})_2(\text{CO})_3]^+$ and dextran conjugate radiolabeling

The $[^{99m}\text{Tc}(\text{OH})_2(\text{CO})_3]^+$ and ^{99m}Tc -cysteine-mannose-dextran radioconjugate were obtained with higher than 99% and $90.7 \pm 0.67\%$ ($n=8$) radiochemical purities respectively. These values were calculated by combination of instant thin layer (ITLC-SG/Methanol/HCl (99:1)) and paper (Whatman 1/acetone) chromatography and confirmed by high performance liquid chromatography [Fig. 2]. The specific activity of the radioconjugate was 3.36 ± 0.02 MBq/ μg or $100.8 \cdot 10^6$ GBq/mol ($n=8$).

3.2. Cysteine and histidine challenge

The ^{99m}Tc -technetium transchelation from dextran radioconjugate to cysteine (1.7% $P=.153$ NS) and histidine (0.6% $P=.342$ NS) and/or re-oxidation to $[^{99m}\text{TcO}_4]^-$ after 90 minutes at plasma concentration of these amino acids and high molar ratio (1000:1) were not detected.

3.3. Lymphoscintigraphy and ex-vivo uptake study in rats. Effects of injection volume and dose

The injection volume effect on the popliteal lymph node uptake was different within dose range under study [Fig. 3A]. At lower dose (0.05 nmol), higher uptake was observed at 50 μl injection volume (2.6 fold increase, $P=.015$). Conversely, at intermediate dose (0.135 nmol), an increase of radiotracer retention in popliteal node was achieved at 100 μl volume, 78% higher ($P=.003$) than 50 μl . However, at 0.22 nmol dose, the injection volume changes did not influence on the popliteal node uptake ($P=.352$ NS).

Popliteal extraction was also modulated by injection volume. Lower volume at 0.05 nmol dose improved significantly (1.73 fold, $P=.009$) this parameter respect to 100 μl injection volume. Similarly, at 0.22 nmol dose, high injection volume decreased in 55% ($P=.003$) the popliteal extraction. No modification in radiotracer popliteal extraction at intermediate dose for both assessed injection volumes was detected ($P=.071$) [Fig. 3B].

Considering as suitable radiotracer performance: high popliteal lymph node uptake and extraction, the radiotracer amounts retained in better injection volume for each dose were 0.0024 ± 0.0008 nmol (0.05 nmol/50 μl), 0.0044 ± 0.0004 nmol (0.135 nmol/100 μl) and 0.0055 ± 0.0008 nmol (0.22 nmol/50 μl). An increase in amount retained was observed ($P=.0050$) from 0.05 nmol to 0.22 nmol but differences were not detected between 0.135 and 0.22 nmol as Turkey's test [Fig. 4]. This suggests popliteal node saturation at 0.135 and 0.22 nmol doses with proper injection volumes.

The radiotracer uptakes in important excretions organs like liver and kidneys were not modified by injection volume in the range of dose evaluated in this study. Similar performance was detected for bone and muscle tissues

