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^{99m}Tc Nanocoll: A radiopharmaceutical for sentinel node localisation in breast cancer—In vitro and in vivo results

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ABSTRACT

This study evaluated labelling efficiency and radiochemical purity of ^{99m}Tc colloid albumin to identify an optimal labelling protocol for sentinel node detection. Results indicate that a 72 h eluate is not recommended for high specific labelling of ^{99m}Tc colloid albumin. Ex vivo, significantly higher count rates were reached using a 2 h eluate in vacuum or nitrogen. Labelling 26 MBq/μg ^{99m}Tc colloid albumin with a 2 h eluate under nitrogen is recommended because of the ease of labelling.

1. Introduction

Cabanas (1977) was the first to describe the concept of a sentinel lymph node (SLN) as the node in the first basin to drain lymphatic flow from a primary tumour, and therefore becoming the primary site of lymphatic metastasis. Krag et al. (1993) were the first to describe a radio-localisation and surgical resection of the SLN in breast cancer using a gamma probe. The technique uses an agent, either a vital dye or a radiopharmaceutical, that enters the lymphatic drainage system after injection and concentrates in one or more sentinel nodes (Borgstein et al., 1998; Haigh et al., 2000). Although various radiopharmaceuticals have been introduced, there is no consensus about the most effective radiopharmaceutical for optimal visualisation. Many research groups have studied methods to optimise radiopharmaceuticals (Kaplan et al., 1979; Valdés Olmos et al., 1999, 2001; Abraham et al., 1999; Imoto et al., 1999; Edreira et al., 2001; Hodgson et al., 2001; Krynyckyi et al., 2001) but to date, no radiopharmaceuticals are registered for sentinel node detection. In the United States, the most commonly used radiopharmaceutical for SLN detection is ^{99m}Tc-labelled sulphur-colloid, and in Europe ^{99m}Tc colloid albumin (Nanocoll®) is most frequently used.

Nanocoll® is available as a kit containing lyophilised human albumin particles and stannous chloride dihydrate. The preparation of ^{99m}Tc colloid albumin involves the addition of pertechnetate to a vial with a lyophilised mixture of human colloid albumin particles, stannous chloride, glucose, polyoxamer 238, and sodium phosphate and sodium phytate. Nanocoll® is registered for intravenous administration for bone marrow scintigraphy, for inflammation scintigraphy in areas other than the abdomen, and for cutaneous administration of lymphatic scintigraphy to demonstrate the integrity of the lymphatic system and differentiation of veins or lymphatic obstruction. After subcutaneous injection, 30–40% of administered ^{99m}Tc albumin colloid particles is filtered into lymphatic capillaries whose main function is the drainage of proteins from the interstitial fluid back into the blood pool. The ^{99m}Tc albumin colloid particles are then transported along the lymphatic vessels and trapped to functionary lymph nodes (Nycomed Amersham Sorin S.r., 1999). In nuclear medicine practice, we typically find 10–15% uptake of activity in the inguinal, para-iliacal or axillary lymph nodes after intra-dermal administration of ^{99m}Tc-colloid albumin in the lower and upper limbs. If used for breast cancer and melanoma, there only is a 1–1.5% uptake in regional lymph nodes (Reintgen, 1998; Pijpers et al., 1998).

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Previously, our group reported experiments using dynamic light scattering techniques for measurement of particle sizes, combined with mathematics of involved particles of colloid albumin. In those reports, we calculated the number of colloid albumin particles contained in a vial of Nanocoll® (Gommans et al., 2000, 2001). We have also confirmed that more than one ^{99m}Tc and/or ^{99}Tc atoms can be labelled to a colloid albumin particle (Gommans et al., 2001; Billinghurst and Jette, 1979; Bergquist and Strand, 1989). This concept is based on the idea that a higher specific activity and/or a higher ratio of ^{99m}Tc versus ^{99}Tc atoms labelled to colloid albumin will lead to a higher density of ^{99m}Tc atoms on individual colloid albumin particles. Unlike many other radionuclides, ^{99m}Tc is never carrier-free and contains varying amounts of its decay product, ^{99}Tc. The amount of ^{99}Tc that is present in the eluate depends on the elution history of the ^{99}Mo/^{99m}Tc generator and increases with time due to decay of ^{99m}Tc (Lamson et al., 1975). For instance, the radiolabelling of colloid albumin using first eluate from a factory fitted ^{99}Mo/^{99m}Tc generator will usually be suboptimal in terms of specific activity, since typically there is a time frame of several days between production and first use of the generator. This may compromise the detection of SLNs in patients.

Several reports state that pertechnetate will be reduced by stannous chloride dihydrate to form Tc (IV), without involvement of a chelator. The chemistry of Tc may be different at high and low concentrations of Tc (Eckelman et al., 1971; Saha, 1992). When labelling colloid albumin, the reduction of Tc (VII) probably occurs in different stages from Tc (VII) to Tc (IV), or from Tc (VII) to a different valence status of Tc (VI, V, IV, or even III) (Saha, 1992; Eckelman et al., 1972)

This study addressed the following hypotheses:

(1) An increased specific activity of ^{99m}Tc-colloid albumin results in a better radiochemical purity (RCP) and a higher labelling efficiency.
(2) Labelling a higher density of ^{99m}Tc atoms to individual colloid albumin particles results in a better RCP and a higher labelling efficiency.
(3) An increased specific activity of ^{99m}Tc-colloid albumin results in higher count rates, as observed during in vivo measurements of radioactivity in SLNs.
(4) RCP of labelled ^{99m}Tc-colloid albumin as seen on ITLC-SG thin layer chromatography ($Rf = 0$) contains different valence states of Tc.

In this report, we present the identification and in vitro and in vivo evaluation of an optimised protocol for labelling ^{99m}Tc to colloid albumin. ^{99m}Tc colloid albumin was either labelled in vacuum or under nitrogen. The batches of colloid albumin were labelled with various ratios of ^{99m}Tc/^{99}Tc, depending on whether 72, 24, or 2 h eluates were used. Also the effects of addition of cysteine and stannous chloride, on the specific activity of radiolabelled ^{99m}Tc colloid albumin, were investigated. In addition, special attention was paid to the effects on specific activity of freezing, storage during several months, defrosting, and subsequent radiolabelling of the colloid albumin.

