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테크네슘-99엠 트리카보닐 시스테인의 제조 및 생물학적 특성 평가

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Preparation and biological evaluation of ^{99m}Tc tricarbonyl cysteine

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Abstract : This paper describes the development of ^{99m}Tc tricarbonyl cysteine as potential renal function diagnostic radiopharmaceutical and evaluation of its biological characteristics using experimental animals. *l*-Cysteine was labeled efficiently with ^{99m}Tc tricarbonyl precursor ($[^{99m}Tc(CO)_3(H_2O)_3]^+$) under 30 min heating at 75°C. Labeling yield and stability were analyzed by high performance liquid chromatography (HPLC). The biodistribution property of ^{99m}Tc tricarbonyl cysteine in mice and its dynamic imaging profiles in rabbits were carried out. To investigate the excretion mechanism of ^{99m}Tc tricarbonyl cysteine, tubular transport inhibition test with probenecid was adopted. ^{99m}Tc tricarbonyl cysteine was obtained with a high labeling yield under the moderate condition. The results of biodistribution experiments of ^{99m}Tc tricarbonyl cysteine in ICR mice at 3 and 90 min provided that ^{99m}Tc tricarbonyl cysteine was very highly accumulated in the kidney and bladder, thereby almost 99% of ^{99m}Tc tricarbonyl cysteine was excreted within 90 min post injection. The same results were confirmed by the whole body dynamic images for 30 minutes and static images in rabbits at given time intervals after injection. Renogram of ^{99m}Tc tricarbonyl cysteine in rabbits showed that its T_{max} and $T_{1/2}$ of ^{99m}Tc tricarbonyl cysteine were 2.33±0.56 and 4.30±0.79 min, respectively. The T_{max} of ^{99m}Tc tricarbonyl cysteine with probenecid pretreatment was 2.30±0.17 min, whereas $T_{1/2}$ of that with probenecid pretreatment was 17.0±32.47 min. $T_{1/2}$ of ^{99m}Tc tricarbonyl cysteine with probenecid pretreatment was significantly different, as compared to the result without probenecid ($p<0.0001$). The results showed that the excretion of ^{99m}Tc tricarbonyl cysteine was extremely affected by probenecid. Therefore, ^{99m}Tc tricarbonyl cysteine was rapidly excreted from the kidney principally by the tubular secretion.

Key words : renal function diagnostic radiopharmaceuticals, ^{99m}Tc tricarbonyl cysteine, tublar secretion

Introduction

Various radiological methods are available to address anatomical profiles about the kidneys. However, to determine the differential function of each kidney, and to detect any obstruction in urine flow, the renography with radiotracers is the method of choice [12]. To date there is no compound excreted completely by kidneys, but only para-aminohippuric acid (PAH) comes close. Tubis *et al*. [15] developed ortho-iodohippuric acid

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(OIH), a radiolabeled analogue of PAH for measurement of ERPF (effective renal plasma flow) and imaging. However, iodine-131-OIH produces poor-images, with a high radiation exposure, especially in patients with urinary obstruction [14]. Because of these disadvantages, many new renal imaging agents labeled with ^{99m}Tc are being introduced. It is well known that the use of ^{99m}Tc as a labeling agent has practical advantages over the iodine isotopes ^{131}I or ^{123}I [11]. Many ^{99m}Tc radiopharmaceuticals are based on ^{99m}Tc(V) oxo or octrahead ^{99m}Tc(III) cores. ^{99m}Tc agents with organometallic low-oxidation state are less common, because of the difficulty of controlling the reduction of the ^{99m}Tc(VII) to ^{99m}Tc(I) oxidation state. Recently, R. Alberto *et al.* synthesized the ^{99m}Tc tricarbonyl complex as a precursor with a low oxidation state ^{99m}Tc(I) for the radiolabeling of biomolecules [1-3, 9, 13]. They reported that it was easily prepared as the organometallic aqua complex $[^{99m}Tc(H_2O)_3(CO)_3]^+$ directly from $[^{99m}TcO_4]^-$ in saline under 1 atm of CO which is stable at the room temperature even when exposed to air [3]. They concluded that this new peptide labeling approach with $^{99m}Tc(CO)_3$ combines the highest possible specific activities with a minimal influence on the biologic properties of the peptide, including receptor affinity and metabolism, and can be transferred to other peptides of choice [9]. On the while, ^{99m}Tc-MAG_3, a currently employed renal function diagnostic, is not ideal owing to its high plasma protein binding and complex synthetic procedure. Thus the development of a new ^{99m}Tc renal function diagnostic is needed.