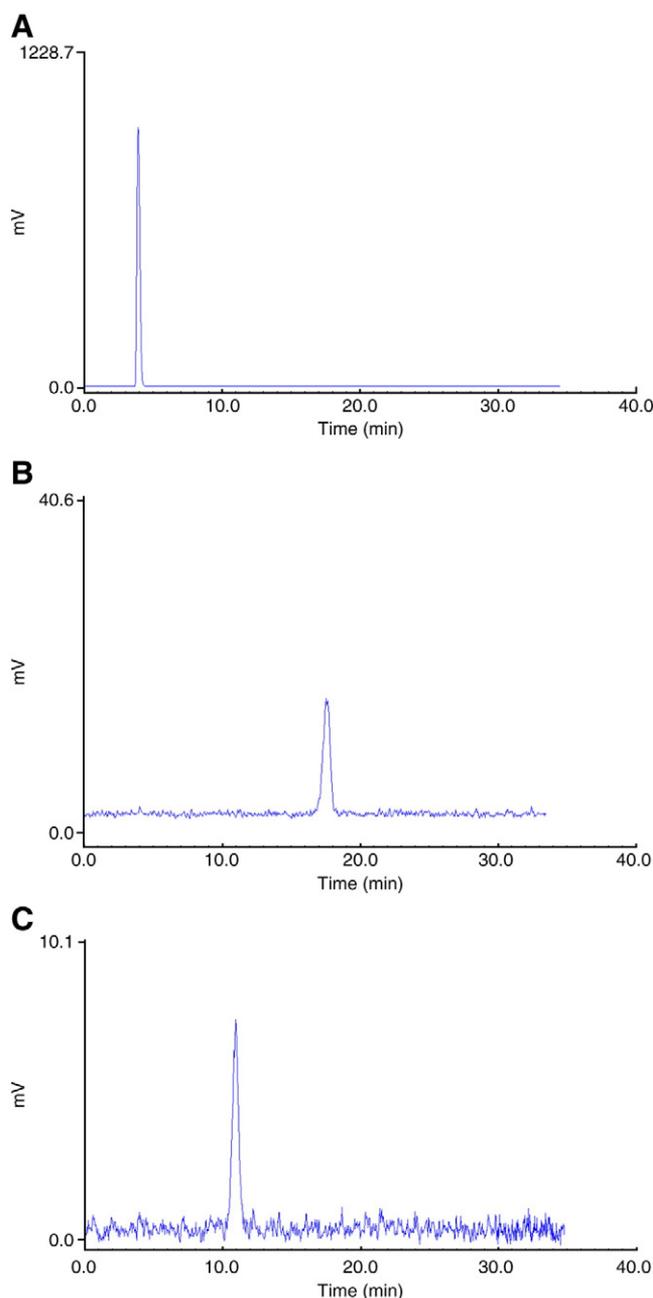


Fig. 2. Phase reverse-high performance liquid chromatography profile of impurities and technetium radioconjugate. A: $^{99m}\text{TcO}_4$ ($t_r=4.0$ min). B: $[^{99m}\text{Tc}(\text{OH})_2(\text{CO})_3]^+$ ($t_r=17.6$ min). C: Technetium dextran-cysteine conjugate ($t_r=11$ min).

whereas in blood, the injection volume effect was only detected at 0.05 nmol dose ($P=.018$). Nevertheless, radioactivities in three last biological compartments were low in all cases [Table 1].

The radioconjugate migration from injection site (% ID) was independent of dose and injection volume ($P=.968$ NS) [Table 1].

Scintigraphic Images corresponding to six combinations of dose and injection volume allowed detecting both