2. Materials and methods

2.1. Mathematics

An integral function (Formula 1a)[1] of disintegrated atoms was used for calculation of the ratio between of ^{99m}Tc and ^{99}Tc in a range of T = 0–72 h. The mathematics (Formula 1b)[2] of the mother/daughter relationship of ^{99}Mo/^{99m}Tc–^{99}Tc were used to calculate an optimal elution time for labelling ^{99m}Tc-colloid albumin.

$$\int_{t=0}^{t_m} A(t)\,dt = \int_{t=0}^{t_m} A_0\, e^{-\lambda t}\,dt = \frac{A_0}{\lambda}(1 - e^{-\lambda t_m}) \quad \text{(formula)} \tag{1a}$$

$$A_{Tc}(t) = \frac{\lambda_{Tc}}{\lambda_{Tc} - \lambda_{Mo}} A_{Mo,0}(e^{-\lambda_{Mo} t} - e^{-\lambda_{Tc} t}) \quad \text{(formula)} \tag{1b}$$

2.2. In vitro design

In all tests, labelling of ^{99m}Tc-colloid albumin was performed under standardised controlled conditions (nitrogen or vacuum). To prepare ^{99m}Tc-colloid albumin, the disposables as used for labelling were first flushed with 10 ml saline. A G21 needle, a 6-ml syringe, and a 3-way stopcock were used. A total of 5 ml saline was added to the original vial of Nanocoll®, with the needle inserted through the thickest part of the rubber plug. Then, the appropriate amounts of mixture were extracted and were supplemented with saline under either vacuum or nitrogen, in order to obtain the concentrations that are given below. The ^{99m}Tc-colloid albumin (standard activity of 1.3 GBq ^{99m}Tc) was prepared using a 2, 24, and 72 h elution, following an initial elution at $T = 0$ h. The in vitro RCP and specific activity of a series of concentrations was tested by evaluating concentrations of 2.6, 5.2, 10.4, 26, 30, 37, and 52 MBq ^{99m}Tc/μg, which resulted from the mixture of 500; 250; 125; 50; 43.3; 35.1; and 25 μg colloid albumin with the standard activity of 1.3 GBq ^{99m}Tc, respectively. The labelling yield was then assessed by ITLC-SG chromatography (see below).

Then we investigated the concentrations that yielded optimal labelling of ^{99m}Tc colloid albumin under nitrogen, for both the 72 and 24 h eluate of ^{99m}Tc. This was done by additional ITLC-SG thin layer chromatography (>95%) within the concentration range between 7 and 9 MBq ^{99m}Tc/μg colloid albumin for the 72 h eluate, and in the range between 11 and 13 MBq ^{99m}Tc/μg for the 24 h eluate. The first concentration range was made using 185–144.4 μg, and the second using 118–100 μg colloid albumin, all with 1.3 GBq ^{99m}Tc.

For further in vitro experiments, residual unlabelled colloid albumin that was left in the original vials was stored in nitrogen containing vials at −20 °C during 4 months. These vials were used to evaluate the effects of storage on the specific activity of ^{99m}Tc colloid albumin. Based on the obtained results, only concentrations of 10.4 and 26 MBq/μg were chosen for this purpose.

To extract the suspected different valence states of technetium from labelled ^{99m}Tc colloid albumin (both at $Rf = 0$), we performed ITLC-SG thin layer chromatography with cysteine [23]. To achieve even further reduction, additional stannous chloride dihydrate was administered.

To summarise, experiments were performed under five distinct conditions:

(1) *Labelling colloid albumin with Tc under nitrogen*: The colloid solutions for all tests were labelled with 1.3 GBq ^{99m}Tc in 5 ml saline in the original vials and prepared in a range of 2.6 MBq/μg (= absolute amount of 500 μg colloid in the vial) to 52 MBq/μg (25 μg colloid in vial).

[1] Formula 1a: A_0 is the activity at time $T = 0$ h and $A(t)$ the activity at time $T = t$; $\lambda = \ln 2/t^{1/2}$.

[2] Formula 1b: A_{Tc} is the activity of daughter 99m-technetium at time $T = t$ h and $A_{Mo,0}$ the activity of mother 99-molybdenum at time $T = 0$ h; $\lambda = \ln 2/t^{1/2}$.

(2) *Labelling colloid albumin after freezing and storage during 4 months at −20 °C*: The colloid solutions for these tests were also labelled with 1.3 GBq ^{99m}Tc in 5 ml and were prepared as concentrations of 10.4 MBq/μg (125 μg colloid in vial) and 26 MBq/μg (50 μg colloid in vial).
(3) *Labelling colloid albumin with Tc under vacuum*: The colloid albumin was removed from the original vial and transferred into vacuum vials (Tyco 11-ml elution vials). Concentrations of colloid albumin identical to those used for preparation under nitrogen were labelled with 1.3 GBq ^{99m}Tc under vacuum using solutions ranging from 2.6 MBq ^{99m}Tc/μg (500 μg colloid in vial) to 52 MBq ^{99m}Tc/μg (25 μg colloid in vial).
(4) *Distinction test with cysteine*: To investigate the involvement of various Tc (VII, V, IV) oxidation states, 20 min after labelling, 0.1 ml cysteine (Fig. 1; valence state V) solution (0.01 M) was added to vials of ^{99m}Tc-colloid albumin. Maximum labelling efficiency concentrations (>95%) were identified. To perform further reduction of the Tc oxidation state to Tc (IV), 20 min after labelling, 0.1 ml cysteine solution (0.01 M) and 0.2 mg stannous chloride dihydrate were injected into duplicate vials with the same concentrations.
(5) *Residual activity of TcO_2 on the inner surface of the glass in preparation vials*: Residual activity at the inner surface of the preparation vials for conditions 1 and 3 was measured at $T = 20$ min and 3 h. Residual activity at the inner surface of the prepared vial for cysteine was measured at $T = 3$ h. Residual activity was measured under standardised controlled geometric conditions in a dose calibrator (VIC 202). All vials were rinsed with 50 ml saline before the measurement (1 min).

2.3. Purity and radiochemical labelling efficiency

ITLC-SG, with 95% methyl ethyl ketone (MEK): Quality assessment was performed using ascending chromatography on ITLC-SG, with 95% MEK as the mobile phase (Rf = 0: ^{99m}Tc-colloid; Rf = 1: free $^{99m}TcO_4^-$). Radiochemical labelling efficiency and purity were tested at $T = 0.5$, 2, 5, 8, and 24 h. To comply with quality requirements, the radiochemical labelling efficiency needed to exceed 95% (minimal RCP >95%, until 6 h after preparation (according to the product leaflet).

