CHAPTER III

MATERIALS AND METHODS

Materials

For Formulation -

- 10-ml Amber glass vial, lyophilize rubber closure and aluminum cap.
- Hydrochloric acid : from E. Merck, Germany.
- Inositol : from Fluka Chemie AG., Switzerland
- L-Ascorbic acid : from E. Merck, Germany.
- Nitrogen gas : from TIG., Samutrprakarn
- pH paper, wide range (1-12) and narrow range (1-4) : from Whatman International, UK.
- Stannous chloride dihydrate ($SnCl_2.2H_2O$) : from Sigma Chemical Company, UK.
- Sodium hydroxide : from E. Merck, Germany.
- 0.45 μm Sterile disposable membrane filter (Acrodisc^R) : from Gelmen Sciences, USA.
- Succimer (meso-2,3-dimercaptosuccinic acid, DMSA) : from Sigma Chemical company. UK.

For Radiolabeling -

- Disposable plastic syringe, 5 ml, from Terumo Corporation. Japan.
- Sodium pertechnetate Tc 99m injection : from Office of Atomic Energy for Peace, Bangkok
- Standard Succimer cold kit : Amerscan Technetium-99m DMSA Agent form Amersham International plc, UK.

For Chromatography -

- n-Butanol : from E. Merck, Germany.
- Hydrochloric acid : from E. Merck, Germany.
- Polysilicic acid gel Impregnated glass fiber sheets (ITLC™ SA) : from Gelmen Sciences, USA.

For Autoradiography -

- 3% v/v Acetic acid.
- Developer and fixer for X-ray film : from Fuji Photo Film, Japan.
- Film cassette
- X-ray film 20.3 x 25.4 cm : from Fuji Photo Film, Japan.

For Biodistribution -

- Disposable latex gloves.
- Disposable plastic syringe with needle,1 and 5 ml from Terumo Corporation, Japan.
- Nembutal injection
- Plastic bag

- Sprague-Dawley female rats, 125-225 g : from Animal Experiment Center, Nakhon Pratom.
- Surgical equipment set
- Thread

Apparatus -

- Automatic pipette (Eppendorf, Germany)
- Gamma counter (model CompuGamma, LKB Bromma, Sweden)
- Lyophilizer (model Lyph-lock-12, Labconco, USA, and model Triphilizer, FTS system , USA.)
- pH meter (model 520A, Orient, USA.)
- Plastic Isolator
- Radioisotope Calibrator (model Amersham ARC-120, Capintec, USA.)
- Radioactive Shield (model Clear-Pb, Victoreen, USA)
- Vacuum Pump (Gast, USA.)

Methods

To protect the hazard from radioactivity the experiments concerning with radioactive ^{99m}Tc were done behind radioactive shield.

Calibration of assay systems

To calibrate the assay systems for analysis of radiochemical purity, the three types of ^{99m}Tc labeled succimer were prepared using the commercially available cold kit and used as standards representing various types of ^{99m}Tc-DMSA complexes, as followed;

1. Standard commercial succimer cold kit was labeled with ^{99m}Tc and used to represent the complex for renal scintigraphy (^{99m}Tc(III)-DMSA). The sodium pertechnetate Tc 99m injection was calibrated to have radioactive concentration of approximately 370 MBq (10 mCi)/ml using Radioisotope Calibrator. In case of high concentration, the solution was diluted with sterile normal saline for injection. The 5 ml sterile disposable syringe was used to inject 1 ml of the solution into each vial of lyophilized DMSA. The vial was carefully inverted a few times until the powder was completely dissolved, allowed to stand for 15 minutes, then processed to analytical step.

2. ^{99m}Tc(V)-DMSA was prepared according to the method purposed by Westera, Gadze and Horst (1985). The standard succimer cold kit was dissolved in

0.4 ml of 7% sodium bicarbonate solution. The vial was shaken and immediately after,10 mCi of sodium pertechnetate Tc 99m injection (1ml) was added. The vial was shaken and incubated for 30 minute at room temperature. The resulting complex was referred to as ^{99m}Tc(V)-DMSA, or the DMSA labeled at alkaline pH.

3. ^{99m}Tc(III)-DMSA labeled in the condition of excess Sn(II) was prepared by adding 100 μl of Sn(II) solution (40mg/ml in 6 M. HCl) into the standard cold kit, follow by radiolabeling as described in 1 (page 30).

