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ORIGINAL RESEARCH ARTICLE

Preparation and biomolecule conjugation of [^{99m}Tc]Tc-MAG3

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ABSTRACT

Introduction: [^{99m}Tc]Tc-MAG3 is one of the routine renal radiopharmaceuticals being used in nuclear medicine centers, throughout the world. This study investigated the synthesis and ^{99m}Tc-labeling of MAG3, as well as the synthesis of the S-acetyl-MAG3-NHS complexing agent, which was used for labeling bovine serum albumin (BSA) as a protein model using technetium-99m.
Methods: S-acetyl-MAG3 was prepared by the reaction of S-acetyl thioglycolic acid and triglycine. It was also activated to its N-hydroxysuccinimide counterpart which was used for preparation of biomolecule conjugates. All compounds and intermediates were characterized by 1H NMR and LC/Mass spectroscopy. Labeling of MAG3 with 99m-technetium was also well performed. The radiochemical purity and stability of labeled products was done by thin-layer chromatography. Also, biodistribution studies in mice was performed.
Results: The spectroscopic results confirmed the structure of compounds. The stability of [^{99m}Tc]Tc-MAG3 and [^{99m}Tc]Tc-MAG3-BSA was determined over 24h .It was found to drop from 90% to 60% and 99% to 80%, respectively. There was no difference between serum and buffer results. Biodistribution studies for [^{99m}Tc]Tc-MAG3 confirmed renal excretion with injected dose per gram (%ID/g) kidney of 41.28 ± 4.70 , 45.63 ± 6.36 and 12.22 ± 2.83 after 1, 4 and 24h respectively.
Conclusion: In this work, the rigorous purification processes were simplified through adjustment of molar ratios of reactants and the crude product obtained with higher yield was directly used for ^{99m}Tc labeling. The prepared labeled biomolecules conjugates showed acceptable radiochemical purity and stability. MAG3 was applicable for renal imaging according to biodistribution results.

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INTRODUCTION

Technetium-99m is used annually in tens of millions of medical diagnostic methods, making it the most commonly used medical isotope in the world. ^{99m}Tc emits detectable gamma rays with a photon energy of 140 kV (these photons have about the same wavelength as those emitted by conventional X-ray diagnostic equipment) and has a half-life of six hours for gamma emission. In mid-1985, Fritzberg et al. introduced mercaptoacetyl triglycine (MAG3) [1]. MAG3, or mertiatide, is a stable complexing agent with technetium activity that is used to scan renal secretion and evaluate renal function [2-5]. The N3S structure of this complex creates a stable complex with technetium. Due to the presence of a sulfur group and the possible formation of disulfide bonds in this complex, the protective substance S-benzoyl is used on the sulfur group. This protective group is removed by heating at 100 °C for 10 minutes, after which the agent is ready to form a stable complex with technetium (Figure 1). S-benzoyl-MAG3 is sold under the brand name Betiatide [6-8]. One of the advantages of labeling with this complex is that it does not require separation and purification [9].

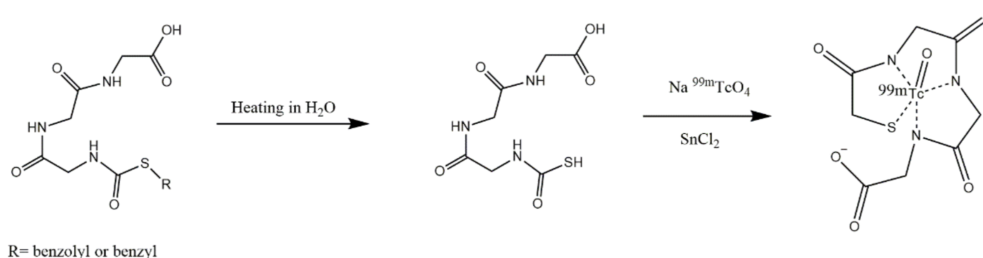

Fig 1. Preparation of [^{99m}Tc]Tc-MAG3 from S-benzoyl or benzyl MAG3

To prevent the alkaline environment and boiling water bath for separating the benzoyl protecting group, a new compound, S-acetyl-MAG3 NHS ester, was synthesized, with the acetyl replacing the benzoyl group. The acetyl group, as a good protecting group for sulfhydryl, can be separated at milder conditions, including normal pH [10]. This evolved S-acetyl-MAG3-NHS can potentially be used to label thermosensitive amine-containing aptamers, oligonucleosides [11, 12], and proteins or peptides with ^{99m}Tc [13, 14] (Figure 2).

