

CHAPTER III

Experimentals

Experiments are composed of different fields. The chemicals and instruments used in this work are separately listed as follow.

3.1 Synthesis of S-Bz-MAG₃

The S-Bz-MAG₃ was synthesized by using the reagents as follow.

reagents

1. Benzoyl chloride (Laboratory grade, Fluka) 97%
2. Thioglycolic acid (Sigma) 98%
3. Toluene (Merck)
4. Concentrate Hydrochloric acid (Merck) 37%
5. N-Hydroxysuccinamide (sigma) 98%
6. N, N'- Dicyclohexylcarbodiimide (sigma) <98%
7. Tetrahydrofuran (Fluka)
8. Ethylacetate (Merck)
9. Sodium hydroxide (Merck)
10. Glacial acetic acid (Merck) 100%
11. Triglycine (sigma) 98%
12. Ethanol (Merck)
13. Acetonitrile (Merck)
14. Methanol (Merck)
15. Iso-propanol (Merck)

Instruments

1. John-Fisher electrothermal melting point apparatus
2. Infrared spectrometer, Juscó, Model A100
3. H^1 NMR spectrometer, Varian, Model 360A
4. Fourier transform NMR spectrometer, Bruker, Model AC200F

Procedure

3.1.1 The synthesis of S-Benzoylthioglycolic acid

Sodium hydroxide 8.8 g (0.22 mol), and thioglycolic acid 9.2 g (0.1 mol) were dissolved in a mixture of 75 ml toluene and 75 ml water. The mixture was cooled in an ice bath to about 10 °C. 14.50 g (0.1 mol) of benzoylchloride was slowly added into the mixture during 30 minutes. The reaction mixture was stirred for an additional 30 minutes at room temperature. The organic layer was removed and extracted four times with water. All the aqueous phase was combined and acidified to pH = 1.5 by concentrated hydrochloric acid. The precipitate was filtered and dried. The product was recrystallized from ethyl acetate to yield 13.74 g (0.07 mol, 70.10%). The product was colorless crystals with a melting point of 104-105 °C. The structure elucidation of the product was obtained by IR and H^1 NMR spectroscopy. The IR and H^1 NMR spectra are shown in Figure 4.1 and 4.2, respectively

3.1.2 Succinimidyl-S-benzoylthioglycolate

S-Benzoylthioglycolic acid 9.89 g (0.05 mol), and N-hydroxy-succinamide 5.75 g (0.05 mol) were dissolved in 60 ml tetrahydrofuran. The mixture was cooled to -5 °C. A solution of 12.38 g (0.06 mol) dicyclohexylcarbodiimide, in 20 ml tetrahydrofuran was then added during 20 minutes. Subsequently the reaction mixture was stirred for 2 hours at -5 °C, then at room temperature for 13 hours. After glacial acetic acid 0.2 ml was adding and stirring for an additional hour. The product was filtered from the N,N'-dicyclohexylurea. The residue was extracted twice with tetrahydrofuran. The combined filtrate was evaporated to dryness. The colorless residue was recrystallized from ethylacetate. The succinimidyl-S-benzoylthioglycolate (11.01 g (0.37 mol 75.15%)) is colorless, needle shape crystal with melting point of 135-136 °C. The IR and H¹ NMR, spectra are shown in Figure 4.3 and 4.4

3.1.3 S-Benzoyl-mercaptoacetylglcylglycylglycine (S-Bz-MAG₃)

2.83 g (15 mmol) of the triglycine, was dissolved in 13 ml water, by dropwise of 1 M sodium hydroxide. The solution was added in one portion to a warm solution (55 °C) of 5.86 g (20 mmol) succinimidyl-s-benzoylthioglycolate in 120 ml ethanol. The mixture was refluxed for 2.5 hours and then stirred at room temperature for 12



hours. After the solvent was evaporated, the colorless residues was extracted twice with warm acetonitrile and recrystallized twice from 20% water in methanol. The product was then recrystallized from isopropyl alcohol to removed residual methanol. The 3.7 g (0.01 mol 67.21%) of product with melting point 193-195 °C was obtained. The IR, H^1 NMR and C^{13} -NMR, spectra are shown in Figure 4.5, 4.6 and 4.7

