LABELLING OF METALLOTHIONEIN WITH ^{99m}Tc

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ABSTRACT

^{99m}Tc-labelled metallothionein (^{99m}Tc-MT) was prepared through both direct and transcomplexation labelling approaches. Buffer systems and a variety of other parameters in the direct labelling procedure were studied in detail. High radiolabelling yields of 93—96 % and the specific radioactivity of 3.7 MBq/μg were obtained under optimal conditions by the direct labelling method. The prepared ^{99m}Tc-MT was stable *in vitro*. The chelate-exchange kinetics of MT with ^{99m}Tc-glucohepatonate and ^{99m}Tc-citrate were also studied. Biodistribution and imaging studies showed that ^{99m}Tc-MT was accumulated in the animal kidneys at an exceptionally high level, indicating that ^{99m}Tc-MT might be a potential renal imaging agent.

Keywords: Metallothionein Technetium-99m Radiolabelling Biodistribution Imaging

1 INTRODUCTION

Metallothionein (MT) is a biochemically and physiologically important metal-binding protein with M. W. about 6500. They play important roles in the aspects of storage, transportation and homeostasis of essential elements, resisting ionizing radiation by eliminating hydroxyl radicals, sequestering toxic heavy metal ions as well as modulating cell metabolism. Mammalian MT is composed of sixty-one amino acids among which twenty are cysteine residues. Zinc, cadmium, and copper could be tightly bound to the metal thiolate clusters of MT, since the -M-S- bonds are thermodynamically stable.

There are a few papers published on the use of MT as a radionuclide carrier in radioimmunodiagnosis and radioimmunotherapy[1-3]. They reported the preparation of

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^{99m}Tc–Zn–MT through transcomplexation radiolabelling of ^{99m}Tc which is an ideal radionuclide for imaging. However, the radiolabelling efficiency reached was low in their work.

In the present work, a satisfactory radiolabelling protocol has been developed and the labelling condition was optimized. High efficiency and stable radiolabel of ^{99m}Tc–MT were achieved. The biodistribution of ^{99m}Tc–MT is also presented.

2 EXPERIMENTAL

2.1 Preparation of Cd—MT

The method to prepare Cd–MT was described in Ref.[4] with some modifications. Two rabbits were SC injected $CdCl_2$ solution in the dose of 2.5 mg Cd/kg for ten times and subsequently an increased dose of 12 mg/kg for the other ten times with the injection intervals of 2—3 days. The animals were then sacrificed. Their livers and kidneys were cut into small pieces and homogenized. Through steps of precipitation, extraction, and centrifugation, a fraction of Cd-binding protein of low molecular weight were separated on a Sephadex G–75 column (5 cm × 80 cm) eluting with 0.010 mol/l pH 8.6 Tris–HCl solution. This raw product was purified into two main peaks of MT (Cd–MT–A and Cd–MT–B) after gradiently eluting with 0.010—0.25 mol/l $(NH_4)_2CO_3$ solution. The amount of Cd–MT was determined by UV–spectrometry and atomic absorption spectrometry. The product was finally lyophilized for the use in the following radiolabelling.

2.2 Radiolabelling procedures

Direct labelling of MT with ^{99m}Tc was accomplished by adding 100 μl fresh prepared $SnCl_2 \cdot 2H_2O$ solution (2.3 mg/ml, $\sim 1\times 10^{-3}$ mol/l) to 500 μl MT solution ($\sim 6\times 10^{-6}$ mol/l), and followed by the addition of 40 μl $^{99m}TcO_4^-$ in saline (0.37—3.7 MBq) eluted from a sterile $^{99}Mo/^{99m}Tc$ generator. Cd–MT solution was dissolved in a buffer and adjusted to required pH values with 0.10 mol/l HCl or 0.10 mol/l NaOH solution. The radiolabelling yield of each batch was determined by paper chromatography and Sephadex gel permeation method.