Amino acid cysteine contains three functional groups composed of carboxyl, sulfhydryl and amino groups which are well known to be an efficient chelating moiety for ^{99m}Tc labeling. Most of ^{99m}Tc-cysteine complexes are synthesized under various reaction conditions as renal scintigraphic agents [5, 7, 8, 10, 16]. However, the ^{99m}Tc cysteine complex as a renal scintigraphic agent is not yet available at the moment.

Therefore, the aims of the present study was to develop ^{99m}Tc tricarbonyl cysteine as a potential renal diagnostic radiopharmaceutical and to study its biological mechanism of excretion.

Materials and Methods

Chemicals

CO gas (99.5%) was obtained from Daehan Gas Co. (Seoul, Korea) and prefiltered with an oxygen trap. $Na^{99m}TcO_4$ was obtained by solvent extraction from $^{99}MoO_3$, which was produced by irradiation of $^{98}MoO_3$ using the High-flux Advanced Neutron Application reactor (HANARO) of the Korea Atomic Energy Research Institute (KAERI). *l*-Cysteine and probenecid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MAG_3, a commercially available labeling kit, was obtained from Mallinckrodt Medical, Inc. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

Labeling yield and radiochemical purity were checked by high performance liquid chromatography (HPLC) coupled with a reversed phase μBondapak C-18 column (3.9×300 mm, Waters, USA), with a gradient system of 0.05 M tetraethylammoniumphosphate (TEAP) buffer and 100% methanol. All solvents for the mobile phase were of HPLC grade and prefiltered with 0.2 μm pore size bottle filter. The radioactivity of ^{99m}Tc complexes in samples was checked by a well-type NaI (Tl) scintillation detector (Canberra Industries Inc., Meriden, CT, USA). The linear regression line for the standard curve of radioactivity (Bq) vs counts per min (cpm) in the radioactivity range of 10~350,000 Bq showed high correlation coefficients (r) of 0.999. For the maintenance of detection efficiency, the standard injected dose radiopharmaceutical was diluted by saline for the calculation in order to be under the 300,000 Bq.

The orbiter gamma camera (Simens, Germany) with a low energy collimator was positioned. The energy gate was set to 140 keV and the window width to 10%. Dynamic image acquisition was performed for 30 minutes according to the dynamic procedure of the Microdelta System (Simens, Germany). The region of interest (ROI) was drawn over the right and left kidney using 1- to 3-min composition image and the computer generated time-activity curve was also obtained.

Animals

Experimental animals were purchased from Bio Genobics, Inc. (Seoul, Korea) that has technical cooperation with Charles River Laboratory, Inc (Wilminton, USA). Mice and rabbits were kept in polycarbonate cages and individual stainless steel cages, respectively, at 22±1°C with relative humidity of 60±10% and a 12 hr light/dark cycle. After acclimation of approximately a week, the healthy

animals were used for the experiments. The animals were allowed free access to fresh tap water and laboratory animal chow. All the experiments with the experimental animals were performed in accordance with the institutional guideline, Standard Procedure for Animal Care and Experiments of the College of Veterinary Medicine, Chungnam National University.