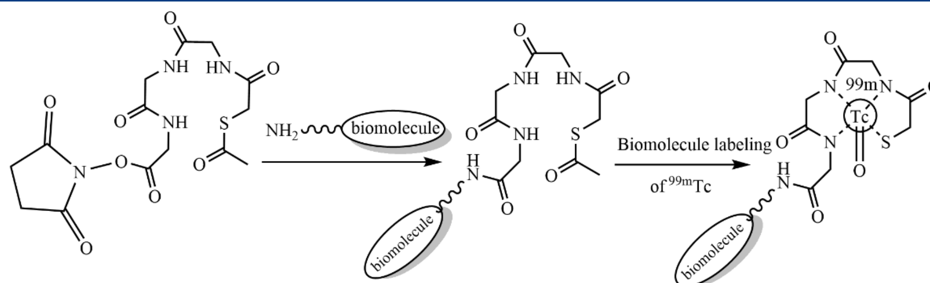

Fig 2. Conjugation S-Acetyl-NHS-MAG3 ester with biomolecules and labeling with ^{99m}Tc

The steps of binding the MAG3-NHS complex to biomolecules to tag 99m-technetium activity was discussed in detail [15, 16].

This study investigated the synthesis and ^{99m}Tc-labeling of MAG3, which is superior to compounds of all classes in general for evaluating kidney function. Previously reported procedures for preparation of MAG3 required time-consuming and yield-lowering purification methods which was simplified in current study [9, 17]. It was also used to prepare S-acetyl-MAG3-NHS for labeling proteins that might bind to the NH2 group of biomolecules and was labeled in milder conditions relative to S-benzolyl-MAG3-NHS.

METHODS

Materials

Reagents including triglycine, NHS-activated S-acetyl thioglycolic acid (SATA), N-hydroxysuccinimide (NHS), and dicyclohexyl carbodiimide (DCC) were purchase from Sigma-Aldrich, USA. All other necessary solvents, dimethyl formamide (DMF), N-methylpyrrolidone (NMP), thin-layer-chromatography (TLC) strips and silica gel G60 were purchased from Merck, Germany.

Synthesis of S-acetyl-MAG3 in water

S-Acetyl-MAG3 was synthesized according to Wang's study [9], with some modifications. Briefly, 72 mg

triglycine (0.378 mmol) was placed into a 10 ml flask, and 4ml of deionized water was added. To improve solubility and create an alkaline environment, 32 mg of sodium bicarbonate was added to the flask, which was then warmed to 60 °C. After the solution cleared, it was passed through a 0.45 syringe filter. Then 100 mg (0.436 mmol) of SATA was added to it in three steps, allowing 15 minutes between each step. The pH of the solution was controlled to be between 7 and 8 during the steps through the gradual addition of a maximum 65 mg sodium bicarbonate. After SATA addition, the transparent solution was acidified with the addition of a few drops of 6 M hydrochloric acid. After overnight in 5°C, the crude yellow precipitate was used directly in the next steps. TLC study with acetonitrile / acetic acid (20:1, v/v) confirmed S-acetyl-MAG3 as the major spot, NHS and traces of unreacted SATA and hydrolyzed SATA. For identification the residue was further purified on a 1 x 20 cm silica gel column eluted with acetonitrile.

Synthesis of S-acetyl-MAG3-NHS in NMP

To 61 mg (0.20 mmol) of the crude product of the previous step were added 1ml of anhydrous NMP. 37mg (0.180 mmol) of DCC at 100µl anhydrous NMP was dissolved and quickly added to the solution at -20 °C. The reaction mixture was stirred at this temperature for 4 h, and then at overnight at 4 °C. The dicyclohexyl urea (DCU) precipitate was removed by centrifugation at 350 rpm for 15 min. 5 ml of anhydrous diethyl ether were added to the reaction. We vortexed the tubes tightly to make the ethereal layer transparent. After removing the ethereal layer a further 5 ml of diethyl ether was added. This was repeated three times. After the ether layer was removed, the crude S-acetyl-MAG3-NHS precipitate was dried. The crude residue was used directly. TLC study with acetonitrile/ triethylamine (50:1. v/v) showed S-acetyl-MAG3-NHS as the main spot, and traces of impurities. For identification purposes, the residue was further purified over a 1 x 20 cm silica gel column (Silica Gel 60, 0.2-0.5 mm particle size) eluted with acetonitrile/methanol (80:20, v/v).