Cd–MT was labelled through transcomplexation, by adding 0.50 ml of either ^{99m}Tc–glucoheptonate (^{99m}Tc–GH) or ^{99m}Tc–citrate (^{99m}Tc–Cit) solution to 0.50 ml of 6×10^{-6} mol/l MT solution at pH 6.3. ^{99m}Tc–GH and ^{99m}Tc–Cit were prepared by adding 40 μl of $^{99m}TcO_4^-$ in saline to 0.50 ml of 0.10 mol/l cadmium glucohepatonate or 0.50 ml of 0.050 mol/l sodium citrate solution after 100 μl of 2.3 mg/ml $SnCl_2$ solution (in 1.0×10^{-3} mol/l HCl solution) was added. The radiochemical purities of both ^{99m}Tc–GH and ^{99m}Tc–Cit used were higher than 95 %.

2.3 Chemical stability test

In determination of the stability of ^{99m}Tc–MT diethylenetriamine–pentaacetic acid

(DTPA) was used as a competitive agent. A batch of ^{99m}Tc–MT (6.0×10^{-6} mol/l) was mixed with equal volume of 1.0×10^{-3} mol/l DTPA solution at pH 6.3. After standing for 15 h at room temperature, the radioactivity bound to DTPA was analysed by paper chromatography.

2.4 Analytical methods

Ascending paper chromatography was performed in saline on each sample with a 1 cm× 15 cm Xinhua No.1 paper strip. The developed paper strip was cut into pieces of 1 cm× 0.5 cm and the radioactivity was counted with a NaI(Tl) well counter. The R_f values for various radioactive species were measured: 0.70 for $^{99m}TcO_4^-$; 0.00 for ^{99m}Tc–MT and reduced and hydrolysed ^{99m}Tc colloid (^{99m}Tc–RH); and 1.00 for ^{99m}Tc complexes with simple ligands, i.e. ^{99m}Tc–GH and ^{99m}Tc–DTPA. On the basis of these parameters, paper chromatography was employed to measure the radioactivity percentage of $^{99m}TcO_4^-$ or simple ligand complex in the sample. If ^{99m}Tc- RH does not form in the system the percentage of ^{99m}Tc bound to MT (^{99m}Tc–MT %) can thus be derived as ^{99m}Tc–MT % = 100 %–[$^{99m}TcO_4^-$ + ^{99m}Tc–GH (and other simple complexes)] %.

When the concentration of MT is very small, the formation of ^{99m}Tc–RH is inevitable. Hence, gel chromatography is additionally employed to distinguish ^{99m}Tc–MT and ^{99m}Tc–RH. ^{99m}Tc–RH can not be eluted from the Sephadex G–15 column whereas ^{99m}Tc–MT does. Therefore, from the recovery efficiency of Sephadex G–15 column, we derive an other formula to calculate the ^{99m}Tc bound to MT: ^{99m}Tc–MT % = (recovery efficiency on gel column)–(percentage of $^{99m}TcO_4^-$ and the ^{99m}Tc–simple–ligand–complex determined by paper chromatography).

It was proved that this analytical method is in satisfactory accordance with the experimental results.

3 RESULTS

3.1 Factors influencing radiolabelling yield in the direct labelling procedure

3.1.1 *Buffer solution* A variety of buffer systems were chosen to test their effects on radiolabelling yield (RY) at the first experiment. They are 0.050 mol/l acetic acid/sodium acetate (pH 5.5, RY 83 %), 0.67 mol/l NaH_2PO_4/Na_2HPO_4 (pH 6.3, RY 80 %), pH 6.3 sodium citrate/HCl (0.010 mol/l, RY 53 %; 0.050 mol/l, RY 46 %), and 0.050 mol/l barbitone (pH 8.3, RY 44 %). Radiolabelling yield is much higher in acetate and phosphate buffer than that in citrate system, because citrate competitively coordinates Tc(V)O core. The low RY in barbitone buffer may result from the alkaline solution, in which ^{99m}Tc–RH and $^{99m}TcO_4^-$ exist due to the fast hydrolysis of tin and thus incomplete reduction of $^{99m}TcO_4^-$. Citrate may minimize the non–sulfhydryl binding of reduced ^{99m}Tc in MT and may consequently increase the stability of the protein, but the resultant RY is much lower.