ITLC-SG, with 12% trichloroacetic acid (TCA) in saline: To prove the involvement of different Tc (VI, V, IV) oxidation states, 0.1 ml cysteine solution (0.01 M) was added. Additional assessment of the radiochemical labelling efficiency and purity at $T = 1$ and 3 h was performed using ascending chromatography on ITLC-SG, with 12% TCA as the mobile phase (Rf = 0: ^{99m}Tc-colloid; Rf = 0,6 ^{99m}TcO-cysteine).

ITLC-SG, with 12% TCA (in saline) after cysteine+stannous chloride dehydrate: To achieve a further reduction of different Tc (VI, V, IV) oxidation states, cysteine+stannous chloride dihydrate was added. Additional assessment of the radiochemical labelling efficiency and purity at $T = 1$ and 3 h was performed using ascending chromatography on ITLC-SG, with 12% TCA as the mobile phase (Rf = 0: ^{99m}Tc-colloid; Rf = 0,6 ^{99m}TcO-cysteine; Rf = 1: $^{99m}TcO_4^-$). No oxygen was involved, and the obtained Tc (V) was expected to be bound to colloid albumin particles.

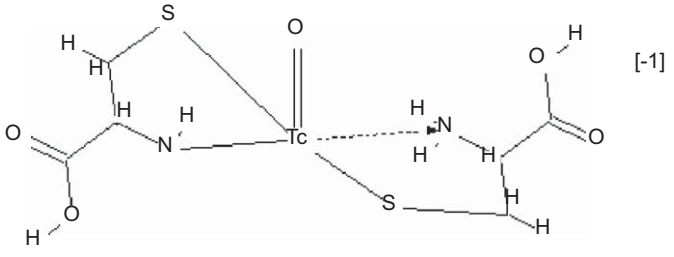

Fig. 1. Chemical formula of Tc (V)+2cysteine.

2.4. In vivo methods

After obtaining informed consent, we tested various batches with different specific activities of ^{99m}Tc colloid albumin in 368 patients with histopathology-proven breast carcinoma (Table 1), comprising five series of patients, including one double-blinded randomised series. In the randomised series of 158 patients, we prepared 26 MBq/μg (50 μg colloid albumin labelled with 1.3 GBq ^{99m}Tc) under vacuum. In 86 patients (group 4), a 2 h elution was used, and in 72 patients (group 3) a 24 h elution was used. In a prospective series with 33 patients (group 1), 8 MBq/μg (162.5 μg colloid albumin in the vial) in a 72 h elution (nitrogen) was used; for 103 patients (group 5), we prepared 26 MBq/μg in a 2 h elution in nitrogen; in 74 patients (group 2), 10.4 MBq/μg (125 μg colloid albumin in vial) in a 24 h elution (nitrogen) was used. All patients were injected along the lateral border of the areola (two injections: intradermally 50 MBq/0.3 ml and parenchymally 50 MBq/2 ml). Ex vivo measurement of count rates was performed (10 s/node, decay corrected). All nodes were measured ex vivo (surgery was performed at 20–24 h after administration) using the same procedure. Holding the probe upward, the surgeon fixed each excised lymph node on top of the probe while searching for the highest count rate. At the highest count rate, a 10 s sample was registered. For all measured nodes, count rates ex vivo were corrected for decay to 24 h after administration.

Table 1
Baseline patient characteristics by group[a].

Group	1	2	3	4	5
N	33	74	72	86	103
Age range (year)	47–70	32–85	35–85	20–79	30–83
Mean age ±SD	57±6.7	55.5±12.4	58.8±11.3	Nitrogen	58.7±10.1
Tumour size (mm): clinical range	8–25	7–20	7–30	5–30	5–30
Tumour size (mm) mean ±SD	15.3±3.4	12.1±2.3	15.3±6.1	15.8±6.1	14.7±6.6
Elution of ^{99}Mo/^{99m}Tc generator (h)	72	24	24	2	2
Concentration colloid albumin (MBq/μg)	7.5	10.4	26	26	26
Labelling condition	Nitrogen	Nitrogen	Vacuum	Vacuum	Nitrogen

SD; standard deviation.
[a] Group 1: 72 h elution-nitrogen; group 2: 24 h elution-nitrogen; group 3: 24 h elution-vacuum; group 4: 2 h elution-vacuum; and group 5: 2 h elution-nitrogen.

2.5. Probe handling and quality control

A physicist performed quality control of the probes (Europrobe, 16 mm CsI) in a biweekly cycle, checking the sensitivity with a ^{57}Co point source.

2.6. Statistical analyses

All quantitative data are expressed as mean ± SD. The Kolmogorov–Smirnov test was used to test data for normal distribution. Differences in data between the subgroups were tested for statistical significance with the two-tailed *t* test in the case of a normal distribution. For all subgroups, we used the ANOVA test to compare mean and variance of age and tumour size. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Mathematics

The minimum difference of ^{99m}Tc ingrown of 0.4% was calculated between $T = 22$ h (99.6%) and 24 h (100%). The maximum ingrown of ^{99m}Tc was calculated at $T = 22$ h, 2 min, and 20 s (Fig. 2).

According to the calculations, elution of the ^{99}Mo/^{99m}Tc generator at 2 h after a previous elution does not lead to reduced activity of ^{99m}Tc the next day (22 h later). ^{99m}Tc at elution time $T = 2$ h contains a ratio 3.3:1 of the involved atoms ^{99m}Tc:^{99}Tc. The ratio of involved atoms ^{99m}Tc:^{99}Tc at $T = 24$ h is 1:2.6, and the ratio of involved atoms ^{99m}Tc:^{99}Tc at $T = 72$ h is 1:12.5 (Fig. 3).

3.2. Purity and radiochemical labelling efficiency

When labelling was performed using the 2 h eluate under either nitrogen or vacuum, a maximum concentration (C_{max}) of 30 MBq/μg (108 μg colloid albumin in the vial labelled with 1.3 GBq ^{99m}Tc) could be achieved (97.2% purity and labelling efficiency). A C_{max} of 12 MBq/μg (43.3 μg colloid albumin in vial) was achieved after using the 24 h eluate under nitrogen (96.0%), and under vacuum conditions, a C_{max} of 25 MBq/μg was achieved (96.6%). A C_{max} of 8 MBq/μg (162 μg colloid albumin in vial) was achieved using the 72 h eluate under nitrogen (96.1%), and under vacuum conditions, a C_{max} of 10.4 MBq/μg (125 μg colloid albumin in vial) was achieved (96.6%). The 72 h eluate under nitrogen conditions resulted in a purity and labelling efficiency of 98–99% for a C_{max} of 10.4 MBq/μg at $T = 2$ h after labelling. At $T = 5$ h, a purity and labelling efficiency of 94% was observed (Table 2).

