CHAPTER III

RESEARCH METHODOLOGY

3.1 Patients

Prosthetic Heart Valve outpatients taking warfarin at Cardiovascular thoracic unit of The King Chulalongkorn Memorial Hospital. One hundred and seven patients were recruited for this study based on the following criteria:

3.1.1 Inclusion criteria

The patients who had all of these characteristics were enrolled in this study.

1. Patients who have received warfarin for at least 2 months
2. Patients who stable with the same warfarin dose for at least 2 visits.

3.1.2 Exclusion criteria

The patients who had either one of these characteristics were excluded from this study.

1. Patients who have at least one of following diseases (CHF, Cancer, hepatic disorder, history of hypo/hyper thyroidism).
2. Lost of follow up

3.1.3 Sample Size Determination

$$N = [(Z\alpha + Z\beta)/Z(r)]^2 + 3$$

$$Z_r = \frac{1}{2}\ln[(1+r)/(1-r)]$$

Significance level (α) = 0.05, $Z_\alpha = 1.96$, $Z_\beta = 1.28$

Calculated the correlation coefficient (r) from Gage et al.[39] $R^2 = 0.39$ and Kamali et al.[38] $R^2 = 0.20$, approximated $R^2 = 0.3$, r is about 0.55

$$Z(r) = \tfrac{1}{2} \ln [(1+0.55)/(1-0.55)]$$

$$= 0.61$$

$$N = [(1.96 + 1.28) / 0.61]^2 + 3$$

$$= 28.2 + 3$$

$$= 31.7$$

Sample size is at least 30 patients

However, there are about 10 factors in this study, ten patients per factor are required and therefore the sample size should be approximately 100 patients.

(The sample size should be at least 6 to 10 cases for every variable in the pool)[51]

3.2 Study design

Study designs was observational study that focuses on the associations between warfarin doses and factors such as CYP2C9 polymorphisms, VKORC1, warfarin concentration etc. Data was collected from outpatients taking warfarin by laboratory blood sample testing, interviewing the patients and collecting data from OPD card. The protocol was approved by the ethic committee of the King Chulalongkorn Memorial Hospital. The flow chart of study shows in figure 3.1.