3.1.2 *pH values of the labelling system* 0.67 mol/l phosphate buffers at pH 6.3, 6.8, 7.3, and 7.9, were used to test pH effect. In addition, 0.050 mol/l acetate buffer at pH 5.5 was used to extend the covering range of pH values. RYs of 83 %, 80 %, 73 %, 68 %, and 44 % were obtained with pH values at 5.5, 6.3, 6.8, 7.3, and 7.9 respectively. With the increase of pH value, RY decreases and falls down sharply at about pH 8. The proposed reason is that $SnCl_2$ can be hydrolyzed readily in a alkaline solution.

3.1.3 *Concentrations of $SnCl_2$ and HCl used to dissolve the reductant* No obvious dependence of RY on the Sn concentration ranging from 2×10^{-5} to 2×10^{-3} mol/l was seen. A small percentage of ^{99m}Tc-RH was found at high tin content in acetate buffer, and a minute percentage of $^{99m}TcO_4^-$ existed at low tin content in phosphate buffer. In addition, RY was higher when the original tin used in the labelling was dissolved in 0.1 mol/l HCl solution than that in 1×10^{-3} mol/l HCl solution, probably due to the easy hydrolysis of stannous chloride in much dilute HCl solution.

Table 1

The effect of concentrations of tin on RY (MT, 6×10^{-6} mol/l)

Buffer	0.05	mol/l	acetate	0.67	mol/l	phosphate
pH value	5.7	5.7	5.0	6.3	6.3	5.7
Conc. of Sn (mol/l)	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-5}	2×10^{-4}	2×10^{-3}
RY (%)	90	93	83	85	90	96

3.1.4 *Reaction time* Table 2 shows the results of labelling batches with preincubation of MT and tin for 5 minutes at room temperature before adding $^{99m}TcO_4^-$, and without preincubation. No significant change of RY was observed.

Table 2

Comparison of RYs with and without preincubation of tin and MT for 5 minutes

	Buffer	0.050 mol/l acetate	0.67 mol/l phosphate		
		pH 5.5	pH 6.3	pH 6.8	pH 7.3
RY (%)	Incubated	85	77	72	67
	Non-incubated	82	82	77	66

3.1.5 *Concentrations of MT* To avoid the formation of ^{99m}Tc-RH, 0.020 mol/l GH, which can bind more than 80 % of reduced ^{99m}Tc alone, was used as a sequestering agent. Fig.1 shows the correlation of RY and the concentration of MT under this condition. It is obvious that MT can not compete with GH till its concentration exceeds 1×10^{-6} mol/l.

3.2 Stability of ^{99m}Tc–MT in competitive DTPA

The prepared ^{99m}Tc-MT was incubated with 1.0 mmol/l DTPA for 15 h at room temperature, and the percentages of radioactivity preserved by MT and chelated by DTPA are listed in Table 3. From paper chromatography analysis, it is found that the

radioactivity of ^{99m}Tc-MT did not lose in both systems, while a small amount of ^{99m}Tc-DTPA was detected in the front part of paper strip. This result proves that ^{99m}Tc-MT is stable in 1 mmol/l DTPA solution.

3.3 Transcomplexation of ^{99m}Tc from ^{99m}Tc—Cit and ^{99m}Tc—GH to MT

The transcomplexation kinetics from ^{99m}Tc-Cit or ^{99m}Tc-GH to MT is shown in Fig.2. The low labelling yield and slow exchange rate show that the transcomplexing method is probably no use in the case of 0.01 mol/l citrate.