The colloid albumin that was stored in nitrogen containing vials at −20 °C during 4 months, was defrosted and then labelled at room temperature. These vials contained either 50 or 125 μg colloid albumin. After labelling with 1.3 GBq ^{99m}Tc, in vitro tests yielded >98% RCP and labelling efficiency (at $T = 0.5$, 5, and 8 h) (Table 3).

3.3. Challenge test with cysteine

The addition of cysteine to concentrations of 2.5, 10.4, 26, and 30 MBq/μg ^{99m}Tc-colloid albumin (respectively, 500; 125; 50, and 43.3 μg, all labelled with 1.3 GBq ^{99m}Tc), resulted in a changed oxidation states in 5.3–8.2% (Tc^{5+}) at $T = 1$ h and in 5.8–16.4% at $T = 3$ h (Table 4).

3.4. Challenge test with cysteine and stannous chloride dihydrate

The addition of cysteine and stannous chloride dihydrate to concentrations of 2.5, 10.4, 26, and 30 MBq/μg (respectively, 500; 125; 50, and 43.3 μg, all labelled with 1.3 GBq ^{99m}Tc), resulted in a further reduction of the valence state Tc^{5+} to Tc^{4+}. At $T = 1$ h, a reduction of almost 100% to TcO_2 was achieved (Table 4).

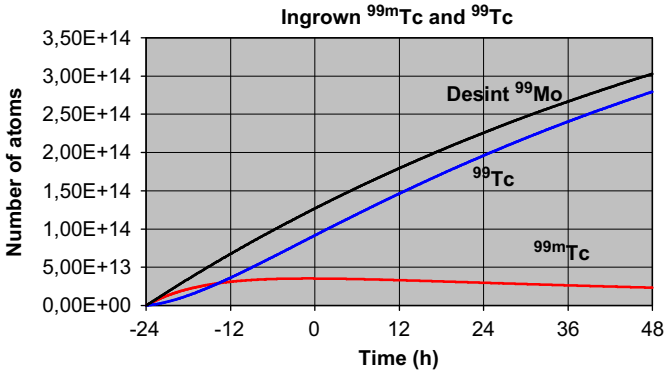

Fig. 3. ^{99m}Tc and ^{99}Tc ingrown of decay of 1.6 GBq ^{99}Mo from $T = 0$–72 h: factory-fitted and shipped generator.

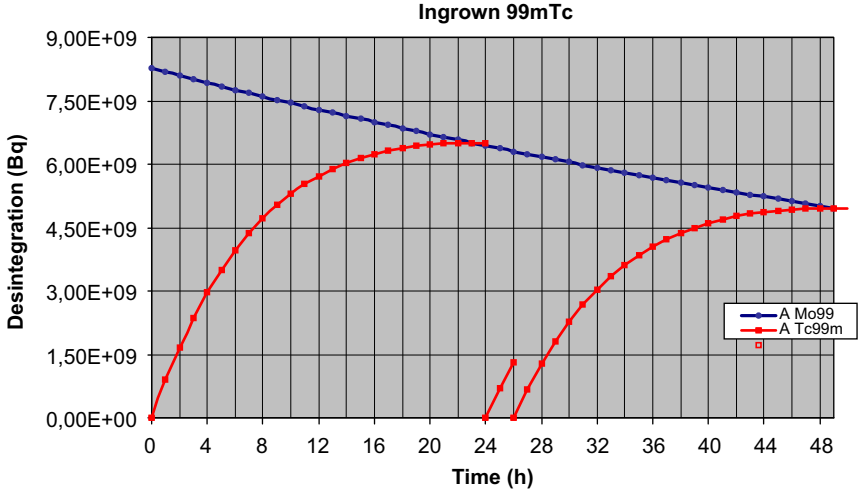

Fig. 2. The ingrown of ^{99m}Tc from ^{99}Mo: 0–24 h ingrown (24-h elution); 24–26 h ingrown (2-h elution), and 26–48 h (22-h elution).

Table 2
The labelling yield (%) ^{99m}Tc-colloid albumin from an elution at $T = 2$, 24, and 72 h (after a previous elution) with different specific concentrations, using ascending chromatography on ITLC-SG, with 95% MEK as the mobile phase (Rf = 0: ^{99m}Tc-colloid albumin; Rf = 1: free $^{99m}TcO_4^-$)[a].

MBq/μg	0.5 h	2 h	5 h	8 h	24 h
Labelling yield under nitrogen, T = 72 h					
2.6	100	100	99.5	99.5	99.3
5.2	100	99.5	98.6	98.5	97.8
7	99.7	99.5	98.0	97.8	96.8
8	99.5	98.0	96.1	96.0	95.5
9	99.5	97.0	94.8	94.3	93.0
10.4	99.3	96.8	94.3	94.2	92.0
26	87.0	83.2	81.7	80.4	78.7
30	85.0	82.3	80.6	78.3	75.2
37	83.2	80.0	78.0	75.2	73.4
52	45.4	40.2	38.1	36.1	34.2
Labelling yield under nitrogen, T = 24 h					
2.6	99.8	99.8	99.9	97.9	97.5
5.2	99.8	99.5	98.9	97.9	97.2
10.4	99.9	98.8	98.5	98.0	97.0
11	99.7	98.5	98.0	97.0	96.5
12	99.5	98.0	97.0	96.5	96.5
13	99.5	97.0	95.5	94.0	93.0
26	96.1	88.7	86.8	85.6	84.0
30	81.5	78.5	76.0	75.4	72.0
37	79.0	74.5	72.0	70.5	68.0
52	52.5	52.5	54	52.7	48.3
Labelling yield under nitrogen, T = 2 h					
2.6	99.6	99.8	99.8	99.7	99.5
5.2	99.6	99.5	99.5	99.5	99.4
10.4	99.5	99.4	99.3	99.2	99.1
26	100.0	98.5	97.7	97.6	97.5
30	97.2	96.8	96.4	95.2	96.0
37	98.6	96.0	94.5	94.8	93.0
52	91.4	85.6	84.4	82.0	81.8
Labelling yield under vacuum, T = 72 h					
2.6	100	99.7	99.7	98.6	98.5
5.2	99.5	99.5	98.5	98.0	97.5
7	99.5	99.0	98.0	97.9	97.0
8	99.5	99.0	98.0	97.0	96.0
9	99.5	98.8	96.8	96.5	95.5
10.4	99.5	98.5	95.8	93.7	92.0
26	88.2	83.2	76.8	74.8	72.7
30	85.2	81.9	76.6	74.4	72.2
37	84.3	78.4	75.2	72.3	70.1
52	46.0	39.6	37.1	35.5	34.5
Labelling yield under vacuum, T = 24 h					
2.6	99.5	99.0	99.0	98.0	98.2
5.2	99.5	99.0	99.0	98.0	97.5
10.4	99.6	99.0	98.5	98.5	96.0
11	99.7	98.5	98.5	97.5	97.0
12	99.5	98.5	98.0	97.5	97.0
13	99.5	98.0	98.0	97.0	96.0
26	98.1	97.8	97.9	97.1	96.6
30	96.5	95.1	93.9	93.7	92.0
37	94.5	88.3	85.5	83.9	82.6
52	94.5	93.4	86.4	85.4	80.1
Labelling yield under vacuum, T = 2 h					
2.6	100.0	100.0	100.0	100.0	100
5.2	100.0	100.0	100.0	100.0	100
10.4	100.0	100.0	100	100	100
26	98.6	99.0	97.5	98.5	98.0
30	98.7	99.3	99.2	98.3	97.6
37	98.8	97.0	94.4	94.2	93.0
52	99	88.6	86.4	85.0	84.6