Synthesis of S-Acetyl-MAG3 in DMF

S-Acetyl-MAG3 was synthesized as previously reported by Winnard et al. with some modifications [17]. In this method, 1 ml of NaOH (0.2M) was mixed with 50 mg triglycine (0.264 mmol) and 10 µl of freshly prepared ethylene diamine tetra-actate (EDTA) 50 mM, then passed through a 0.45µ filter. In another flask, 90 mg (0.390 mmol) of SATA was mixed with 1 ml of dry DMF and added dropwise to the solution prepared in the previous step. At first, the solution became slightly cloudy, but after 15 minutes it became clear. After 15 minutes at ambient temperature, the pH of the reaction was reduced from 8.9 to 2.7 using 6 M hydrochloric acid. A pH 8.9 was used for triglycine deprotonation. The SATA acetyl group might be hydrolyzed if the pH was not controlled; thus the pH needed to be reduced immediately. After overnight at 5°C, the crude yellow precipitate was used directly in the next steps. TLC study with acetonitrile/acetic acid (20:1, v/v) showed S-acetyl-MAG3 as the major spot, NHS and traces of unreacted SATA and hydrolyzed SATA.

For identification, the residue was further purified on a 1 x 20 cm silica gel column with acetonitrile.

Synthesis of S-Acetyl-MAG3-NHS in DMF

The crude S-acetyl-MAG3 88.5mg (0.290mmol) prepared above was added to 60 mg (0.290 mmol) DCC and mixed with 3.6 ml of dry DMF. The reaction solution was cloudy due to dicyclohexyl urea (DCU) precipitation. It was stirred at room temperature for 3 hours, placed at -20 °C for 1 h, and centrifuged at 4 °C for 15 minutes at 350 rpm. This operation was repeated three times to completely remove the DCU deposits. We reduced the reaction mixture volume using a rotary. The concentrated DMF solution of S-acetyl-MAG3-NHS was stored at -20 °C until it was used. TLC study with acetonitrile/triethylamine (50:1, v/v) showed S-acetyl-MAG3-NHS as the main spot with traces of impurities.

For identification purposes, the residue was further purified over a 1 x 20 cm silica gel column (Silica Gel 60, 0.2-0.5 mm particle size) eluted with acetonitrile/methanol (80/20, v/v).

Synthesis of MAG3-protein conjugate

BSA 20 mg (0.289µmol) was dissolved in 400 µl of phosphate buffer, and 100 µl of S-acetyl-MAG3-NHS DMF solution (1mg/ml) containing MAG3-NHS 0.1mg (0.248 µmol) was added to this solution. It became a bit cloudy. After one hour, the solution was transferred to a dialysis bag to remove any possible unreacted S-acetyl-MAG3-NHS.

The solution that was removed from the dialysis bag contained MAG3-BSA. The final product was freeze-dried. The total volume of the solution before freezing was 500 µl.

Preparation of [^{99m}Tc]Tc-MAG3-BSA

A 1 mg/ml solution of MAG3-BSA in acetonitrile and water (50:50) was prepared. A mixture of 100 µl of this solution, 100 µl of citrate buffer (0.1 M), 300 µl of disodium tartrate solution (40 mg/ml aqueous solution), and 100 µl of $SnCl_2$ (4 mg/ml in hydrochloric acid 0.05 N) was poured into a microtube. About 1 mCi $^{99m}TcO_4^-$ (in normal saline) was added to the microtube, which was then placed in a water bath at 50 °C for 10 minutes.

Quality control of [^{99m}Tc]Tc-MAG3

The potential radiochemical impurities in the preparation of [^{99m}Tc]Tc-MAG3 are free pertechnetate $^{99m}TcO_4^-$), colloidal technetium [^{99m}Tc]$(TcO_2)_n$, and

[^{99m}Tc]Tc-tartrate (precursor) [11]. To control the quality of the complex by the TLC paper. Two solvent systems were used. The first system was composed of 40% methyl ethyl ketone and 60% ethyl acetate. The second was 90% ethanol and 10% water. We prepared 10 cm papers with a width of 1 cm. The sample (5 µl) was loaded at the one-centimeter distance of the lower part of the TLC.