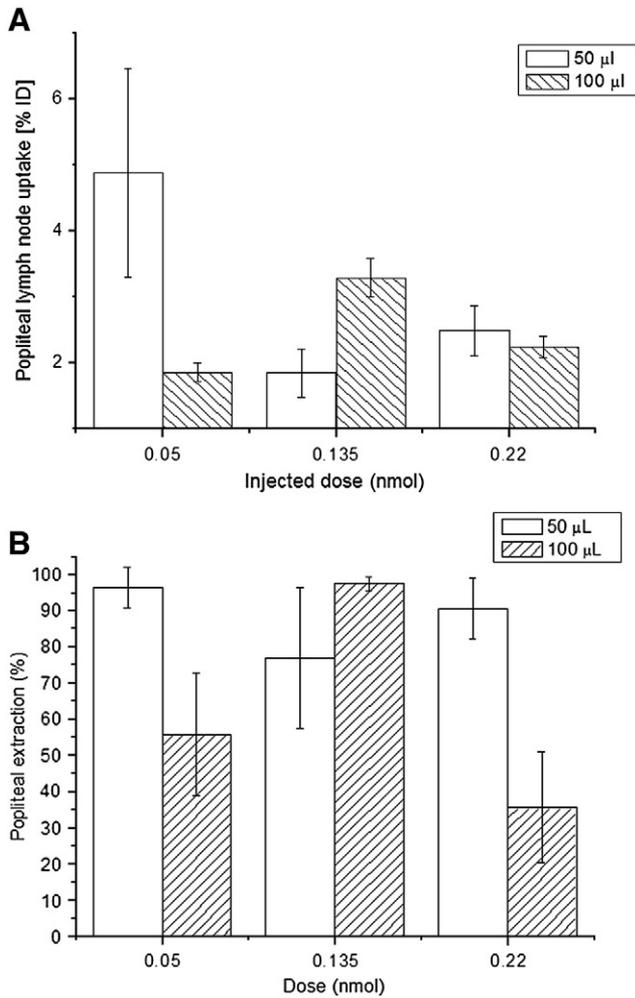


Fig. 3. Influence of injection volume (50, 100 µl) at different doses on popliteal lymph node uptake (A) and popliteal extraction (B) at 30 minutes post-injection. Variables averages±standard deviations are represented (n=3).

popliteal and inguinal node [Fig. 5]. These images confirmed the ex-vivo results regarding popliteal lymph node uptake and extraction.

3.4. Ex-vivo uptake study in rats. massage effect on radiotracer biodistribution over the course of 24 hours post-injection

The massage effect on the biological performance of ^{99m}Tc -dextran conjugate was assessed at 0.05 nmol dose and 50 µl injection volume because suitable results were obtained with these conditions without massage.

The massage effect was significant for improving radiotracer migration from the injection site ($P=.017$). After 30 min post injection, a 15.79% ID decrease from injection site was observed when massage was carried out [Tables 1-2]. However, neither popliteal lymph node uptake ($P=.518$ NS) nor popliteal extraction ($P=.251$ NS) were enhanced with massage. The other organs, tissues and blood

did not also show differences in uptakes with or without massage [Fig. 3,6] [Tables 1-2].

A similar uptake profile for popliteal node and liver over 24 hours post-injection was confirmed. The maximum uptakes for both anatomical elements had a 30 minutes delay and the uptake decrease at the end of study time was more pronounced in popliteal lymph node than liver [Fig. 6]. The radiotracer retention in kidneys, blood, and bone and muscle tissues was stable during 24 hours post-injection. Similar behavior showed the injection site over the course of 90 minutes after injection.

3.5. Influence of radiochemical purity on radiotracer biodistribution

No statistical differences between lymph nodes and main excretion organs uptakes as well as radiotracer migration from injection site for purified and non purified radioconjugate were detected (Table 3). The blood, bone and muscle uptakes for purified product were 0.10 ± 0.04 %ID/ml; 0.06 ± 0.01 %ID/g; 0.01 ± 0.01 %ID/g respectively. These values are similar to the observed for non purified radiotracer, 30 min p.i. (Table 2).

4. Discussion

Site-specific radiocompounds to demonstrate occupancy of a target site such as receptor-binding radiotracers has become a major component in drug development process and nuclear medicine diagnostic procedures [18,19]. Specifically, for lymph node detection, a novel molecular imaging agent based on receptor-binding property, Lymphoseek, has been designed. This radiopharmaceutical opens new possibilities for lymphatic system agents, until now they have been primarily designed taking into account their particle size (radiolabeled colloids) together with functional and mechanical properties of the lymphatic system [20]. Therefore, the suitable tracer performance in sentinel node