2.6, 5.2, 7, 8, 9, 10.4, 11, 12, 13, 26, 30, 37, and 52 MBq ^{99m}Tc/μg represents the labelled colloid albumin of 500; 250; 186; 162.5; 144.4; 125; 118;108;100; 50; 43.3; 35.1; and 25 μg.

[a] The average is based on a 10-fold iteration of each chromatography. Results of maximum found RCP: 72 h nitrogen, 8 MBq/μg; 72 h vacuum, 9 MBq/μg; 24 h nitrogen, 12 MBq/μg; 24 h nitrogen, 26 MBq/μg; 2 h nitrogen 30 MBq/μg; and 2 h nitrogen, 30 MBq/μg. ^{99m}Tc/μg.

Table 3
Labelling yield of colloid albumin stored at −20 °C.

MBq/μg	$T = 2$ h			$T = 24$ h		
	0.5 h	5 h	8 h	0.5 h	5 h	8 h
10.4	100	99.8	99.4	99.7	99.5	99.3
26	100	99.4	99.2	×	×	×

Mean labelling yield (%) ^{99m}Tc-colloid albumin from an elution at $T = 2$ and 24 h (after a previous elution) with specific concentrations of 10.4 and 26 MBq/μg (represents labelled 125 and 50 μg), using ascending chromatography on ITLC-SG, with 95% MEK as the mobile phase. (Rf = 0: ^{99m}Tc-colloid albumin; Rf = 1: free $^{99m}TcO_4^-$). The average is based on a 16-fold iteration of each chromatography in a period of 16 weeks after preparing the colloid albumin concentrations.

3.5. Residual activity adhering to the inner surface of the glass vials

Residual radioactivity after rinsing the glass vial with 50 ml of saline yielded at $T = 20$ min 16–18% of ^{99m}Tc and at $T = 3$ h 17–30% ^{99m}Tc (most probably in the form of TcO_2). The addition of 0.1 ml (0.01 M) cysteine to a vial resulted in a residual activity of ^{99m}Tc of 3–12% at $T = 3$ h. Adding 0.1 ml (0.01 M) cysteine+0.2 mg stannous chloride dihydrate to a vial resulted in a residual activity of ^{99m}Tc at $T = 3$ h that was only 1.5–2% ^{99m}Tc (Table 5).

3.6. In vivo results

In all series, a normal distribution of age and tumour size was found.

Group 1: In 30 patients (72 h elution under nitrogen), the SLN was successfully localised, and 41 nodes (mean 1.37; range 1–2) were removed. The count rates varied by a factor of 14, from 31 to 412 cps (counts per second) (mean for first node, 179 cps). In 11 patients (33%), it was difficult to localise the SLN. In three patients, the SLN could not be visualised. Immunohistochemistry (IHC) and haematoxylin–eosin (HE) staining revealed tumour-negative nodes in 22 patients (Table 6).

Group 2: In 71 patients (24 h elution under nitrogen), the SLN was successfully localised, and 114 nodes (mean 1.61; range 1–4) were removed. The count rates varied by a factor of 100, from 35 to 3495 cps (mean for first node, 1071 cps). In three patients, the SLN could not be visualised in the axillary region probably because of a large prior biopsy. In this group, IHC and HE staining revealed tumour-negative nodes in 51 patients. Comparing groups 1 and 2 in terms of count rate, there was a significant difference ($P = 0.001$).

Group 3: In the third group (24 h elution under vacuum), the SLN was successfully localised in 70 patients, and 117 nodes (mean 1.67; range 1–5) were removed. The count rates varied by a factor of 88, from 78 to 6902 cps (mean for first node, 1108 cps). In three patients, the SLN could not be visualised (the SLNs of two of these patients were fully infiltrated by the tumour). IHC and HE staining revealed tumour-negative nodes in 44 patients. A comparison of groups 1 and 3 in terms of count rate yielded a significant difference ($P = 0.001$). The comparison of groups 2 and 3, however, showed no difference ($P = 0.833$).

Group 4: In 83 patients (2 h elution under vacuum), the SLN was successfully localised, and 130 nodes (mean 1.60; range 1–4) were removed. The count rates varied by a factor of 88, from 90 to 6213 cps (mean for first node, 1738 cps). In three patients, the SLN could not be visualised (SLN of all three patients revealed >80% tumour infiltration). IHC and HE staining revealed tumour-negative nodes in 47 patients. Comparing groups 1 and 4 in terms of count rate, there was a significant difference ($P = 0.001$), as was

Table 4
The yield of different valences of Tc-cysteine at Rf = 0.6 with maximum labelled specific activity when performing a $T = 2$, 24, and 72 h elution after the previous elution of a $^{99}Mo/^{99m}Tc$ generator.