Preparation of ^{99m}Tc tricarbonyl precursor

^{99m}Tc tricarbonyl precursor was prepared using a modified procedure described by Alberto *et al*. [3]. A 10 m*l* vial containing Na_2CO_3 (8 mg, 0.076 mmol) and $NaBH_4$ (10 mg, 0.26 mmol) was capped with a rubber stopper and then flushed with a stream of CO gas (99.5%) at room temperature for 30 min. Six m*l* of sodium pertechnate ($Na^{99m}TcO_4$) with up to 37 GBq was added by a syringe and then heated at 75°C for 30 min under the bubbling of CO gas. For safety reasons, the syringe was kept on the stopper during the procedure to avoid any overpressure in the reaction vial. After the rapid cooling down to room temperature, 0.6 m*l* of phosphate buffered saline (0.05 M phosphate buffer, pH 7.4) was added to neutralize the ^{99m}Tc tricarbonyl precursor. Radiochemical purity and the stability of the ^{99m}Tc tricarbonyl precursor were analyzed every hour for 8 hrs by HPLC.

Preparation of ^{99m}Tc tricarbonyl cysteine

One m*l* of ^{99m}Tc tricarbonyl precursor was added to 0.1 m*l* of *l*-cysteine solution (10 mM in saline) for 30 min at a reaction temperature of 75°C. After cooling to room temperature, labeling yield and stability were checked by HPLC.

Lipophilicity of ^{99m}Tc tricarbonyl cysteine

The octanol-water partition coefficient of the complexes was measured by determining their *n*-octanol/buffer partition coefficients in triplicate. One m*l* of nitrogen-purged 0.05 M phosphate buffered saline (PBS, pH 7.4) was mixed together with 1.0 m*l* of *n*-octanol. After adding 10 μ*l* of ^{99m}Tc tricarbonyl cysteine (or ^{99m}Tc tricarbonyl precursor), the samples were vortexed for 3 min, centrifuged at 3,000 g for 10 min using a Marathon 22K centrifuge (Fisher Science Co., USA) and then the two phases were separated. Each 200 μ*l* of the PBS and the octanol phases were measured in a well-type NaI(Tl) scintillation detector. The calculation was performed according to the following equation.

$$\text{Lipophilicity (\%)} = \frac{\text{Octanol (cpm)}}{\text{Water (cpm)} + \text{Octanol (cpm)}} \times 100$$

Plasma protein binding test

The extent of plasma protein binding was measured in triplicate. Three male New Zealand White rabbits (3,045±458 g) were injected with 3.7 MBq of test radiolabeled complexes per head through the left ear vein. Blood samples were collected at 5 and 60 min after administration. The fresh heparinized whole blood was centrifuged using a swing-out rotor at 4,120 g for 10 min. The count rates of plasma was measured in a well-type NaI(Tl) scintillation detector.

For the determination of plasma protein binding, the plasma protein was precipitated by a method using trichloroacetic acid [17]. The plasma protein is precipitated by adding 0.2 m*l* of 50% trichloroacetic acid to 1 m*l* of plasma. The supernatant and the precipitate were separated by centrifugation at 13,000 g for 10 min. The activities of both phases were measured separately in a well-type NaI(Tl) scintillation detector.

The percentage of protein binding was determined by the following equation.

$$\text{Plasma protein bound (\%)} = \frac{\text{Plasma (cpm)} - \text{Supernatant (cpm)}}{\text{Plasma (cpm)}} \times 100$$

Biological characterization studies

(1) Biodistribution study

Biodistribution studies were carried out using 6-week-old ICR male mice (25.0±2.7 g, n=6, SPF grade). The mice were injected with 0.74±0.07 MBq radiopharmaceuticals per head (20±2 μCi) through a tail vein. At each interval (3 and 90 min post injection), 3 mice were sacrificed with ethylether. The liver, spleen, kidney, lung, heart, blood, bladder, total gastrointestinal track and carcass were excised, weighed and counted, along with the diluted standard injected radiopharmaceuticals in a well type gamma counter (Canberra Industries Inc., CT, USA).