3.2 The preliminary preparation of Tc^{99m} -MAG₃ complex and determination of suitable separation method.

Chemicals

1. S-Bz-MAG₃ (synthetic Ligand)
2. Stannous chloride dihydrate (Analar grade, Merck)
3. Sodium gluconate (sigma)
4. Normal saline for injection (General Hospital)
5. Sterile water for injection used throughtout this experiment (General Hospital)
6. Instant thin layer chromatography (ITLC SG, Gelman)
7. Whatman paperchromatography number I (WT No.1, Whatman)
8. Sodium pertechnetate (Amersham)

Instrument and Apparatus

1. pH meter, Suntex, Model SX-600
2. Thin layer scanner gamma-ray detector, Berthold
3. Multi channel analyser, Ortech

Procedure

3.2.1 Preparation of MAG_3 kit

(0.1 g) S-Bz- MAG_3 and (2.0 g) sodium gluconate were dissolved in deoxygenated sterile water for injection. Stannous chloride dehydrated (0.002 g in 0.08 ml conc. HCl) was added dropwise. The volume of mixture solution was made up to 100 ml with water. The solution was filtered through 0.22 μm membrane and then dispensed 1.0 ml into each of 10 ml sterilized vial.

3.2.2 Labeling of MAG_3 kit with Tc-99m

The freshly eluted 25 mCi sodium pertechnetate (Tc-99m) solution was introduced into the MAG_3 kit and shaken to mix. The labeling reaction was performed in the boiling water bath for 10 minutes and then cooled in running tap water.

3.2.3 Quality control of $^{99\text{m}}\text{Tc-MAG}_3$ complex

The radiochemical purity of complex, reactant and hydrolysis species were determined by paper-thin layer chromatography. Radiochemical purities were assayed by thin layer scanner and each interested zone was counted by multichannel analyser. The separate conditions are tabulated in table 3.1

Table 3.1 Separation condition of $^{99m}\text{Tc-MAG}_3$

media	solvent
ITLC SG,	0.9% NaCl
ITLC SG,	85% Methanol
ITLC SG,	n Octanol
WT I	Acetonitrile
WT I	50-100% Acetonitrile in water

3.3 Complex Formation Study of $^{99m}\text{Tc-MAG}_3$ for Renal Imaging Instrument and Chemical

The instruments and Chemicals used were the same as in 3.2. The preparation, labeling and quality control process were also the same as in 3.2. The radiochemical purities were analysed by paper chromatography (WT No.1 and developpe with 65% Acetonitrile in water).

Procedure

3.3.1 Effect of Stannouse ion

Stannous chloride dihydrate was used as reducing agent in a reaction. MAG_3 kit preparation process was performed as in 3.2.1. The content of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in each kit for studying were 20,40,60,80 and 100 μg . The labeling

conditions were the same as in 3.2.2. The present chemical species were assayed by paper chromatography. The results, percentage of labeling of each kit and stannous ion quantities were plotted in Figure 4.8.

3.3.2 Effect of pH

A series of kit solutions containing 60 µg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ at various pH from 3 to 8, was studied as conditions in 3.3.1. The radiochemical purity of all species were plotted in Figure 4.9

3.3.3 Effect of Standing time

The percentage of radiochemical purities of the complexes were measured periodically at specific time interval after labeling. Ingredient in a kit containing 60 µg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ and other as 3.2 were used. The standing time was varied from 10, 30, 60, 120 and 180 minutes. Percentage of labeling changing were plotted in figure 4.10.

3.4 Study of Complex Formation of Copper(II) ion with MAG_3

3.4.1 Potentiometric titration

Chemicals

Normal saline for injection was used continually this experiment. A 0.02 M. solution of the synthetic ligand was prepared in normal saline. The Copper (II)

chloride of analar grade was used to prepare 0.10 M. of metal ion. Standard solution of hydrochloric 1.00 M. and sodium hydroxide 1.00 M (Merck) were also dilute with normal saline to optimized concentration.

Instruments and Apparatus

1. Potentiometer, Orion Research, Model 940/960 pH electrode
2. Personal Computer, NEC, Power mate II.

Procedure

The combination pH electrode was calibrated by three standard buffer of pH 4.01, 7.00 and 10.00. Accuracy of the instrument was indicated by the slope value based on the isopotential point of pH 7.00 = 0.0 mV, exceeded 99%. The dispenser of the automatic potentiometer was calibrated regularly.