Table 3

Stability of ^{99m}Tc—MT in competitive DTPA (1 mmol/l)

Solution		0.050 mol/l acetate, pH 5.5	0.010 mol/l citrate, pH 6.3	0.050 mol/l citrate, pH 6.3	0.67 mol/l phosphate, pH 6.8
^{99m}Tc-MT	Original	91	70	61	82
	Incubated	90	82	75	90
^{99m}Tc-DTPA		4	–	–	2

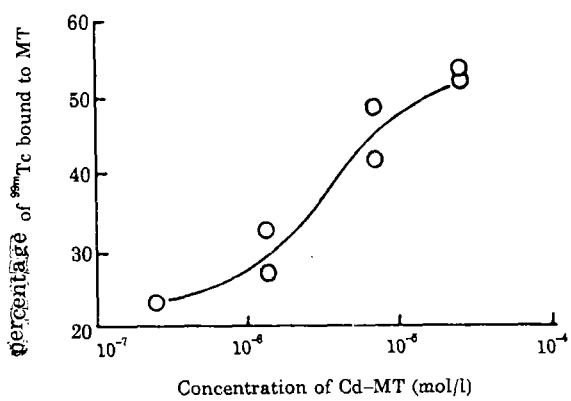

Fig.1 Percentage of ^{99m}Tc coordinated with MT related to the concentration of MT in 0.020 mol/l GH

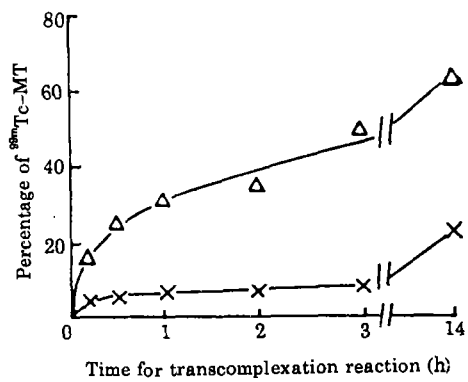

Fig.2 Kinetics of transcomplexation of MT and ^{99m}Tc—Cit, ^{99m}Tc—GH

MT 3×10^{-6} mol/l × 0.010 mol/l citrate, pH 6.3 △ 0.010 mol/l GH, pH 6.3

3.4 Biodistribution

^{99m}Tc-MT prepared in 0.67 mol/l phosphate buffer, pH 6.3, was used for biodistribution study without further purification (radiochemical purity > 90 %). The Swiss mice weighing 17— 20 g were I.V. injected 0.07— 0.74 MBq ^{99m}Tc-MT (0.10— 0.40 μg MT), and then killed and dissected. Radioactivity was determined with NaI (Tl) crystal well counter. Data were expressed as the percentage of total injected dose per gram of tissue (%ID/g).

More than 50 % radioactivity of injected ^{99m}Tc-MT is found in kidneys at the first half an hour after I.V. administration. The uptake in kidneys remains higher than 174 ± 10 % ID/g within 1 h. On the other hand, the uptakes of liver, spleen, and other organs are quite low. The blood clearance is rapid (Table 4). It is speculated that ^{99m}Tc-MT is uptaken by renal tubular cells for further catabolic breakdown, similar

to the fate of other low molecular wight proteins[5].

3.5 Renal imaging study

Two rabbits weighing 2.4 and 2.0 kg were injected 1.8 MBq ^{99m}Tc–MT (5.0 μg MT), which was prepared in 0.67 mol/l phosphate buffer. Scintiphotos were taken at 5 and 20 min after ear IV injection under 501 s digital large–field–of–view gamma camera (Toshiba, Japan). The rabbits were anesthetized with pentobarbitone.

The kidney images are evidently clear with a contrast of very low background (Fig.3). The quick imaging of rabbit kidneys is in good compatibility with the biodistribution result in mice.

Table 4

Biodistribution of ^{99m}Tc–MT in unanesthetized Swiss mice % ID/g±SD, $n=5$

Tissue	10 min	20 min	30 min	60 min	6 h	24 h
Blood	11.95±2.48	3.88±0.64	4.77±2.02	1.14±0.23	0.486±0.064	0.067±0.070
Heart	3.03±0.38	1.13±0.34	1.21±0.19	0.362±0.028	0.217±0.058	0.08±0.15
Lung	6.42±0.92	1.97±0.28	1.58±0.16	0.702±0.035	0.344±0.064	0.08±0.13
Liver	3.86±0.71	1.63±0.70	2.74±0.69	0.94±0.17	0.752±0.053	0.75±0.43
Kidney	358±49	211±25	255±32	174±10	73±12	45.0±7.1
Spleen	2.82±0.37	1.14±0.33	1.38±0.55	0.57±0.19	0.75±0.67	0.27±0.26
Stomach	1.96±0.27	0.96±0.25	1.2±1.7	0.65±0.34	0.43±0.31	0.17±0.25
Muscle	2.53±0.39	1.54±0.69	0.885±0.089	0.473±0.066	0.190±0.032	0.027±0.061
Intestines	2.88±0.43	1.84±0.63	1.15±0.22	0.67±0.15	1.22±1.21	0.08±0.13