Labelled yield ^{99m}Tc+cysteine (Rf = 0.6)	2 h nitro	2 h vac	24 h nitro	24 h vac	72 h nitro	72 h vac
$T = 1$ h, 2.6 MBq/μg used (500 μg)	100–94.7	100–93.0	100–92.1	100–91.7	100–91.0	100–90.8
$T = 1$ h, 10.4 MBq/μg used (125 μg)	100–94.7	100–93.0	100–92.0	100–91.8		
$T = 1$ h, 26 MBq/μg used (50 μg)	100–94.8	100–93.1		100–91.8		
T = 1 h, 30 MBq/μg used (43.3 μg)	100–94.8	100–93.2				
$T = 3$ h, 2.6 MBq/μg	100–94.2	100–89.0	100–91.7	100–83.6	100–82.0	100–82.1
$T = 3$ h, 10.4 MBq/μg	100–94.2	100–89.0	100–91.6	100–83.5		
$T = 3$ h, 26 MBq/μg	100–94.1	100–89.1		100–83.4		
$T = 3$ h, 30 MBq/μg	100–94.0	100–89.1				
Labelled yield ^{99m}Tc+cysteine+stannous chloride dihydrate (Rf = 0.6)						
$T = 1$ h, 2.6 MBq/μg	100–100	100–100	100–99.5	100–100	100–99.2	100–99.1
$T = 1$ h, 10.4 MBq/μg	100–100	100–100	100–99.6	100–100		
$T = 1$ h, 26 MBq/μg	100–100	100–100		100–100		
$T = 1$ h, 30 MBq/μg	100–100	100–100				
$T = 3$ h, 2.6 MBq/μg	100–99.9	100–99.9	100–99.2	100–99.5	100–99.0	100–99.1
$T = 3$ h, 10.4 MBq/μg	100–99.9	100–99.9	100–99.2	100–99.5		
$T = 3$ h, 26 MBq/μg	100–99.8	100–99.8		100–99.4		
$T = 3$ h, 30 MBq/μg	100–99.8	100–99.8				

2.6, 10.4, 26, and 30 MBq ^{99m}Tc/μg represents the labelled colloid albumin of 500; 125; 50; and 43.3 μg.
Results of thin layer chromatography are presented quantitatively as all valences of Tc (first number) and pure labelled TcO_2-colloid albumin (second number).The quality of the solutions was assessed by testing the labelling efficiency and RCP after $T = 1$ and 3 h. The average is based on a 10-fold iteration of each chromatography. Ascending thin layer chromatography on ITLC-SG with 12% trichloroacetic acid (TCA in saline) as the mobile phase (Rf = 0: ^{99m}Tc-colloidal; Rf = 06: ^{99m}TcO-cysteine) were used. Ascending thin layer chromatography on ITLC-SG, with 12% TCA acid (in saline) after cysteine+stannous chloride dihydrate as the mobile phase (Rf = 0: ^{99m}Tc-colloidal; Rf = 0.6: ^{99m}TcO-cysteine; Rf = 1: $^{99m}TcO_4^-$) was used.

Table 5
Results of the percentage of residual activity of $^{99m}TcO_2^+$ adhered to the inner surface of the preparing glass vial left from maximum labelled specific activity when performing a $T = 2$, 24, and 72 h elution after the previous elution of a $^{99}Mo/^{99m}Tc$ generator.

Labelling condition (under)	2-h elution		24-h elution		72-h elution	
	Nitrogen	Vacuum	Nitrogen	Vacuum	Nitrogen	Vacuum
$T = 20$ min, 2.6 MBq/μg	16	17	17	18	18	18
$T = 20$ min, 10.4 MBq/μg	17	18	17	18		
$T = 20$ min, 26 MBq/μg	17	18		18		
$T = 20$ min, 30 MBq/μg	17	18				
$T = 3$ h, 2.6 MBq/μg	17 (16)	18 (17)	17 (17)	18 (18)	18 (18)	18 (18)
$T = 3$ h, 10.4 MBq/μg	17 (17)	18 (18)	18 (18)	22 (18)		
$T = 3$ h, 26 MBq/μg	17 (17)	18 (18)		30 (18)		
$T = 3$ h, 30 MBq/μg	17 (17)	18 (18)				
$T = 3$ h, 2.6 MBq/μg+cysteine	3.5 (16)	4 (17)	3 (17)	12 (18)	3 (18)	3 (18)
$T = 3$ h, 10.4 MBq/μg+cysteine	3.5 (17)	4 (18)	3 (18)	12 (22)		
$T = 3$ h, 26 MBq/μg+cysteine	3.5 (17)	4 (18)		12 (30)		
$T = 3$ h, 30 MBq/μg+cysteine	3.5 (17)	4 (18)				
$T = 3$ h, 2.6 MBq/μg+cys+Sn (II)	1.5 (16)	2 (17)	2 (17)	2 (18)	2 (18)	2 (18)
$T = 3$ h, 10.2 MBq/μg+cys+Sn(II)	1.5 (17)	2(18)	2 (18)	2 (22)		
$T = 3$ h, 26 MBq/μg+cys+Sn(II)	1.5 (17)	2 (18)		2 (30)		
$T = 3$ h, 30 MBq/μg+cys+Sn(II)	1.5 (17)	2 (18)				

2.6, 10.4, 26, and 30 MBq ^{99m}Tc/μg represents the labelled colloid albumin of 500; 125; 50; and 43.3 μg.
Vials were rinsed with 50 ml saline. Rest activity at $T = 20$ min (without cysteine+stannous chloride dihydrate) and at $T = 3$ h (with and without cysteine+stannous chloride dihydrate).

also the case for comparisons of groups 2 and 4 ($P = 0.002$) and groups 3 and 4 ($P = 0.005$).

Group 5: In 102 patients (2 h elution under nitrogen), the SLN was successfully localised, and 158 nodes (mean 1.56; range 1–4) were removed. The count rates varied by a factor of 83, from 103 to 8582 cps (mean for first node, 1873 cps). In one patient, the SLN could not be visualised (SLN revealed >80% tumour infiltration). IHC and HE staining revealed tumour-negative nodes in 65 patients. Comparing groups 1 and 5 in terms of count rate, a significant difference ($P = 0.001$) was found, as was also the outcome for comparisons of groups 2 and 5 ($P = 0.001$) and groups 3 and 5 ($P = 0.003$), but not groups 4 and 5 ($P = 0.481$) (Fig. 4).

4. Discussion

We have evaluated different labelling conditions and concentrations of colloid albumin on the RCP and stability of ^{99m}Tc-labelled Nanocoll®, in order to identify the optimal labelling procedure for this radiotracer. We also tested the effects of specific activity of the tracer on the ex vivo count rates of SLNs, which

Table 6
Results in the different patient groups[a].