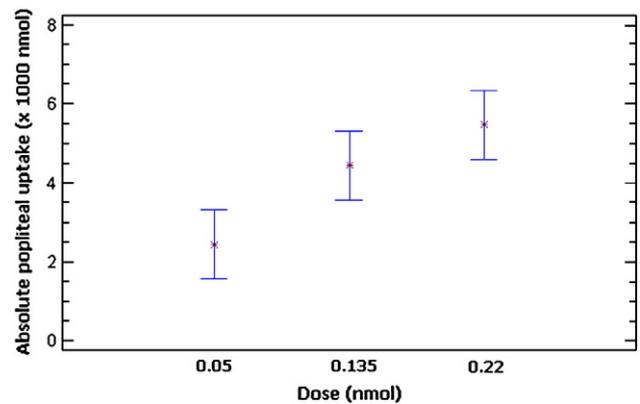


Fig. 4. Absolute popliteal uptakes for better radiotracer performances in each dose. The injection volumes for 0.05, 0.135 and 0.22 nmol doses were 50, 100, 50 µl respectively. Bars represent the confidence intervals for Tukey test ($\alpha=0.05$)

Table 1
Biodistribution study for different doses and injection volumes of the radioconjugate at 30 minutes post injection

Dose (nmol)	Volume (μl)	Uptakes					
		Injection site (% ID)	Liver (% ID)	Kidneys (% ID)	Blood (% ID/ml)	Bone (% ID/g)	muscle (% ID/g)
0.05	50	64,73±2,24	3,14±0,42	0,68±0,18	0,26±0,03	0,09±0,01	0,04±0,01
	100	66,77±2,62	2,71±0,18	0,46±0,09	0,20±0,02	0,08±0,03	0,04±0,01
0.135	50	64,40±3,19	2,42±0,48	0,42±0,04	0,24±0,04	0,07±0,02	0,03±0,01
	100	63,42±10,58	2,93±0,42	0,40±0,08	0,13±0,06	0,12±0,03	0,04±0,01
0.22	50	62,59±7,85	2,72±0,37	0,39±0,12	0,16±0,04	0,11±0,07	0,02±0,01
	100	64,52±3,31	3,43±0,83	0,44±0,06	0,15±0,03	0,10±0,05	0,04±0,03

The results were expressed as average±standard deviation are shown (n=3).

detection does not only depend on its own features, other parameters related with lymphatic functioning like injection volume and dose must be considered [21,22]. In literature, it can find different radiotracers for this purpose with good clinical results by means of a proper selection of the related variables with the biological performance, despite of pronounced molecular differences.

In addition to specificity elements, site-specific radiotracers also need an appropriated synthetic route for radionuclide incorporation. In that sense, our group previously optimized radiolabeling conditions for 2-propylene-cysteine-mannose-dextran with precursor [$^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3$] $^+$. The criteria taken in consideration were high radiochemical purity and concentration, rapid radiolabeling and high specific activity.

The radiochemical yield was 91%, this value is within reported range for other ^{99m}Tc -mannose-dextran conjugate using mercapto-acetyl-tri-glycine chelator (MAG3) [23] but it is lower than values usually reported for Lymphoseek (>98%) [17]. Specific activity (101×10^6 GBq/mol), another important radiochemical parameter, was higher than those observed for radiotracers using MAG3 (22.3×10^6 GBq/mol)

[23] but inferior than values shown for diethylenetriamine-pentaacetic acid-mannose-dextran conjugate (Lymphoseek) (5.3×10^9 GBq/mol) [17]. In addition, the radiotracer ^{99m}Tc -2-propylene-cysteine-mannose-dextran avidity for mannose receptors in lymph node cells was *in vivo* demonstrated [24].

The ^{99m}Tc -technetium transchelation absence to cysteine and histidine in plasma concentrations at high molar ratio (1000:1) suggests a poor background effect in image studies for this radiotracer. In fact, the protein concentration of lymph is usually less concentrated than, that of blood plasma [25]. In this experiment was also confirmed no chelating reaction between both amino acids because [$^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3$] $^+$ percentage kept constant. Thus, protein radiolabeling in biological fluids (*in situ*) is unlikely over first 90 min post-injection. In addition, low protein lymph binding and rapid migration from the injection site are expected because of the marked hydrophilicity of the radiotracer (Log $P=-2.144$) (property previously determinate by our group) [24].