After the solvent rose to 1 cm from the top edge of the paper, we took the paper out of the solvent, dried it, and cut it in half. The amount of activity in each area of the paper was measured with a gamma counter device. Radiochemical purity (RCP) was measured as follow:

$$\%\ \text{impurities strip 1} = \frac{\text{Activity top part}}{\text{Activity top+bottom part}} \times 100 = {}^{99m}TcO_4$$

$$\%\text{impurities strip 2} = \frac{\text{Activity bottom part}}{\text{Activity top+bottom part}} \times 100 = ({}^{99m}TcO_2)\ n + [{}^{99m}Tc]Tc\text{-Tartrate}$$

RCP (%) = 100% - (%impurities strip 1 + % impurities strip 2)

Preparation of [^{99m}Tc]Tc-MAG3

For the MAG3 kit, we prepared [^{99m}Tc]Tc-MAG3 as before. We diluted 5 µl of this sample with the same amount of acetonitrile / water (50:50) and performed the rest of the preparation method as described above.

Stability of [^{99m}Tc]Tc -MAG3 and its conjugate by TLC

We prepared two separate samples in human serum and phosphate buffer for the [^{99m}Tc]Tc-MAG3 and [^{99m}Tc]Tc-MAG3-BSA kits. For each sample, we evaluated TLC quality at 0, 1, 2, 3, and 24h.

Purifiction of [^{99m}Tc]Tc -MAG3 with Sephadex column

We prepared the [^{99m}Tc]Tc-MAG3 kit and the [^{99m}Tc]Tc-MAG3-BSA kit and performed the following procedure for each one separately: the sample was added to the column, eluted by the phosphate buffer. The contents of the column were milked into 25 tubes, with 2ml in each tube, and the amount of activity in each tube and column was measured with a dose calibrator.

Biodistribution study

The biodistribution of the synthesized material was examined to confirm renal secretion. Nine mice were prepared in the laboratory. Each was injected with 100 µCi (for 1, 4h studies) and 300µCi (for 24h study) [^{99m}Tc]Tc MAG3 activity through the tail vein. The organs (intestine, heart, lung, spleen, kidney, liver, tail) were separated after 1, 4 and 24h (n=3), and then each placed in a gamma counter tube. The weight of each isolated limb was recorded and the activity of each organ in the gamma counter tube was measured.

RESULTS

Synthesis of S-acetyl-MAG3 and its NHS ester

In general, reaction of triglycine with SATA in the absence of moisture resulted in the intermediate formation of S-acetyl-MAG3. The MAG3 carboxylate group was activated in NMP or DMF with DCC in the presence of NHS to obtain the active form of S-acetyl-MAG3-NHS (Figure 3).

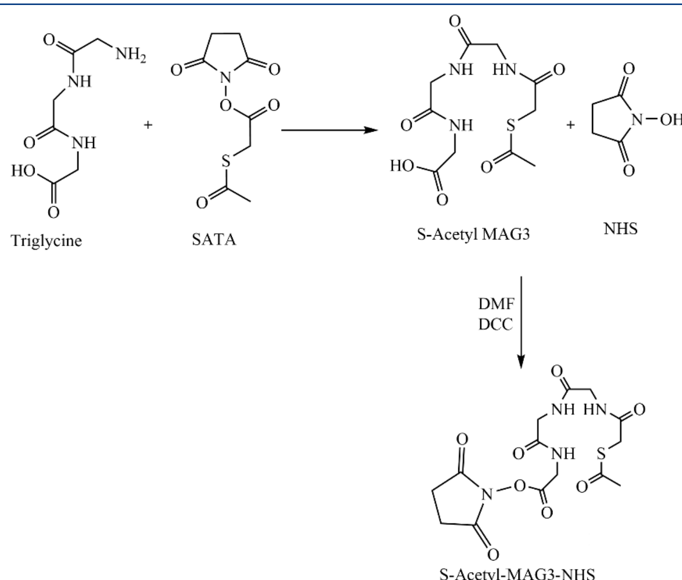

Fig 3. Steps for synthesis of S-Acetyl-NHS-MAG3

TLC study was performed to follow progress of reaction and purity of product. The crude S-acetyl-MAG3 and NHS-adduct were almost pure and were used directly without further purification.

To confirm the synthesis of the crude compounds of S-acetyl-MAG3 and its NHS ester, a small amount of crude sample was purified and characterized using NMR and LC/Ms spectrum.

The ^{1}H-NMR analyses were performed in D_2O at 300 MHz (Bruker-300). The chemical shifts for S-acetyl-MAG3 were 2.36 ppm (s, 3H, SCOCH3) and 3.66-3.78 ppm (m, 8H, COCH2). The chemical shifts for S-acetyl-MAG3-NHS were 2.38 ppm (s, 3H, SCOCH3); 2.80 ppm (s, 4H, succinimidyl); and 3.68-3.80 ppm (m, 8H, COCH2).