(2) Dynamic imaging studies and probenecid blocking studies

Dynamic imaging studies were performed with 6-week-old male New Zealand White rabbits (1987.4±45.7 g, n=3) which were anesthetized with 25 mg/kg of

ketamine (Ketamine 50 inj., Yuhan Co., Korea) and 6 mg/kg of xylazine (Rompun inj, Bayer Korea, Korea). The effect of probenecid was studied by the injection of probenecid (20 mg/kg) into the right ear vein of rabbits (2004.6±72.3 g, n=3) at 10 min prior to the injection of ^{99m}Tc tricarbonyl cysteine. The rabbits were placed in a posterior position, and injected with 37 MBq of test complex per head (1.0 mCi) via the left ear vein. In addition, the static images of ^{99m}Tc tricarbonyl cysteine were obtained at 5, 10 and 30 min post injection with a microdot imager (Simens, Germany).

Data were expressed as mean±SD. Statistical analyses were performed by the Students *t*-test. Differences were considered to be significant at $p<0.05$.

Results

Preparation of ^{99m}Tc tricarbonyl precursor and radiolabeling with *l*-cysteine

A ^{99m}Tc tricarbonyl precursor was successfully prepared with 98% of radiolabeling yield using a modified procedure described by Alberto *et al*. [9]. The radiolabeling of *l*-cysteine with a ^{99m}Tc tricarbonyl precursor was also successful (yield>95%). The typical chromatograms of free $^{99m}TcO_4^-$, a ^{99m}Tc tricarbonyl precursor and ^{99m}Tc tricarbonyl cysteine showed that their retention times are 10, 4, and 17 min, respectively (Fig. 1).

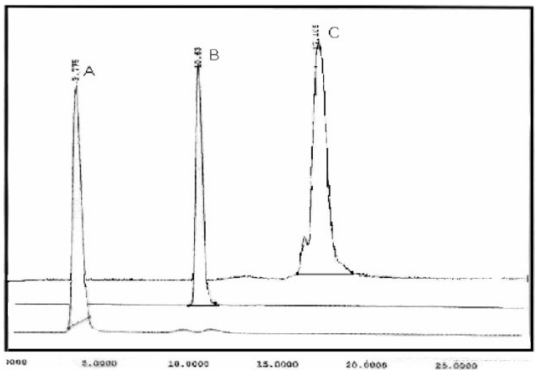

Fig. 1. The chromatogram of ^{99m}Tc tricarbonyl precursor (A), free $^{99m}TcO_4^-$ (B), and ^{99m}Tc tricarbonyl cysteine (C). HPLC condition: Mobile phase, gradient system based on 0.05 M TEAP buffer and 100% MeOH; Column, μBondapak C-18 column (3.9×300 mm); Flow rate, 1 ml/min.

Table 1. Lipophilicity of ^{99m}Tc tricarbonyl precursor and ^{99m}Tc tricarbonyl cysteine

	Counts per min		Lipophilicity (%)
	Water	Octanol	
^{99m}Tc tricarbonyl precursor	823,360	99,594	10.79
	755,330	90,258	10.67
	752,160	81,801	9.81
Mean±SD			10.42±0.54
^{99m}Tc tricarbonyl cysteine	770,558	17,826	2.26
	740,255	16,993	2.24
	776,417	16,638	2.10
Mean±SD			2.20±0.09

(1) Lipophilicity of ^{99m}Tc tricarbonyl precursor and ^{99m}Tc tricarbonyl cysteine

The lipophilicity of the ^{99m}Tc tricarbonyl precursor and ^{99m}Tc tricarbonyl cysteine is summarized in Table 1. Lipoplilicity of ^{99m}Tc tricarbonyl cysteine was about 2.2±0.1%, which was higher than those of ^{99m}Tc-MAG_3 (0.07%) and ^{131}I-OIH (1.61%) in 0.1 N phosphate buffer (pH 7.4) [6]. ^{99m}Tc tricarbonyl cysteine was revealed to be more lipophilic than ^{99m}Tc-MAG_3 and ^{131}I-OIH.