Group	1	2	3	4	5
N	33	74	72	86	103
No. of successful procedures (SP)	30 (90%)	71 (96%)	70 (97%)	83 (97%)	102 (99%)
No. of second injections (SI)	4	3	2	3	1
No. of failed procedures after (SI)	3	3	2	3	1
Removed nodes	41(1–2)	114(1–4)	117 (1–5)	130 (1–4)	158 (1–4)
Lymph nodes, mean ± SD	1.37 ± 0.49	1.61 ± 0.83	1.67 ± 0.90	1.60 ± 0.75	1.56 ± 0.75
cps (SP)	31–412	35–3495	78–6902	93–6213	103–8582
cps (mean ± SD), first node	179 ± 115	1071 ± 949	1108 ± 1039	1738 ± 1596	1873 ± 1599
95% confidence interval for mean (lower bound–upper bound)	136–222	846–1295	836–1380	1385–2092	1557–2189
IHC and HE staining negative	22 (73%)	51 (72%)	44 (63%)	47 (58%)	65 (64%)

SD; standard deviation.
[a] Group 1: 72-h elution-nitrogen; group 2: 24-h elution-nitrogen; group 3: 24-h elution-vacuum; group 4: 2-h elution-vacuum; and group 5: 2-h elution-nitrogen. Mean cps rates between 72–24 h and 2 h elution are 179 cps: 1090 cps: 1806 cps. Mean cps rates between a 72 h (nitrogen) and 24 h elution (nitrogen/vacuum) is 6 times fold higher. Mean cps rates between a 24 and 2 h elution vacuum/nitrogen is 1.7 times fold.

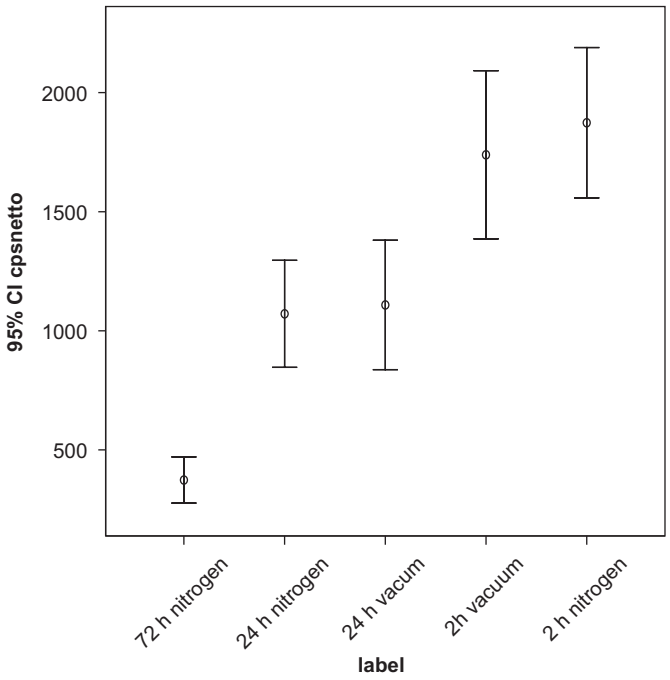

Fig. 4. Results of series 1–5; 95% cpsnetto (2a) and 95% confidence interval for mean (2b). Comparing series 1 and 2 (72 h nitrogen/24 h nitrogen) in terms of count rates, a significant difference ($P = 0.001$) was found; between series 1 and 3 (72 h nitrogen/24 h vacuum), $P = 0.001$; between series 2 and 4 (24 h nitrogen/2 h vacuum), $P = 0.002$; between series 3 and 4 (24 h vacuum/2 h vacuum), $P = 0.005$; between series 2 and 5 (24 h nitrogen/2 h nitrogen), $P = 0.001$; and between series 3 and 5 (24 h vacuum/2 h nitrogen), $P = 0.003$. Comparing series 2 and 3 (2 h nitrogen/2 h vacuum) in terms of count rates, no further significance was found ($P = 0.833$), as was also the case between series 4 and 5 (2 h vacuum/2 h nitrogen; $P = 0.481$).

proved to be higher for SLNs when using Nanocoll® with a high specific activity. The count rates of SLNs that were evaluated using the 24 h eluate were approximately 6-fold higher as compared to the 72 h eluate, whereas the rates were 2-fold higher for the 2 h eluate as compared to the 24 h eluate (Gommans et al., 2003).

Theoretically, 1.3 GBq of ^{99m}Tc is equivalent to 40×10^{12} atoms (Ballinger 2004). From an integral function of disintegrated atoms starting with standard activity of 1.3 GBq of ^{99m}Tc at preparation time $T = 0$ h (24 h eluate), a total of 4.08×10^{13} atoms ^{99m}Tc and 8.5513 atoms ^{99}Tc can be calculated (Gommans et al., 2001). There would be 1.44×10^{14} atoms of Tc, and 5.2×10^{13} atoms ^{99m}Tc in a 2 h eluate (Ballinger, 2004). If this is added to 10% of a standard kit, which equals 5.5×10^{13} particles of colloid albumin (50 μg), we observe in a 2 h eluate an average of 1 atom of ^{99m}Tc per particle of colloid albumin (24 h; average ~2.7 atoms/particle of colloid albumin). Thus, the same amount of ^{99m}Tc has been added to the same number of particles (Ballinger, 2004).

The difference in RCP between a labelling performed under nitrogen and under vacuum is interesting. Although in both systems oxygen is removed, vacuum shows better results. The reason behind this is not totally clear, but we speculate that oxygen is introduced in the vial in two ways. The first is that air is dissolved in the (diluted) eluate, introducing oxygen into the vial during the addition of pertechnetate. A second possibility is that energy transfer in the aqueous solution, due to the radioactive decay, causes radiolysis. One of the products formed in radiolysis is oxygen. In case of a vacuum vial, the dissolved air will be transferred from the liquid phase into the vacuum and cannot or is less able to react with the dissolved stannous ions. This pressure difference is not present in the preparation vial under nitrogen. The stannous content in vials under vacuum above the liquid is evidently higher.

Our results indicate that the stannous chloride content is critical with respect to the RCP facilitation of or reduction from Tc (VII) to Tc (IV). Because it is critical to exclude oxygen, labelling a high specific activity of ^{99m}Tc colloid albumin under vacuum should be reserved only for experienced personnel. Moreover, extracting the colloid albumin solution from the original vial, transferring it into a vacuum vial, and subsequently adding the ^{99m}sodiumpertechnetate has to be performed in a very short time. On the other hand, the presence of small amounts of oxygen during the radio-labelling process of colloid albumin can still yield a radiochemical labelling of typically 98–100% (when measured 30 min later by TLC). Such percentages of labelling are typically achieved in daily nuclear medicine practice. The effects of time on the specific activity of ^{99m}Tc colloid albumin, in the presence of small amounts of oxygen, have not been described clearly in the literature. In the present study, we performed TLC at $T = 2.5$, 8 and 24 h after labelling of colloid albumin (exposed to small amounts of oxygen) and we observed a sharp decline of the specific activity of ^{99m}Tc-colloid albumin to $<80\%$ at 2.5 h after the labelling process. This is probably due to oxidation of stannous chloride. At later time points, we observed only a slight, further decline in specific activity.