Besides the three kinds of standard preparations, the sodium pertechnetate Tc 99m was also used to represent the free pertechnetate in the chromatographic separation procedures.

The four preparations were then analyzed for radiochemical purity by two chromatographic systems. The chromatographic bands were visualized by autoradiography. The procedures were described as follows:

1. Chromatographic system I

System I was modified from that described in USP XXII under Technetium Tc 99m Succimer Injection. Thus, this system was an official system for used in the step of evaluation of the formulations. The instant thin layer chromatographic paper coated with silicic acid (ITLC-SA) was cut into 25x200 mm strip with the length of the paper parallel to the machine direction. The origin position was marked with soft

pencil at 25 mm from paper end. Approximately 1 μl of sample was applied and the paper was allowed to dry. The chromatogram was developed over a period of 45 minutes by ascending chromatography using n-butanol saturated with 0.3 M. HCL and then air dried. The band was localized by autoradiography. The radioactivity distribution on the chromatographic patterns were measured by strip cut-and-count method. The chromatographic paper was cut into 1 cm strips and the radioactivity of each strip was counted by well gamma counter for 1 minute (the half-life correction was calculated automatically by the counter). The percentage of radioactivity in each strip was calculated by using sum of the counts from every strips as a total counts. The ratio of the distance from the edge of strip to the distance from start point to solvent front, which was mentioned as relative Rf value or simplified as Rf in this study, was calculated by the following formula:

$$Rf = \frac{Distance\ (the\ edge\ of\ strip - start\ point)}{Distance\ (solvent\ front - start\ point)}$$

After calculation the % radioactivity was plot versus Rf value. The total radioactivity in the ^{99m}Tc-DMSA band and the other bands of interest, with respected to the standard bands, was calculated.

USP Limit : not less than 85.0 % of the labeled amount of ^{99m}Tc as the succimer complex.

Theoretical Rf value : pertechnetate = 1

^{99m}Tc(III)-DMSA = 0.45-0.7

Hydrolyzed ^{99m}Tc = 0-0.15

2. Chromatographic system II

System II was modified from that described by Westera et al (1985) as a system for determination of ^{99m}Tc(V)-DMSA. This assay system has no acceptant limit for ^{99m}Tc(III)-DMSA and was used for characterization of the labeled ^{99m}Tc-DMSA complexes in case the difference among the three formulations was found and the system I could not give satisfactory results. The assay procedure was done by the following methods. The 200x200 mm aluminum foil-backed Silica gel G_{60} TLC plate was used. The plate was separated into 25X200 mm portions by streaking the gel with needle. The original position was marked with soft pencil at 20 mm from the bottom. Approximately 1 μl of sample was applied and the plate was allowed to air dried. The chromatogram was developed by ascending chromatography using n-butanol/acetic acid/water 3/2/1 (V/V) as a mobile phase until the mobile phase moved to 11 cm from the bottom. The plate was air dried and the chromatographic bands were localized by autoradiography. The gel layer was sealed by self adhesive tape and the plate was cut into 5 mm strips and counted for the radioactivity by using well gamma counter. The calculations were done in the same way as for system I.

Theoretical Rf value : pertechnetate = 0.7

^{99m}Tc(III)-DMSA = 0-0.44

^{99m}Tc(V)-DMSA = 0.5

3. Autoradiography

The chromatographic paper or TLC plate was wrapped with plastic wrap and placed in the film cassette, then brought into the dark room. A sheath of X-ray film was placed on the paper, the cassette was closed firmly and leaved for 2 hours. After the exposure step the film was developed in the X-ray film developer for 1 minute, washed with 3 % acetic acid, fixed in the fixer solution for 15 minutes, washed with running tap water for 1 hour and air dried. The position of chromatographic bands were seen on the film as dark bands on blue background. The radiochromatographs were use for inspection of the number and position of the chromatographic bands. The Rf values were also calculated by using the data measured from the radiochromatographs by the following formula:

$$Rf = \frac{Distance\ (\ spot\ center - start\ point)}{Distance\ (solvent\ front - start\ point)}$$

Formulation

The three Technetium Tc99m succimer injection "cold kits" were formulated by using the two basic components, succimer and $SnCl_2.2H_2O$. The mole ratio of Succimer:$SnCl_2.2H_2O$ is 3:1, as described by Lin, Khentigan and Winchell (1974). The formulation 1 contained no other active ingredient. Ascorbic acid was added into the formulation 2 and 3 and inositol was added into the formulation 3. The pH of the 3 formulations was adjusted to 2.5 prior to lyophilization. After lyophilization the vials were filled with nitrogen gas, closed tightly and stored at 4°C. The formulation

procedures were as follows:

1. Formulation 1

Fifty milligram (0.27 mM) of meso-2,3-dimercaptosuccinic acid was dissolved in 25 ml of 0.1 M NaOH. Stannous chloride solution was prepared by dissolving 206 mg (0.913 mM) of $SnCl_2.2H_2O$ in 5 ml of 6 M HCl and was put into boiling water bath until clear solution was obtained. Both solution were degassed under vacuum and were brought into the isolator filled with nitrogen gas. 0.5 ml of stannous chloride solution (0.09 mM) was added into DMSA solution and mixed thoroughly. The pH was adjusted to 2.5 by 1 M NaOH and total volume was adjusted to 50 ml with distilled water. After mixing the solution was filtered through 0.45 μm. sterile membrane filter to get rid of microorganism and particulate matter. To each of 10-ml vial 1 ml of filtrate was transferred to a vial and rubber stopper was placed loosely on the top of the vial. Then the product was transferred to lyophilizer and lyophilized (see method of lyophilization on page 36). After the process finished the vial was capped under nitrogen atmosphere and stored at 4°C for further experiments.

2. Formulation 2

The formulation 2 was modified from formulation 1 by adding ascorbic acid as an antioxidant. Ascorbic acid solution was prepared by dissolving 35 mg of ascorbic acid in 1 ml of distilled water. After the pH of the Succimer-stannous chloride mixture was adjusted to 2.5, 1 ml of ascorbic acid solution was added. The volume was adjusted to 50 ml by distilled water. The final concentration of ascorbic

acid is 0.07 % (W/V). The solution was aliquoted and lyophilized were performed in the same manner as described in 1.

3 Formulation 3

The formulation 3 was modified from formulation 1 by the addition of ascorbic acid as an antioxidant and inositol as a suspending medium for lyophilization. The 35 mg/ml ascorbic acid solution was prepared and added into the succimer-stannous chloride mixture, pH 2.5. The solution of 2.5 g inositol in 15 ml of distilled water was prepared and added. The pH was checked and adjusted to 2.5 if necessary and the volume was adjusted to 50 ml. The resulting formulation was aliquoted and lyophilized in the same way as described in 1.

4. Lyophilization

The lyophilization process was performed by using the lyophilizer equipped with stoppering tray dryer. The three succimer formulations were lyophilized by using Lyph-lock 12^{R}. The product vials were loaded on the shelves and were frozen at -40°C. After the ice collecting coil temperature reached -55°C the vacuum pump was started and the vacuum was maintained at 100 microns at the temperature of the product chamber was - 25°C. When the products were completely dried the product temperature was brought up to 20°C.The product chamber was then filled with nitrogen gas and the vials were stoppered by air bag mechanism. After finishing the vials were taken out and sealed with aluminum caps. The products were stored at 4°C for further experiments.

Evaluation of the formulations

To evaluate the various properties of the cold kit formulations, the formulations were sampling for analyzed at various time interval according to the schedule. The formulations were labeled with ^{99m}Tc prior to analysis by the same method as described for the standard in topic 1 of page 30.

1. Properties of succimer cold kit formulations

The three formulations of succimer cold kits were sampling for analysis at time 0, 1, 2 and 3 month(s) after preparations. The kits were radiolabeled and assayed for radiochemical purity using system I. The assay procedures were performed in the same manner as for standards. After radiolabeling the mixtures were taken out for analyze at 15, 30, 60, 90, 120 minutes and 24 hours respectively. The autoradiography and determination of radioactivity distribution by strip cut-and-count method were also performed. The percentage radioactivities of the strips were plotted versus relative Rf values. The resulting plots were devided into segments coresponding to the position of the main peaks and the total radioactivity in each segment was calculated. The assay results were evaluated as follows:

1.1 Labeling property The labeling property of the three formulations were determined by USP limit. It should contain not less than 85 % of the labeled amount of ^{99m}Tc-DMSA. The differences among the 3 formulations were determined by ANOVA.

1.2 Stability of the formulations The stability of the formulations were evaluated by comparing the different among the % labeling at various storage time.The comparison was done by ANOVA.

1.3 Stability of labeled products The stability of the labeled products was determined by comparing the % labeled amount at various time after labeling using mean and standard deviation.