The injection of radiotracers into the foot pads of healthy mice, rats or rabbits has been useful to determine the lymphatic movement of these products directed to sentinel node detection [17,26,27]. In general, these models in healthy animals are used during the initial phase of preclinical studies where a new radiocompound is contrasted with one or various established radiopharmaceuticals. Three parameters are mainly considered when injection is performed in rear foot pads: popliteal lymph node uptake, popliteal extraction and remaining radioactivity in injection site. The assessed radiotracer performance in this model defines the continuity of preclinical studies in more complex animal models to investigate lymph node metastases. In the present work, a variant of this model in rat was used to quantify the effects of dose and injection volume for a specific sentinel node radiotracer on its lymphatic movement and lymph nodes uptakes. Influences of injection volume and dose have been investigated in preclinical and even in clinical assays [28,29] but combined effects of both parameters in radiotracers for lymph node detection have not still explored.

In vivo experiments demonstrated that the uptake in popliteal lymph node, the nearest node from the injection site, was influenced by the injection volume, radiotracer concentration and dose. To define the actual effect of each variable, experimental data was analyzed by injected dose.

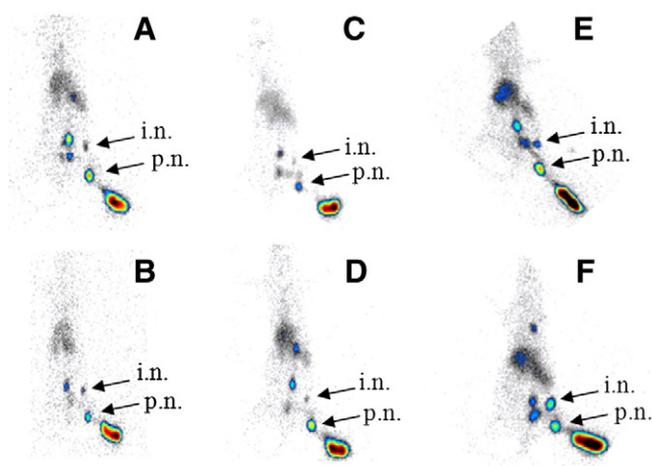


Fig. 5. Static images for six combinations of injection volume and dose after 30 minutes. Dose of 0.05 nmol and injection volumes of 50 and 100 μl respectively are represented in A and B. Dose of 0.135 nmol and similar increasing volumes are showed in C and D respectively. Dose of 0.22 nmol and 50 and 100 μl injection volumes correspond to E and F images. Popliteal node (p.n.), inguinal node (i.n.).

Table 2
Radioconjugate biodistribution over the course of 24 hours (1440 min) post injection

Time (min)	Popliteal Extraction (%)	Uptakes				
		Injection site (% ID)	Kidneys (% ID)	Blood (% ID/ml)	Bone (% ID/g)	muscle (% ID/g)
15	91,40±8,19	54,35±4,64	0,36±0,06	0,22±0,10	0,12±0,04	0,04±0,01
30	88,07±9,63	48,94±9,02	0,47±0,27	0,25±0,13	0,13±0,05	0,04±0,02
60	91,05±9,65	46,35±3,16	0,41±0,09	0,22±0,11	0,11±0,04	0,03±0,00
90	91,10±6,63	48,24±3,41	0,37±0,11	0,20±0,09	0,10±0,07	0,03±0,01
1080	88,68±9,56	-	0,51±0,13	0,11±0,09	0,05±0,04	0,02±0,00
1440	87,87±14,05	-	0,51±0,25	0,12±0,09	0,04±0,02	0,01±0,00

Dose and injection volume were 0.05 nmol and 50 μ l respectively. Gentle massage was performed for 1 minute after injection. The results were expressed as average±standard deviation are shown (n=5).

At low dose, the injection volume effect displayed as a rapid radiotracer migration and retention from injection site for 100 μ l, it was not observed [Fig. 3A]. This phenomenon could be justified by the low radioconjugate concentration (0.5 μ M). The concentration effect has been mentioned in literature related with non-specific radiopharmaceutical for lymph node detection [30]. In specific receptor-binding radiotracer for this purpose, the concentration effect is also important because the radiotracer binding to cellular receptor depends on free concentration of the radiomolecule [18].