Preparing 10 MBq/μg ^{99m}Tc colloid albumin in a vacuum and under nitrogen using a 72 h elution resulted in a labelling efficiency of $<95\%$ at $T = 5$ h. This finding is not in accordance with product specifications, and a 72 h elution is therefore not to be recommended for high specific labelling of ^{99m}Tc colloid albumin.

The reduction of Tc (VII) is a multi-stage process, and it can be assumed that further reduction of Tc (V) to Tc (IV) can be achieved by an increase of ionic concentrations in the equilibrium. In the first phase of labelling ^{99m}Tc colloid albumin, Tc (VII) reduces to Tc (V and IV) with stannous chloride dihydrate. Table 5 of the manuscript shows the percentage of residual $^{99m}TcO_2^+$ that is adhered to inner surface of the glass wall and is demonstrating that under all conditions, this percentage is similar (16–18%). After this first reduction phase, no further spontaneous reduction takes place. The different valence of Tc (V) in solution can be demonstrated through complexing with cysteine (Stalteri et al., 1999). Addition of cysteine to the vials (without changing the stannous concentration) promotes a further reduction of Tc^{5+} to Tc^{4+}. Addition of $SnCl_2$ at $T=3$ h only slightly improves further reduction of TcO_2^+ to TcO_2 except in the 24 h vacuum samples. Therefore, a decrease of stannous chloride concentration makes no differences for the experiment (see Table 5). It is likely that during the labelling of ^{99m}Tc colloid albumin under $SnCl_2$, the involved Tc (VII) does not fully reduce to valence state IV, but to valence state V and IV (see Table 5). To reach complete reduction of technetium from valence state VII to IV, additional cysteine or even ascorbin acid may be helpful. In the present experiments, addition of $SnCl_2$ at $T=3$ h only slightly improved further reduction of TcO_2^+ to TcO_2 in the 24 h vacuum samples.

In this experiment, cysteine was added to separate Tc^{5+} from other valences. Adding cysteine to the solution might result in acidification because of the low pKa of cysteine. H^+ atoms might influence the behaviour of the TcO_2 (Eshima et al., 2000; Schűmichen et al., 1979); e.g., the TcO_2 can be dissolved partly by the acid forming Tc^{4+} and water. Acid solutions are also known for reducing absorption to glass surfaces. The lower amount of activity of $^{99m}TcO_2$ at the glass surface of a preparation vial as measured before and after adding cysteine will probably therefore be caused by the phenomenon of adhesion of H^+ atoms to the active sites at the glass surface. Dissolving $^{99m}TcO_2$ from the glass surface will bring it into solution, and it will also be directly labelled in addition to colloid albumin particles.

Eluting a ^{99}Mo/^{99m}Tc generator twice daily leads to different ratios of ^{99}Tc/^{99m}Tc atoms. A mother/daughter relationship of ^{99}Mo and ^{99m}Tc/^{99}Tc showed a 1:2.6 ratio assigned to an elution obtained 24 h after a previous elution (14% of ^{99}Mo decays directly to ^{99}Tc). The advantage of labelling ^{99m}Tc colloid albumin with a 2 h elution is the increased number of ^{99m}Tc atoms (^{99m}Tc/^{99}Tc ratio 3.3:1). However, a disadvantage for high specific labelling using a 2-h eluate is the need for a high activity generator.

Raising the number of ^{99m}Tc atoms and therefore the less-involved ^{99}Tc atoms in solution leads to a higher labelled number of ^{99m}Tc atoms per single colloid particle. Eluting a ^{99}Mo/^{99m}Tc generator twice a day ($T=0$ and 2 h) will heighten the daily production of ^{99m}Tc by 20% without influencing the daily ingrowth of ^{99m}Tc from the ^{99}Mo generator.

In vitro testing of the effective specific activity, using an increased ^{99m}Tc:^{99}Tc ratio in labelling ^{99m}Tc-colloid albumin, showed improved labelling efficiency. Lymphatic transport of an equal concentration of colloid albumin particles labelled with a higher effective specific activity (MBq/μg colloid albumin) can be performed to increase the cps of ^{99m}Tc-colloid albumin in sentinel nodes. For labelling colloid albumin using the maximum specific concentration, an excess quantity of ^{99m}Tc is necessary. Labelling at $T=2$ h with a maximum specific activity of 1.3 GBq ^{99m}Tc to a tenth part of an original vial of Nanocoll® gives the same results in vitro as labelling of 13 GBq ^{99m}Tc. Although not in accordance with product specifications and currently not a registered radiopharmaceutical for labelling colloid albumin specially designed for SLN detection, highly effective specific labelled ^{99m}Tc colloid albumin is recommended for detection of SLNs. Given that significance was achieved between 72 h nitrogen, 24 h nitrogen/vacuum and 2 h vacuum/24 h vacuum with no further significance in comparing 2 h vacuum/2 h nitrogen, we recommend a labelling protocol for preparing ^{99m}Tc colloid albumin: performing a 2 h elution after an initial elution and labelling under nitrogen with 26 MBq/μg. This protocol is easy to perform and ^{99m}Tc colloid albumin can be labelled in any department of nuclear medicine.

Further studies on this not-yet-registered radiopharmaceutical for the use of sentinel node localisation in breast cancer is recommended. These data support the development of a colloid albumin specially designed for sentinel node detection.

5. Conclusion

In view of patient care, to avoid patient re-injection, unnecessary axillary lymph node dissection, and/or false-negative diagnostics, we have described an optimal labelling procedure of colloid albumin with ^{99m}Tc.

Labelled ^{99m}Tc colloid albumin with a 2 h elution of a ^{99}Mo/^{99m}Tc generator yields optimal labelling efficiency and RCP. Although it is not yet a registered radiopharmaceutical for sentinel node localisation, we recommend that the protocol for preparing ^{99m}Tc colloid albumin should be a 2 h elution under nitrogen with a specific activity of 26 MBq/μg.

In contrast to the product information, we found that labelling 10.4 MBq/μg ^{99m}Tc colloid albumin in nitrogen using a 72 h elution, as the first elution, commonly occurring after a weekend or as a first elution from a factory-fitted generator, yields unacceptable RCP.

Therefore, a 72 h elution is not recommended for high specific labelling of ^{99m}Tc colloid albumin. Ex vivo, significantly higher count rates were reached using a 2 h elution in vacuum or nitrogen. Labelling 26 MBq/μg ^{99m}Tc colloid albumin with a 2 h elution under nitrogen did not achieve further significance but is recommended because of the ease of labelling.

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