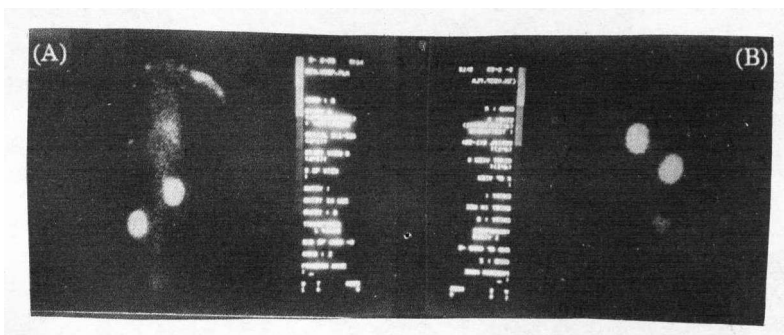

Fig.3 Two scintiphotos taken from rabbits at 5 min (A) and 20 min (B) after IV injection of ^{99m}Tc–MT

4 DISCUSSION

The labelling of MT with ^{99m}Tc in very low concentrations is strongly influenced by the labelling protocols and a variety of parameters of the solution. In transcomplexing labelling of MT from ^{99m}Tc–GH as well as ^{99m}Tc–Cit, the insufficient transcomplexing efficiency and slow transcomplexing rate limit its practical application. It is found that the direct labelling approach may provide a useful route accessible efficient and fast labelling of MT with ^{99m}Tc . The choices of buffer systems

and the pH value of labelling system are two critical parameters in the direct labelling method because the buffer may also play a ^{99m}Tc-chelating role in the labelling and the pH values of the labelling solutions strongly affect the hydrolysis of reductant agent $SnCl_2$. The optimized system for the labelling of MT with ^{99m}Tc is a HAc/NaAc or NaH_2PO_4/Na_2HPO_4 buffer at pH 6.3 (5.5—6.5). Radiolabelling efficiency of 93—96 % can be obtained in these systems when 6×10^{-6} mol/l of MT and 2×10^{-4} mol/l of $SnCl_2$ solutions are used and the labelling reaction proceeds for 15 min. Efficient radiolabelling could be carried out even if MT solution is as dilute as 1×10^{-6} mol/l. The radiolabelled ^{99m}Tc-MT has been proven to be stable *in vitro*.

The biodistribution in mice and rabbit imaging studies showed that ^{99m}Tc-MT accumulated in kidneys at an exceptionally high level, while the background was very low. Since technetium-99m dimercaptosuccinic acid (DMSA) has replaced organoradiomercurial renal agent as a major ^{99m}Tc renal imaging agent[6], a number of ^{99m}Tc-radiopharmaceuticals have appeared to be valuable for renal imaging. These agents include calcium gluconate, carlidin, acetylcysteine, TPEN, as well as ^{99m}Tc-Fe-ascorbate[7]. However, only a few of them have been practically used, i.e. ^{99m}Tc-DMSA and ^{99m}Tc-gluconate, while the majority of them have not yet clinical value due to their cumbersome preparations or not having significant merits compared with the common renal imaging agents. Our new findings on ^{99m}Tc-MT indicate that it would be a good candidate for renal imaging agent.

Furthermore, the conjugation of ^{99m}Tc-MT with anti-tumor monoclonal antibody leads to the formation of a radiolabel ^{99m}Tc-MT-MoAb which would be a potential imaging agent in radioimmunodiagnosis for malignant tumors.[8]

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