At intermediate dose (0.135 nmol), the injection volume effect is well observed, because 50 μ l injection volume showed a lower uptake respect to 100 μ l volume [Fig. 3A]. The concentration in 100 μ l was 1.35 μ M, it was similar to that presented by 0.05 nmol and 50 μ l injection volume (1.0 μ M). We hypostatize concentrations above 1 μ M of ^{99m}Tc -2-propylene-cysteine-mannose-dextran are necessary for suitable sentinel lymph node detection.

At 0.22 nmol dose, receptors saturation is suggested because similar popliteal lymph node uptakes were detected with both injection volumes [Fig. 3A].

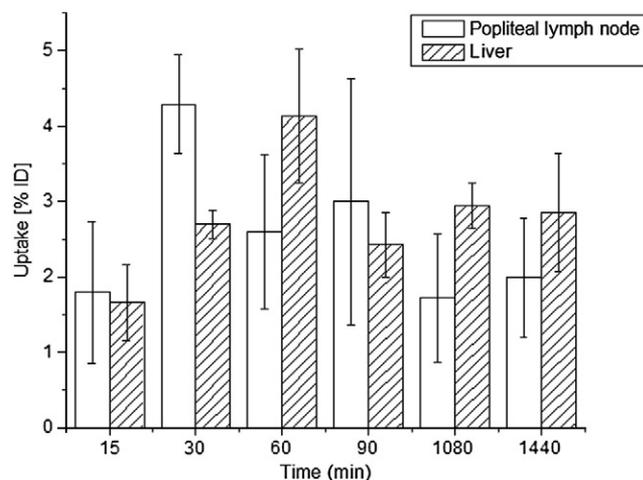


Fig. 6. Popliteal lymph node and liver uptakes of the radiotracer over the course of 24 hours (1440 min) post-injection. Injected dose: 0.05 nmol, injection volume: 50 μ l and gentle massage during 1 min post-injection. Variables averages±standard deviations are represented (n=5).

Summarizing, the expected positive effect for 100 μ l volume on popliteal lymph node uptake was not always confirmed for three doses under study. Higher volumes favor the radiotracer migration because pressure outside of lymphatic capillary increase, allowing fluid to enter the lymphatic capillary [29]. However, effects of radiotracer concentration and amount (dose) modified the hoped performance.

Popliteal extraction defines the preferential uptake in sentinel lymph node respect to the second lymph node in lymphatic chain. Therefore, high values of this parameter are desirables. In rat model, this can be translated as higher popliteal node uptake respect to inguinal lymph node.

Popliteal extraction at 0.05 nmol dose was improved at low injection volume (50 μ l). This could be explained by the lower concentration and rapid migration at 100 μ l volume. In these conditions the radiotracer is more likely to migrate to inguinal lymph node and present similar uptakes in both lymph nodes.

At intermediate dose, no differences were detected at both injection volumes. The mannose receptors in popliteal lymph node could not be totally filled by the radiotracer; hence it is just observed a high popliteal node uptake at 100 μ l injection volume. However, at 0.22 nmol dose, as probably the popliteal lymph node saturation is reached, then the radiotracer can better migrate to inguinal node at 100 μ l volume. Thus, the popliteal extraction decreases in these conditions.