The liquid chromatography system, Shimadzu UFLC LC-AD20 system (Shimadzu, Japan) was equipped with DGU-20A$_{3R}$ Degasser, a Binary pump (LC-20AD), an auto sampler (SIL-20AC HT), and a column oven (CTO-20 AC). Analyte were separated with a SUPELCO analytical Discovery HS C18 column (150mm×4.6 mm, 3µm, PA, USA). The mobile phase were 0.1 % formic acid in methanol. The flow rate and injection volume were 0.25mL/min and 5 µL, respectively. The experiment was carried out at 30°C temperature. The chromatogram was shown in Figure 4 confirmed the purity of S-acetyl-MAG3-NHS which was eluted as a single peak at 5.28 min. Mass analysis was performed using 3200 QTRAP mass spectrometer instrument (AB Sciex, MA,USA) operated in positive (ESI$^+$) electrospray ionization mode. Analysis was performed with nitrogen using the following setting: gas supply was set at pressure of 10 psi, ion source gas 1 with a pressure of 40 psi and ion source gas 2 with a pressure of 40 psi. The source temperature and ion spray voltage (IS) were set at 400°C and 4.5kV, respectively. The molecular weight for $C_{14}H_{18}N_4O_8S$ was determined to be 403.7(M^++1).

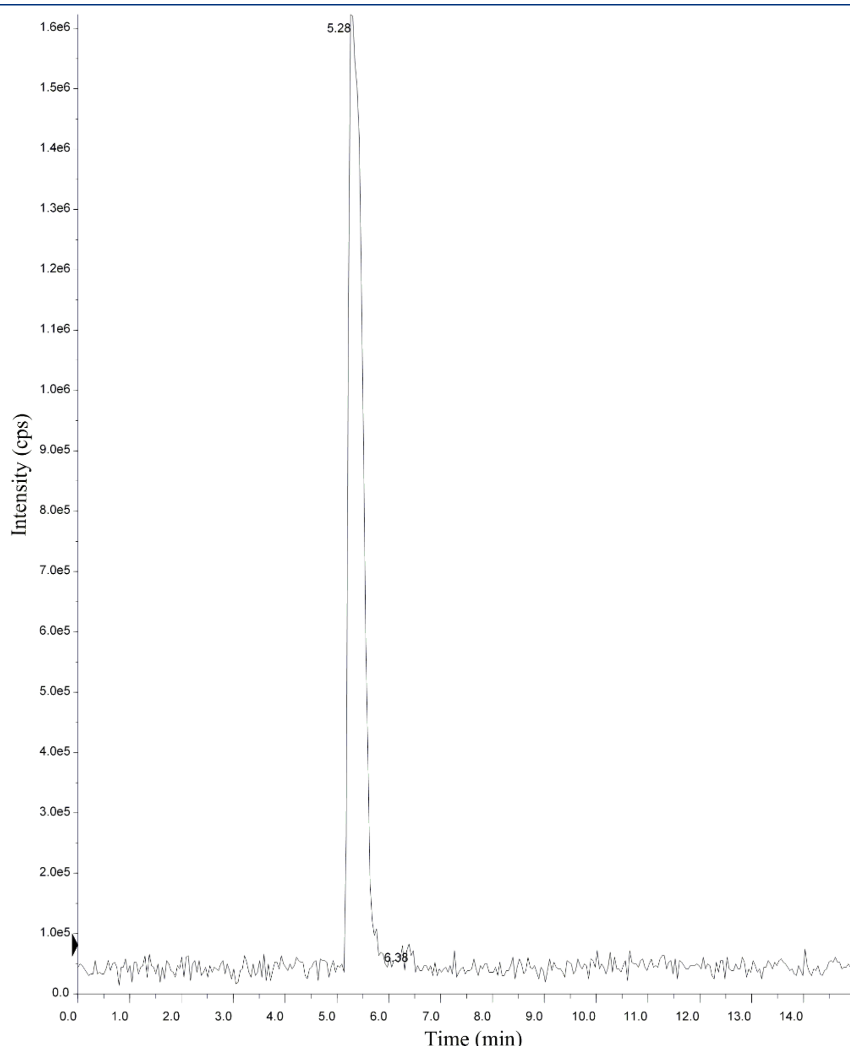

Fig 4. Chromatogram of S-acetyl-MAG3-NHS eluted with acetonitrile and formic acid 1% by LC-MS

Stability of [^{99m}Tc]Tc -MAG3 and its conjugate as measured by TLC

According to the data obtained from the TLC test in solvent systems one and two, the stability of the labeled compound [^{99m}Tc]Tc-MAG3 in serum and buffer up to 3 h were significant (Table 1). In the first solvent system, $^{99m}TcO_4^-$ free technetium impurities were located at the top of the paper. In the second

solvent system, colloidal technetium impurities [^{99m}Tc](TcO_2)n and [^{99m}Tc] Tc-tartrate were located at the bottom of the paper (Figure 5).