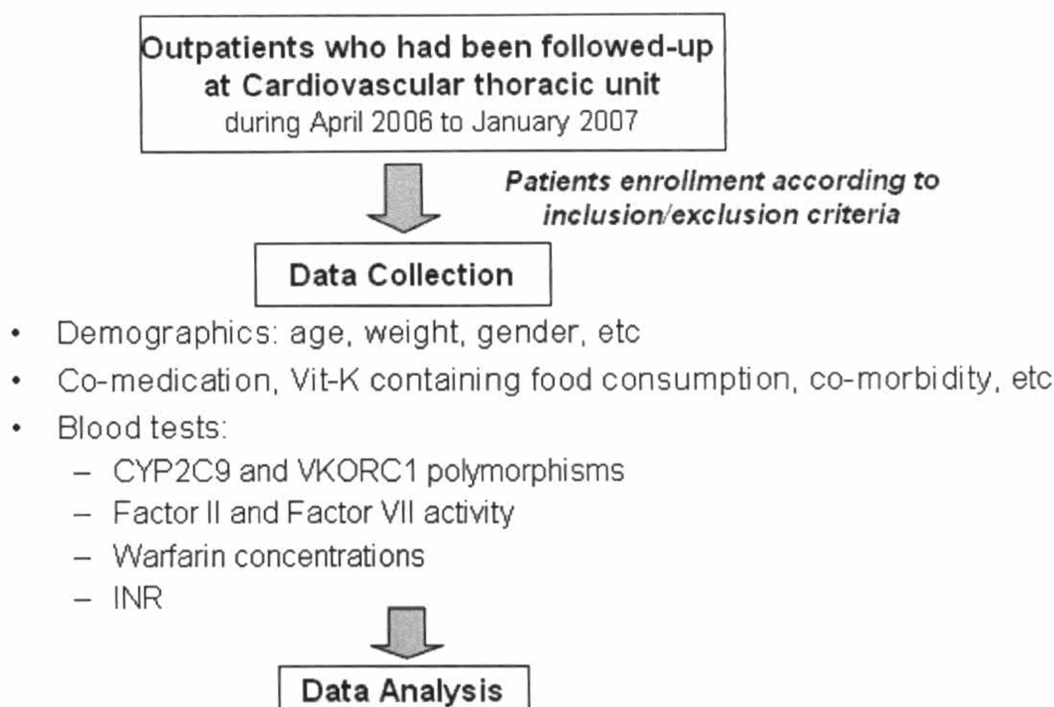

Figure 3.1 Flow chart of the study

3.3 Sampling

One hundred and seven patients who met the inclusion criteria were participated in this study. As routine of patient care, without intervention, warfarin dosage regimens were prescribed by physician. All patients were treated with Orfarin® (Orion Pharma, Finland).

Eighteen milliliters of whole blood was drawn from patients before the next warfarin dose in the morning. Blood samples were collected in 2 tubes of 4.5 mL of 3.2% sodium citrate tube and 9 mL of EDTA tube. Centrifuge at 3,000 rpm for 10 minutes then plasma from sodium citrate tube were transferred into 1.5 mL of microcentrifuge tubes and stored at -20°C (for warfarin concentration analysis) and snap frozen by liquid nitrogen then stored at -80°C (for clotting factor analysis) until analysis. The samples were analyzed within one month for warfarin concentration. Buffy coat in the EDTA tube was prepared for genomic DNA extraction.

3.4 Bioanalysis

3.4.1 DNA extraction

Peripheral blood lymphocytes were used for DNA extraction by standard Phenol-Chloroform extraction method. Blood is composed of white blood cells and red blood cells. The erythrocytes were lysed by hypotonic solution. Proteins were precipitated by Phenol-Chloroform.

3.4.1.1 Materials

Chemical and reagents

1) Lysis buffer I
2) Lysis buffer II
3) 10% SDS
4) Proteinase K
5) 1% Triton X-100
6) Phenol-chloroform-isoamylalcohol (25:24:1)
7) 7.5 M Ammonium acetate
8) 70% Ethanol
9) 100% Ethanol (Cold)

Instruments

1) Autoclave
2) Centrifuge
3) Microcentrifuge
4) Water bath
5) Vortex mixer
6) Micropipettes
7) UV-Vis spectrophotometer

3.4.1.2 Extraction methods

1. Pipette 1,000 μL of buffy coat into polypropylene tube size 15 ml.
2. Add about 10 ml of cold Lysis buffer I and shake well then incubate at -20°C for 5 min
3. Centrifuge at 4,500 rpm for 8 min and discard supernatant
4. Add about 3 ml of cold Lysis buffer I and shake well
5. Centrifuge at 4,500 rpm for 8 min and discard supernatant (RBC lysate). Repeat step 4 and 5 for 2-3 times.
6. Add 900 μL of Lysis buffer II, 50 μL of 10% SDS and 20 μL of Proteinase K then vortex for 15 seconds.
7. Incubate in water bath at 50°C overnight
8. Add 1 ml of Phenol-chloroform-isoamylalcohol (25:24:1) and vortex for 15 seconds.
9. Centrifuge at 6,000 rpm for 5 min.
10. Pipette 500 μL of supernatant into a new microcentrifuge tube
11. Add 250 μL of 7.5 M Ammonium acetate and 500 μL of 100% Ethanol (Cold) and gently mix. (DNA Appear)
12. Centrifuge at 14,000 rpm for 20 min and discard the supernatant
13. Add about 300μL of 70% Ethanol
14. Centrifuge at 14,000 rpm for 15 min and discard the supernatant
15. Place the microcentrifuge tube opening face down to dry at room temperature overnight.
16. Dissolve DNA by about 50 μL of autoclaved distilled water and incubated at 37°C
17. Determine OD at 260 nm by UV-Vis Spectrophotometer and calculate DNA concentration from following equation.

DNA concentration (ng/μL) = OD. x 50 x dilution factor

(OD 1.0 is equivalent to approximately 50 ng/μL of double stranded DNA)

3.4.2 Genotyping

CYP2C9 polymorphisms and VKORC1 genotypes were analyzed by Real time PCR using fluorogenic hybridization probes.