2. Biodistribution studies

The biodistribution studies were performed using samples at 0 and 3 month(s) after preparations. The experimental procedures were modified from the procedures stated in the USP XXII under Technetium Tc 99m Succimer Injection. Three 125-g to 225-g Sprague-Dawley female rats were anesthetized by injecting 60 mg/kg of Nembutal intraperitoneally. A dose between 3.7 MBq and 92.5 MBq (100 μCi and 2500 μCi of Technetium Tc 99m Succimer injection, in a volume of 0.2 to 0.25 ml, was injected into the caudal vein of each rat. The opening of the urethra was clamped with a hemostat. The animal was sacrificed 1 hour after injection by heart puncture. The kidney, bladder, liver, spleen, heart, head and legs were carefully removed by dissection. Each organ and the remaining carcass (excluding the tail) were placed in plastic bags. The radioactivity in each organ was measured by radioisotope calibrator, The time of measurement was recorded for calculation of dose calibration.

The dose at time t was calculated from equation:-

$$D_t = D_0 e^{-\lambda t}$$

Where D_t = dose at time t

D_0 = dose at time 0

t = time in minute

λ = 0.693/half-life of ^{99m}Tc

= 0.693/(6.08 hr x 60 min)

After the dose correction was made the % radioactivity in each organ and the ratio of kidney/(liver and spleen) were calculated.

USP limit : not less than 40 % of the administered radioactive dose is found in the kidneys and a ratio of not less than 6:1 of the administered dose is found in the ratio kidney/(liver and spleen), in not fewer than two of the animals.

The statistical difference in organ distribution among the three formulations and within formulation at different storage times were determined by ANOVA.

Studies on the factors affecting the quality of the formulations

1. Study on the degradation patterns of the three formulations

When the experimental data from three months were analyzed the differences among the three formulations were found. The attempt had been made to

differences among the three formulations were found. The attempt had been made to investigate the different degradation patterns. The three formulations from the third month were labeled and analyzed by system II at 15 minutes, 180 minutes and 24 hours after labeling. The % radioactivity distributions were measured by strip cut-and-count method and plot versus Rf values. The resulting radiochromatograms from the 3 formulations were compared.

2. Determination of the optimum amount of ascorbic acid

The experimental results from the previous experiment demonstrated that the theoretical amount of ascorbic acid had an effect on stabilizing the formulations. The following experiments were done to determine the optimum amount of ascorbic acid suitable for this formulation methods. Five preparations of succimer cold kits with various amounts of ascorbic acid were prepared in solution. The solutions of succimer and Sn(II) were prepared using succimer:Sn(II) equal to 3:1 with the various concentrations of ascorbic acid ie. 0.175, 0.35, 0.7, 1.4 and 2.8 mg/vial.

Each of 5 portions of 50 mg of meso-2,3-dimercaptosuccinic acid (DMSA) was dissolved in 25 ml of 0.1 M NaOH. Stannous chloride solution was prepared by dissolving 206 mg of $SnCl_2.2H_2O$ in 5 ml of 6 M HCl and was put into boiling water bath until clear solution was obtained. Then 0.5 ml of stannous chloride solution was added into each DMSA solution and mixed thoroughly. The pH was adjusted to 2.5 by 1 M NaOH. The solutions of 8.75, 17.5, 35, 70 and 140 mg of ascorbic acid per one ml of water were prepared and one ml of each solution was added into previous solution. The volumes were adjusted to 50 ml with distilled water. The 1 ml of

solutions were aliquoted into 10 ml vials. The vials were capped with rubber and aluminum cap and leaved overnight. The 5 preparations were allowed to expose to oxygen and moisture in the closed vials for 12 hours at room temperature. After 12 hours each preparation was labeled with 1 ml of ^{99m}Tc in the same way as in previous experiments. The labeled preparations were sampling to asay for radiochemical purity using system II at 15, 180 minutes and 24 hours after labeling. The radioactivity distributions of the resulting chromatographic bands were measured by strip cut-and-count methods. and plotted against relative Rf value. The total % radioactivity in each band was calculated and the difference among five formulations were determined by ANOVA.

3. Effect of efficiency of equipment on the quality of the formulations

From theoretical point of view, oxygen has remarkable effect on the properties of the Succimer cold kits. At the late phase of study the new type of lyophilizer (Triphilizer^{R}) that had better efficiency than the former one was available. The new batch of the three succimer formulations were prepared and lyophilized using this machine. The lyophilization process was automatically monitored. After the process finished the product chamber was filled with nitrogen gas and the vials were stoppered by using motor drive stoppering system. After preparation the products were analyzed by system II and the radiochromatographic patterns were made in the same manner as 2.