Table 1. Stability of [^{99m}Tc]Tc-MAG3 and [^{99m}Tc]Tc-MAG3-BSA complex in serum and phosphate buffer

Incubation	0	1h	2h	3h	24h
[^{99m}Tc]Tc-MAG3 serum	90.85	75.73	76.90	75.90	61.40
[^{99m}Tc]Tc-MAG3 buffer	90.85	79.00	76.76	75.41	51.00
[^{99m}Tc]Tc-MAG3-BSA buffer	99.87	98.86	97.52	96.70	80.00
[^{99m}Tc]Tc-MAG3-BSA serum	99.87	99.81	99.80	98.68	79.00

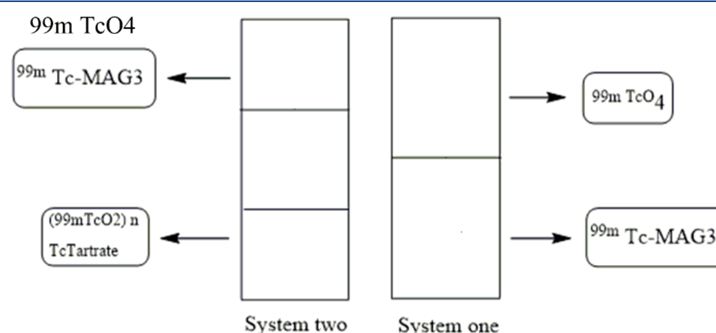

Fig 5. Location of the complex on TLC paper in systems one (40% methyl ethyl ketone and 60% ethyl acetate) and two (90% ethanol and 10% water)

According to the data obtained from the TLC test in solvent system one, the stability of the labeled compound [^{99m}Tc]Tc-MAG3-BSA in serum and buffer were 79 and 80% at 24h (Table 1).

The [^{99m}Tc]Tc-MAG3-BSA showed a better radiochemical purity versus [^{99m}Tc]Tc-MAG3 (99.8% versus 90%) . The stability of the ^{99m}Tc-MAG3-labeled complex in serum and buffer was over 90% at the beginning of a 24h period, and dropped to 60% and 50% in buffer and serum, respectively after 24h. The stability of [^{99m}Tc]Tc-MAG3-BSA in serum and buffer was approximately the same. The stability of [^{99m}Tc]Tc-MAG3-BSA was acceptable even after 24h, dropped from 99.87% to 80%.

Purity as measured by sephadex column

BSA protein contains 583 amino acids with MW of 69 kDa. Due to the fact that the ^{99m}Tc-MAG3-BSA was larger in size than the impurities, it was milked from the column sooner than other compounds, in tube numbers 7, 8, and 9. Other tubes' activity was related to impurities. Column activity included the free form, which was smaller in size than the other compounds. The radiochemical purity of [^{99m}Tc]Tc-MAG3-BSA was calculated to be 96% (Figure 6).

The [^{99m}Tc]Tc-MAG3 was expected to be milked later (tubes 8,9, and10) than the [^{99m}Tc]Tc-MAG3-BSA (tubes 7, 8, and 9) under similar conditions because of its smaller size. The purity of [^{99m}Tc]Tc-MAG3 was calculated to be 76.59% (Figure 7).