3.4.2.1 Materials

Chemical and reagents

1) LightCycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics Corp.)
2) Light Mix (TIB MOLBIOL, Berlin, Germany) (Primer & probes mix and Control DNAs for human, wild type, mutant and heterozygous of CYP2C9*2 and wild type, mutant and heterozygous of CYP2C9*3)
3) Genomic DNA (20-25 ng/µL)

Instruments

1) LightCycler 2.0 (Roche, Mannheim, Germany)
2) Glass capillary tubes
3) LightCycler Carousal Centrifuge 2.0

3.4.2.2 Analytical Methods

Single reaction (15 µL) consisted of 4 µL of primer and probes, 2 µL of FastStart DNA mix and 9 µL of water (PCR grade) in pre-cooled glass capillaries tube. Add 5 µL of genomic DNA (20-25 ng/µL) to each capillary given 20 µL of final reaction volume. Control DNA (supplied with kit) as positive control and water (supplied with kit) as negative control were analyzed with patient DNA sample in each batch. The genotypes were identified

by melting curve in different melting points (Tm). As shown in Table 3.1, fluorescence was acquired once per cycle at the end of the annealing phase in the cycling program. Melting curves were analyzed by slowly increasing temperature (0.2°C per second) from 40°C to 85°C. During melting program, fluorescence emissions were acquired continuously.

Table 3.1 Real time PCR conditions

Program	Denaturation	Cycling			Melting			Cooling
Analysis mode	None	Quantification			Melting curves			None
Cycles	1	45			1			1
Segment	1	1	2	3	1	2	3	1
Target (°C)	95	95	60	72	95	40	85	40
Hold	10 min	5 sec	10sec	15sec	20sec	20sec	0 sec	30 sec.
Ramp rate (°C/s)	20	20	20	20	20	20	0.2	20
Acquisition mode	None	None	Single	None	None	None	Cont.	None

Cytochrome P450 2C9 polymorphisms

CYP2C9*2 was detected with SimpleProbe in channel 530 and CYP2C9*3 was detected with LightCycler Red 640 labeled hybridization probes in channel 640. For CYP2C9*2, Tm of wild type and mutant were 58.5°C and 50.5°C, respectively. For CYP2C9*3, Tm of wild type and mutant were 48.3°C and 58.3°C, respectively.

VKORC1 C1173T and VKORC1 G-1639A

Tm 51.5°C for VKORC1 C1173 and 58.1°C for VKORC1 1173T was detected with SimpleProbe in channel 530. Tm 52.6°C for VKORC1 G-1639 and 61.3°C for VKORC1 -1639A were detected with probe labeled with LightCycler Red 640 in Channel 640.

3.4.3 Warfarin concentration

Determination of warfarin concentration was performed using HPLC with UV detection method modified from Locatelli et al.[52]

3.4.3.1 Materials

Chemical and reagents

1) Warfarin (Sigma) Lot no.104K1261
2) Naproxen (as the internal standard)
3) Phosphate buffer (Potassium dihydrogen phosphate)
4) Sulfuric acid (Merck) Lot no. K34041831
5) Diethylether
6) Acetonitrile (Fisher Scientific)
7) Methanol (Fisher Scientific)

Instruments

1) High-Performance Liquid Chromatography (HPLC) system (Dionex) consisted of P680 HPLC pump, ASI-100 automated sample injection and UVD 170U detector.
2) Column HyperClone C18-BDS 5 μm, 150x4.6 mm (Phenomenex)
3) SecurityGuard Guard Cartridge KJ0-4282, AJ0-4287 (Phenomenex)
4) pH meter S-20K (Mettler Toledo)
5) Digital balance AB 204-S (Mettler Toledo)
6) Sonicator and Degasser (Elmasonic, Elma S100H)
7) Vacuum pump and compressor (Gast manufacturing)
8) Vortex mixer (Scientific Industries)
9) Sample concentrator DB-3 Dri-Block (Techne)

10) Freezer -20°C (Sanyo)

3.4.3.2 Analytical Methods

Samples were prepared using liquid-liquid extraction. Total plasma warfarin concentration was determined by adding 50 μl of internal standard (naproxen 100 μg/ml), 0.7 ml of 1 M sulfuric acid and 5 ml of diethyl ether to 1 ml of plasma sample , vortex mixing for 5 second and shaking the sample at 300 rpm for 30 min. Centrifuge the resulting solution at 600 rpm for 10 min. and kept in a freezer at -20 °C for 3 hours. The diethyl ether layer was decanted from a frozen aqueous layer to a clean tube and then evaporated to dryness in a heat box at 45 °C. The residue was dissolved in 250 μL of acetonitrile in water (25:75 v/v). A volume of 100 μL of the solution was injected into the chromatograph.