(2) Plasma protein binding rates

The binding rates of ^{99m}Tc tricarbonyl cysteine with plasma protein in rabbits at 5 and 60 min post injection are summarized in Table 2. The plasma protein binding rate of ^{99m}Tc tricarbonyl cysteine was about 70% to 80%, and similar to that of ^{131}I-OIH (70%) but lower than that of ^{99m}Tc-MAG_3 (90%) [6].

Table 2. Plasma protein binding of ^{99m}Tc tricarbonyl cysteine

Unit: %

	5 min		60 min	
	Plasma protein	Free form	Plasma protein	Free form
1	69	31	79	21
2	68	32	79	21
3	71	29	79	21
Mean± SD	69.20± 1.74	30.80± 1.74	79.01± 0.27	20.99± 0.27

Table 3. Biodistribution of ^{99m}Tc tricarbonyl cysteine in ICR mice at 3 and 90 min after 0.74 MBq/0.2 m*l* intravenously administration

	% Injected Dose±SD	
	3 min	90 min
Liver	32.50±3.34	0.41±0.13
Spleen	0.24±0.06	0.01±0.00
Kidney	17.23±2.43	0.08±0.03
Lung	0.82±0.23	0.01±0.03
Heart	0.27±0.02	0.01±0.01
Blood	8.76±3.01	0.03±0.01
Bladder	4.30±6.98	0.02±0.02
GIT	6.04±2.00	0.16±0.02
Carcass	25.16±3.98	0.16±0.03

Biological characterization studies

(1) Biodistribution study

The results of the biodistribution experiments of ^{99m}Tc tricarbonyl cysteine in ICR mice at 3 and 90 min are shown in Table 3 as a percentage of injected dose (%ID). The group of 3 min post injection showed that the %ID of kidney and bladder were 17.2% and 4.3%, respectively. The group of 90 min post injection showed that the %ID of the liver and kidney were 0.4% and 0.08%, respectively. No more than 1% remained in the whole body untill 90 min post injection. ^{99m}Tc tricarbonyl cysteine very rapidly concentrated in the kidney and bladder. Additionally, ^{99m}Tc tricarbonyl cysteine was rapidly cleared from the whole body without any concentration.

(2) Dynamic imaging studies and probenecid blocking studies

The static image of ^{99m}Tc tricarbonyl cysteine at 5 min post injection showed high concentrated activity in the kidney of rabbits. In the image of 15 and 30 min post injection, most activity appeared in the bladder without a residue in other organs (Fig. 2). The renograms of ^{99m}Tc tricarbonyl cysteine with or without probenecid pretreatment were summarized in Table 4. The T_{max} of ^{99m}Tc tricarbonyl cysteine in the kidney without probenecid pretreatment was 2.33±0.56 min and the elapse time of the T_{max} to $T_{1/2}$ was 4.30±0.79 min. The renogram of ^{99m}Tc tricarbonyl cysteine in rabbits showed that the activity of the kidney was rapidly reduced within 30 min post injection when it reached the background level (Fig. 3). But the T_{max} of ^{99m}Tc tricarbonyl cysteine in the kidney with probenecid pretreatment was 2.30±0.17 min and the elapse time of T_{max} to $T_{1/2}$ was 17.03±2.47 min. This study proved that the excretion of ^{99m}Tc tricarbonyl cysteine was highly affected by probenecid and this is excreted rapidly through the tubular secretion of the kidney as ^{99m}Tc-MAG_3 or ^{131}I-OIH.

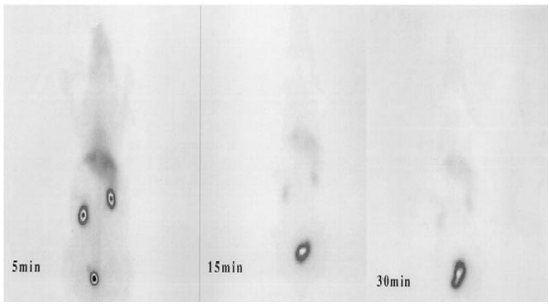

Fig. 2. The images of a rabbit administered ^{99m}Tc tricarbonyl cysteine at 5, 15, and 30 minutes after injection.