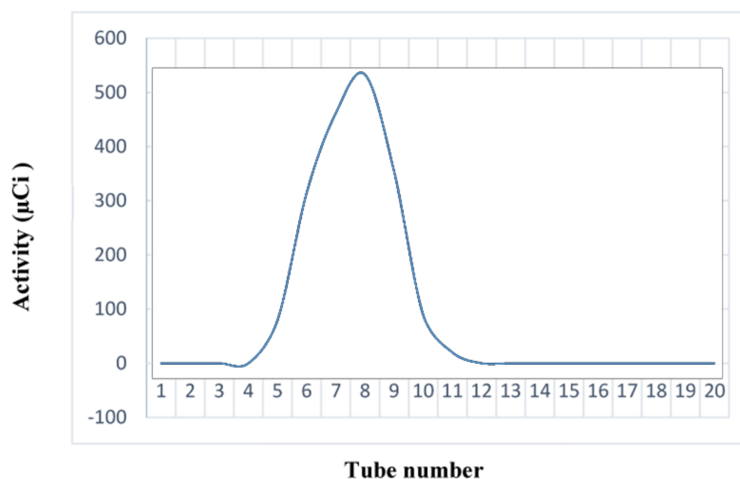

Fig 6. Activity values obtained from [^{99m}Tc]Tc-MAG3-BSA complex. Column activity: 370µCi; free form percentage: 3.6%; purity based on sephadex column peak (tubes 7, 8, and 9): 96%

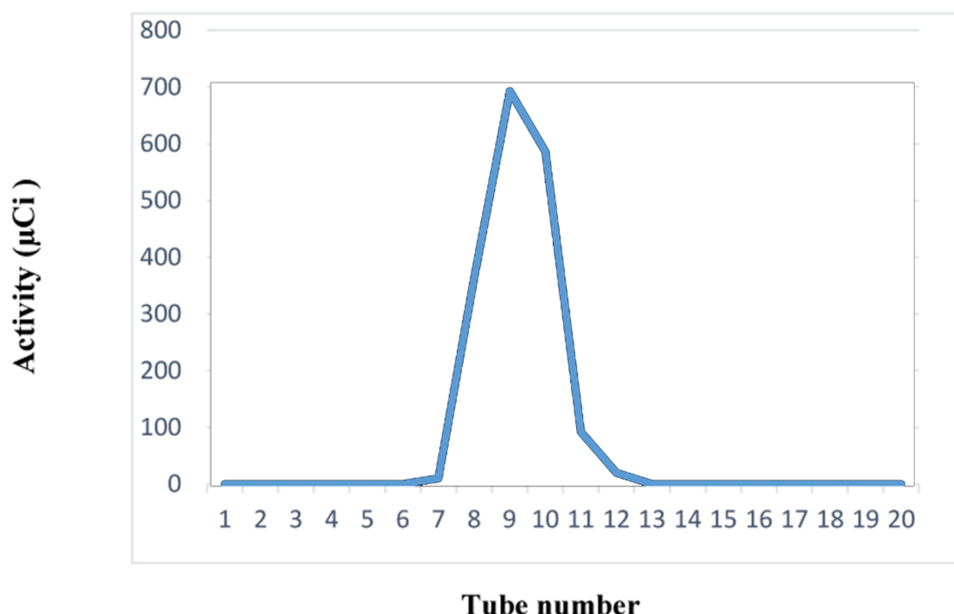

Fig 7. Activity values obtained from [99mTc]Tc-MAG3 complex. Column activity: 377 µCi; free form percentage: 17%; purity based on sephadex column peak (tubes 8,9, and 10): 76.59%

Biodistribution study

In this study we used nine mice. [99mTc]Tc-MAG3 (100 µCi t 1,4 h and 24 h 300 µCi) was injected into each mouse's tail. The results of the percentage of the injected dose (ID) per gram of organ weight (intestine, heart, lung, spleen, kidney, liver, tail) were calculated after 1, 4 and 24h, n=3 for each time (Table 2). The results confirmed the specific excretion of [99mTc]Tc-MAG3 in kidneys with %ID/g of 41.28 ± 4.70 , 45.63 ± 6.36 and 12.2± 2.83 after 1, 4 and 24h respectively ; hence, the prepared [99mTc]Tc-MAG3 can be used in renal imaging, as expected.

Table 2. Tissue distribution of [99mTc]Tc-MAG3 in mice (%ID/g, n = 3) at 1, 4 and 24 hours after injection [99mTc]Tc-MAG3

% ID/g of [99mTc]Tc-MAG3			
Organ	**1h**	**4h**	**24h**
Muscle	0.76 ± 0.08	1.09 ±0.09	1.77 ± 1.60
Intestine	0.56 ± 0.30	1.61 ± 1.23	0.97 ± 0.12
Heart	0.34 ± 0.15	1.49 ± 1.10	0.74 ± 0.18
Lungs	0.51 ± 0.03	1.74 ± 1.51	1.01 ± 0.63
Spleen	0.28 ± 0.20	1.37 ± 1.42	1.80 ± 0.60
Kidneys	41.28 ± 4.70	45.63 ± 6.36	12.20 ± 2.83
Liver	1.32 ± 0.09	7.6 ± 2.08	4.18 ± 1.17
Tail	0.10 ± 0.03	0.41 ± 0.06	3.19 ± 1.52

*ID/g the injected dose (ID) per organ gram weight

DISCUSSION

Due to the lack of S-acetyl-MAG3's market availability (in contrast to S-benzolyl-MAG3) and the very high cost of S-acetyl-MAG3-NHS (about USD$1,080 for 50mg of S-acetyl-MAG3-NHS), in this study we aimed to modify the synthesis protocol for easier and more cost-effective outcome.