All potentiometric titrations were carried out at 37.0 ± 0.5 °C and ionic strength of 0.15 M sodium chloride. The pH of solution was adjusted by sodium hydroxide solutions (0.05 M NaOH in 0.15 M NaCl for ligand and 0.15 M NaOH in 0.15 M NaCl for complex system). The titration were performed under nitrogen atmosphere by flowing nitrogen gas through the titration chamber. The pH range and the concentration of each titration were shown in table 3.2

Dissociation and basicity constant of S-Bz-MAG₃ were attained together with the dissociation constant of water (k_w) by simultaneous optimization using SUPERQUAD computer program. The calculations were done by personal computer. The formation constants of S-Bz-MAG₃ acquired from the calculations were shown in Table 4.11 and the dissociation constant of water refined from the optimizing process was a reasonable value of $\log k_w = -13.56$ at 37 ± 0.5 °C.

Table 3.2 Experimental Data used in Refinements.

Titration	initial concentration (mM)			pH range	Data
	Cu (II)	MAG ₃	Proton		
1	-	2.00	2.00	2.99-9.85	57
2	-	1.20	1.20	3.32-10.25	48
3	-	0.80	0.80	3.41-10.51	50
4	1.96	1.96	1.76	3.22-11.20	80
5	0.99	1.98	1.78	3.33-11.21	69
6	0.59	1.98	1.78	3.36-11.22	65

Formation constants of complex species from the computer refinements were shown in Figure 4.12. This stability constant was optimized together with the previously gainable dissociation constant of the ligand

alone. Metal hydroxide species $M(OH)^+$, and k_w were also included in the optimizing process in order to precisely obtain the stability constants of the complex system.

3.4.2 Stoichiometric by UV/visible spectroscopy

Chemical and Instrument

The 0.1 M glycine buffer pH 8 and 12 were used throughout this experiment. Solution of the copper(II) chloride and synthetic ligand were prepared in glycine buffer 8 and 12. The absorption spectrum of sample were measured with UV/visible spectrophotometer, Perkin Elmer, Model lambda 15

Optimum Wavelength of Cu-MAG₃ complex

Optimum wavelength of complex, Cu-MAG₃ was determined in glycine buffer pH 8 and 12 respectively by keeping ligand concentration at 1×10^{-3} M and varying Cu(II) concentration from 0 to 7×10^{-3} M. The spectral changes were detected at pH 8 and 12 compare with the reactant by UV/visible spectrophotometer as shown in Figure 4.14 and 4.15 respectively.

Job's Method (Mole fraction)

A series of Cu(II) and MAG₃ ligand mixture in glycine buffer pH 12 containing various mole fractions of Cu(II) from 0-1 were prepared. The sum of the concentration of Cu (II) and ligand was kept constant at 1×10^{-3} M.

The pH of solution was adjusted to 12. The absorbance of Cu(II)-MAG₃ mixture was measured at 494 nm. The result shown in figure 4.16.

Mole ratio

A series of solution containing equal concentration of the ligand was fixed at 1×10^{-3} M and the different concentration of ion from 0 to 4×10^{-3} M were prepared. The pH of the solution was adjusted to 12. The absorbance of the complex was measured at 494 nm. The plotted result is shown in figure 4.17

The Slope Ratio Method

Two series of solutions were prepared for studying. One was in the conditions of excess Cu(II), concentration fixed at 1×10^{-3} M, whereas the concentration of the ligand varied from 0 to 8×10^{-4} M. Another one was in the condition of excess ligand concentration fixed at 1×10^{-3} M whereas the concentration of the Cu(II) varied from 0 to 8×10^{-4} M. The pH of each solution was adjusted to 12. The absorbance of these complexes were measured at 494 nm. The data acquisition is shown in Figure 4.18

3.5 Influence of Cu(II) on $^{99m}\text{Tc-MAG}_3$

Chemicals

MAG₃ kit was prepared as in 3.3.3. A 0.24 g in 100 ml of copper chloride solution was prepared in 0.9% sodium chloride as a stock solution.

Instrument

The chromatograms and percentage labeling were counted and calculated by gamma-ray thin layer scanner and Multichannel Analyser.

Procedure

A series of Cu(II) 0, 90, 325, 450, 675 and 900 µg/ml added into the solution of $^{99m}\text{Tc-MAG}_3$ (Single dose for human, 5 mci/ml, prepared as in 3.3.3 under nitrogen pressure). The radiochemical purities were determined from 10 minutes to 180 minutes after the preparation by paper chromatography as 3.3 only $^{99m}\text{Tc-MAG}_3$ interested zone (Rf 0.3-0.4) used for calculated. The percent radiochemical purity of $^{99m}\text{Tc-MAG}_3$ in presence Cu(II) ion against times are plotted and shown in Figure 4.19. Summarized slope of its are shown in Table 4.13.