Statistical significant differences

The statistical significance was determined by Analysis of Variances (ANOVA) at a significance level of 0.05 using Microsoft Excel[R] computer program.

The flow chart of experimental procedures has been summarized as displayed in figure 4.

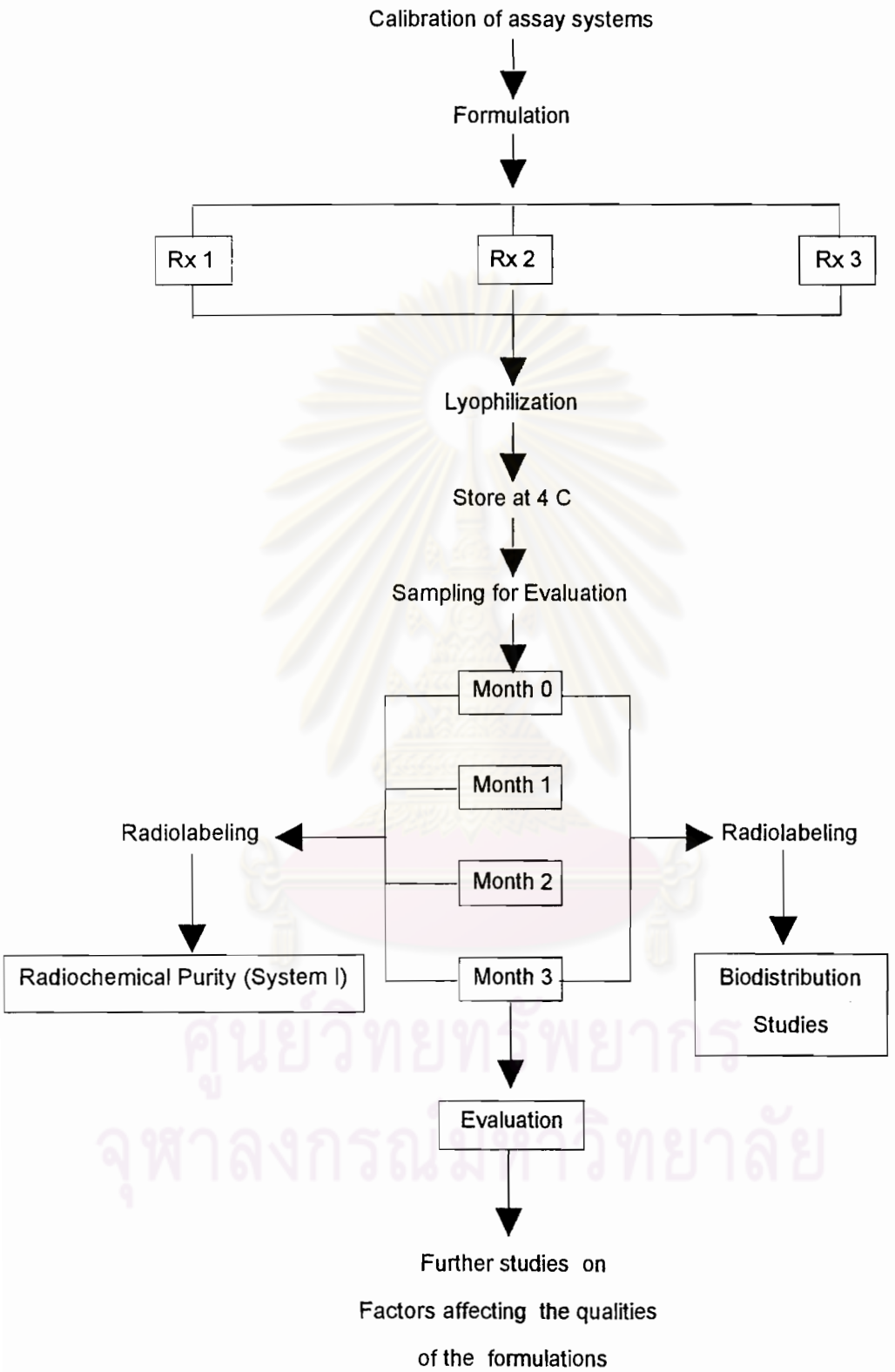

Figure 4 The flow chart of experimental procedures