In mid-1985, Fritzberg and colleagues introduced MAG3 [1].The first experiments performed on mice with [99mTc]Tc-MAG3 showed complete renal removal due to their rapid excretion and high excretion quality compared to traditional ortho-iodohippuric acid or [131I]I-OIH [18]. The [99mTc]Tc-MAG3 was marketed by Fritzberg et al. [1, 19]. [99mTc]Tc-diethylene-triamine-pentaacetate or [99mTc]Tc-DTPA is also used to diagnose kidney problems. Image quality with [99mTc]Tc-MAG3 is higher than that with [99mTc]Tc-DTPA in the presence of decreased renal function [20-24]. [99mTc]Tc-MAG3 is excreted due to tubular extraction, in contrast to DTPA which are mostly filtered [25].

Wang et al. reported on the synthesis of S-acetyl-MAG3-NHS esters to label different types of oligonucleosides in various studies [9]. The synthesis of this set can be done using the method mentioned in the Winnard study [26]. Shabani et al. reported

synthesis of S-benzoyl-MAG3 which is available in the market [27].

The [^{99m}Tc]Tc-succinimidyl-6-hydrazinonicotinate hydrochloride (SHNH) has also been used to bind to DNA [28, 29]. High performance liquid chromatography (HPLC) showed that the SHNH-DNA complex was rapidly and non-specifically bound to serum proteins, but in the case of MAG3-DNA, binding to serum proteins was very low even after 24h, and it showed significant stability in binding to the desired biomolecule [10].

The synthesis of the S-acetyl-MAG3 was accomplished with a few excess of SATA to ensure that all triglycine had been consumed. The reaction monitoring by TLC showed that S-acetyl-MAG3 was almost pure with trace amount of unreacted SATA. In the synthesis of NHS-ester intermediate equimolar amounts of reactant was used and DCC was filtered out. Hence we expected almost pure product as it was determined by TLC which showed S-acetyl-MAG3-NHS as one spot with traces of impurities.

In order to confirm the synthesis of the of S-acetyl-MAG3 and its NHS ester, a small amount of crude sample was purified and characterized using NMR and LC/Ms spectrum; then the crude product was complexed with ^{99m}Tc, and its purity and stability determined according to TLC and Sephadex column tests. The [^{99m}Tc]Tc-MAG3-BSA showed a high percentage of purity (96 versus 76.59%). This might be due to application of excess BSA and application of dialysis bag in purification process which caused all S-acetyl-MAG3 to be consumed and any unreacted small molecule was separated out. Radiochemical purity would not have been influenced by contamination of unlabeled BSA.

In stability study determined by TLC the observed lowering of radiochemical activity for [^{99m}Tc]Tc-MAG3- BSA during study 0 to 24h in serum or buffer (dropping from 99.87 to 79 or 80%) was higher than [^{99m}Tc]Tc-MAG3 (dropping from 90.85 to 61.4 or 51%). This might be due to BSA conjugation which increased stability. There was found no difference between serum and buffer and hence serum constituents did not cause instability.

According to biodistribution studies, the prepared [^{99m}Tc]Tc-MAG3 kit can be used for quantitative renal function studies. Our results for organ distribution in mice was comparable to previous study [25].

CONCLUSION

The ratio of reactants was adjusted so that S-acetyl-MAG3 and its intermediate NHS ester were almost pure and hence the troublesome purification processes was simplified and the crude product was used directly. The purification procedure lowered the yield and wasted time. After ^{99m}Tc labeling, the radiochemical purity of labeled products was determined and they were above 90%. We also did biodistribution studies on mice and renal excretion was acceptable. Hence we could prepare ^{99m}Tc-labeled MAG3 without rigorous purification processes. We also used acetyl-S-MAG3 for preparation of acetyl-S-MAG3-NHS ester which was used for conjugating proteins. Furthermore, because of simplified cheaper and less time consuming synthesis method, it might be possible to prepare the NHS-ester with relative and apply this modification for future labeling of different biomolecules.

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