Table 3.2 Chromatographic conditions

Column	:	HyperClone C18-BDS 5 μm, 150x4.6 mm
Mobile Phase	:	15mM of Phosphate buffer (pH 3.0, adjusted by 1M HCl): Methanol : Acetonitrile (52 : 32 : 16 v/v)
Internal Standard	:	Naproxen
Detector	:	UV 306 nm
Retention time	:	warfarin 16 min., naproxen 11 min.
Flow Rate	:	1.2 mL/min
Injection volume	:	100 μl

Validation of HPLC method including linearity, specificity, selectivity, precision, accuracy, and stability were performed (See in appendix). Standard solutions were prepared by dissolved in phosphate buffer solution (pH 7.4).

3.4.4 Clotting factor activities

Clotting factor activities (FII and FVII) were determined by Photo-optical clot detection method (scattered light detecting method) on CA-500 Automated Coagulation Analyzer (Sysmex).

3.4.4.1 Materials

Chemical and reagents (Dade Behring, Marburg, Germany)

1) Factor II deficient plasma
2) Factor VII deficient plasma
3) Control plasma N (Negative, Positive)
4) Standard Human plasma
5) Thromborel S
6) CA clean
7) Owren's Veronal Buffer

Instruments

1) CA-500 series Automated Coagulation Analyzer (Sysmex)
2) Microcentrifuge (Sorvall Legend RT)
3) Reaction tubes SU-40
4) Vortex mixer (Scientific Industries)
5) Freezer -80°C (ThermoForma)

3.4.4.2 Analytical Methods

Program was automatically running followed this process.

1. Rinse probe with CA clean
2. Add 5 µL of sample plasma in 45 µL of Owren's Veronal Buffer into the reaction tube.
3. Incubate at 37 °C
4. Add 50 µL of Factor deficient plasma and mix
5. Incubate at 37 °C for 30 seconds
6. Add thromborel S 100 µL then mix
7. Incubate at 37 °C for 210 seconds
8. Detection

3.5 Pharmacokinetic parameter calculation

Clearance, pharmacokinetic parameters was calculated using the following equations:

$$Cl = Dose/Cssav*interval$$

3.6 Statistical analysis

Analysis of descriptive statistics and inferential statistics data were performed using the SPSS program version 14. A p-value of less than 0.05 was considered statistically significant for all analyses.

1. Patients' characteristics: gender, age, weight, social history (alcohol consumption, smoking), underlying diseases, dose of warfarin, number of co-medication, bleeding and thrombosis history, and history of INR values, self-medication, vitamin K-containing food consumption will be shown as frequency, percentage, mean range and standard deviation.

2. Dose and clearance of warfarin will be shown as mean range and standard deviation.
3. Prevalence of CYP2C9 and VKORC1 genotypes will be shown as frequency, percentage and 95%CI of the proportion.
4. Comparison of warfarin doses and warfarin clearance in different CYP2C9 and VKORC1 genotypes will be shown as mean, standard deviation and 95%CI of the difference were analyzed by independent t-test and one way ANOVA.
5. Association of all factors and doses of warfarin will be analyzed by forward stepwise multiple linear regressions. Multicollinearlity of independent factors were determined.
6. Comparison of genotypes and frequency of complications of warfarin will be analyzed by Chi-square test and odds ratio.

3.7 Ethical consideration

In this study, the blood samples of patients who are taking or start to take warfarin were analyzed for VKORC1, CYP2C9 Polymorphisms, coagulation factors activity and warfarin concentration. All patients were written informed consent. Patient's medical information and DNA were protected confidentially. Results of this study may be published in scientific journals or presented at medical meetings but patient were not be personally identified.