Since radiotracer amount retained in popliteal lymph node kept stable in better conditions (high popliteal node uptake and extraction) for 0.135 and 0.22 nmol despite the concentration increase, 1.35 μ M and 4.4 μ M respectively,

Table 3
Radioconjugate biodistribution with high radiochemical purity (99.5%), and statistical comparison with radioconjugate lacking further purification step (90.7±0,67%) (Table 2 and Fig. 3A)

Organ	Uptake (% ID)	P values (t-student)
Popliteal lymph node	3.41±1.77	0,3844
Inguinal lymph	1.10±1.17	0,3602
Kidneys	0.48±0,20	0,9605
Liver	2.58±1.10	0,8318
Injection site	59.89±7.43	0,0901

Assessed dose, injection volume and time p.i. were 0.05 nmol, 50 μ l and 30 min respectively.

probably the receptors saturation was achieved. Another fact confirming this hypothesis is the similar absolute uptake reported for sentinel lymph node saturation in a clinical trial with Lymphoseek (11.0 ± 8.5 pmol) [28]. Making an analogy with in-vitro saturation binding assay, free radiotracer concentrations in lymph for both situations are in plateau region of the saturation binding curve [31], where the maximum number of receptors on the cells (B_{\max}) detected by radiotracer is a 4.4–5.5 pmol range.

All the partial conclusions related to dose and injection volume for this subcutaneous injection can change for other tissues in the same specie (rat) or even in a similar tissue for humans because lymphatic flow modifications depend on tissue density [29]. Nevertheless, radiotracer concentrations in injection solution higher than 1 μM are recommended regardless tumor anatomical site, injection site or specie. For this concentration range, the mannose receptor-radiotracer complex formation is favored and an increase of product uptake in sentinel lymph node could be observed. The accurate definition of these three parameters in human tumors needs more experiments in other animal models, while considering the present difficulties in the establishment of appropriated animal models for lymph node metastases [32]. However, it was demonstrated that different combinations of doses and injection volumes for a bio-specific radiotracer could show proper performances in sentinel node detection.

The high relative uptake of the liver respect kidneys in spite of radiotracer hydrophilicity is due to similar mannose receptors are found in hepatic cells [33].

As good definition images were obtained at three doses under study with proper injection volume [Fig. 5A,D,E], we decided to continue the study with low dose.

When massage was applied in injection site at 0.05 nmol and 50 μl injection volume, the most important change was detected in injection site clearance of radioconjugate. The lymphatic flow was favored by massage without affect popliteal lymph node uptake and extraction. As radiotracer concentration kept constant, the lymphatic environment conditions did not also change, so both parameters remained constant. The mechanical action of massage may also just lead a radiotracer migration by lymphatic vessels through lymph nodes or their surface without discharging lymph into the node [13].

The similar uptake profile for popliteal lymph node and liver could be attributed to similar receptor-mediated endocytosis [34] and the 30 min delay may be due to the anatomical distances and physiological way from injection site.

Finally, it was demonstrated that the radiotracer purity for values higher than 90 % do not change the biodistribution pattern, 30 mi p.i. This means the pertechnetate ion, $[\text{}^{99\text{m}}\text{Tc}(\text{OH})_2(\text{CO})_3]^+$ precursor and any labeled macromolecule in situ are not significantly retained neither lymph nodes nor main excretion organs, 30 mi p.i. This outcome allows the radioconjugate use without purification step after radiolabel-

ing and confirms the previous conclusions related with injection volume and dose.

5. Conclusion

Similar suitable performances could be reached by different combination of dose, injection volume and concentration for a specific radiotracer used in lymph node detection. When a critic concentration (1 μM) in administrated dose is achieved for $^{99\text{m}}\text{Tc}$ -cysteine-mannose-dextran (30 KDa), combinations of high doses/low injection volume or low dose/high injection volume are recommended. This methodology used and the derived results could serve as a guide for improving receptor specific radiopharmaceuticals performance in sentinel lymph node detection both animals and humans. However, the adjusted parameters in this study could change according to the radiotracer properties like size and receptors affinity as well as tissue density around injection point.

Acknowledgments

The authors are indebted to the National Commission for Nuclear Energy (CNEN, São Paulo, Brazil), National Counsel of Technological and Scientific Development (CNPq, Brasília, Brazil), the International Atomic Energy Agency (IAEA, Vienna, Austria), Covidien and Guerbet for scientific grants and reagents donation.

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