Table 4. T_{max} and $T_{1/2}$ of ^{99m}Tc tricarbonyl cysteine renography in rabbits with or without probenecid pretreatment

Unit: min

Non-probenecid pretreatment		Probenecid pretreatment	
T_{max}	$T_{1/2}$	T_{max}	$T_{1/2}$
2.33±0.56	4.30±0.79	2.30±0.17	17.03±2.47*

*: $p<0.0001$

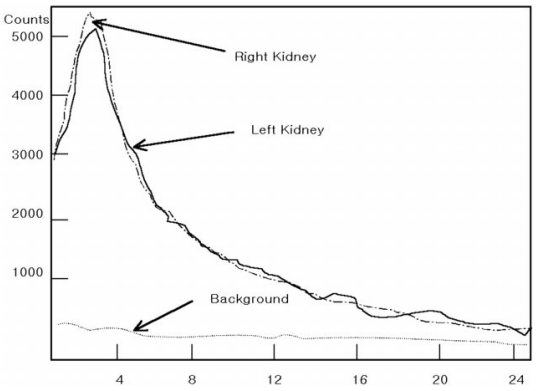

Fig. 3. Renogram of ^{99m}Tc tricarbonyl cysteine in New Zealand White rabbit for 24 min after injection.

Discussion

^{99m}Tc tricarbonyl precursor was simply prepared CO gas flushing and heating. ^{99m}Tc tricarbonyl cysteine was simply prepared only by heating with high yield (>95%) and stable in saline and serum within 6 hrs. ^{99m}Tc tricarbonyl cysteine showed more lipophilic property than ^{99m}Tc-MAG_3 and ^{131}I-OIH. The plasma protein binding rate of ^{99m}Tc tricarbonyl cysteine was lower than that of ^{99m}Tc-MAG_3. Thus we suggested that excretion of ^{99m}Tc tricarbonyl cysteine was less effected by the plasma protein than ^{99m}Tc-MAG_3.

Our results of biodistribution experiments using ICR mice showed that ^{99m}Tc tricarbonyl cysteine was very rapidly excreted through the urinary system without residue in other organs throughout the whole body.

Renograms of ^{99m}Tc tricarbonyl cysteine in rabbits showed that it was rapidly excreted via the kidney and reached the background level within 30 min after injection. The $T_{1/2}$ of ^{99m}Tc tricarbonyl cysteine (4.30± 0.79 min) was longer than those of ^{99m}Tc-MAG_3 (2.43±0.7 min) or ^{131}I-OIH (3.71±0.89 min) but shorter than those of ^{99m}Tc-ECD (7.00±1.35 min) and ^{99m}Tc-EC (4.83±0.67 min) [4].

The excretion mechanism of ^{99m}Tc tricarbonyl cysteine was confirmed by the probenecid blocking study in rabbits. The T_{max} of ^{99m}Tc tricarbonyl cysteine in probenecid pretreated rabbits was similar to that of the untreated rabbits. However the $T_{1/2}$ of ^{99m}Tc tricarbonyl cysteine in rabbits pretreated with probenecid was increased four-fold in comparison with that of untreated rabbits, which means the excretion of ^{99m}Tc tricarbonyl cysteine was highly affected by probenecid.

This study proved that the excretion of ^{99m}Tc tricarbonyl cysteine was more extremely affected by probenecid than ^{99m}Tc-MAG_3. Thus we concluded that ^{99m}Tc tricarbonyl cysteine was principally excreted by the active tubular transport, probably by the same carrier proteins responsible for the renal excretion of ^{131}I-OIH and/or ^{99m}Tc-MAG_3 [6, 12].

The labeling procedure of the ^{99m}Tc tricarbonyl cysteine is very simple and ^{99m}Tc tricarbonyl cysteine has an excellent renal excretion property. We concluded that our synthetic method and the ^{99m}Tc tricarbonyl cysteine described here appear to offer a promising approach toward developing a renal agent as a replacement of ^{131}I-OIH and/or ^{99m}Tc-MAG_3.

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