CHAPTER III

MATERIALS AND METHODS

Materials

1. TDx® Digoxin II

1.1 No. 9511-01, Digoxin Calibrators

Six vials with accurately measured amounts of digoxin in human serum at the following concentrations :

Vial	*Digoxin Concentration (ng/ml)*
A	0.0
B	0.5
C	1.0
D	2.0
E	3.0
F	5.0

1.2 No. 9511-10, Digoxin Controls

Three vials of digoxin in human serum within the following ranges :

Vial	*Digoxin Concentration (ng/ml)*
L	0.55-0.95
M	1.30-1.70
H	3.15-3.85
	(Preservative : 0.1% Sodium Azide)

1.3 No. 9511-31, Digoxin II Precipitation Reagent (3% 5 -sulfosalicylic acid in 50% aqueous methanol)

1.4 No. 9519, Dilution Buffer

The dilution buffer, a 0.1 M phosphate buffer, contains a protein stabilizer and 0.1% Sodium Azide as a preservative.

1.5 No. 9511-60, Digoxin II Reagent Pack
There are three vials of reagents

Vial	*Components*
P	Pretreatment solution : Surfactant in buffer. (Preservative : 0.1% Sodium Azide)
S	<1% Digoxin Antiserum (Rabbit) in buffer with protein stabilizer. (Preservative : 0.1% Sodium Azide)
T	<0.01% Digoxin Fluorescein tracer in buffer with protein stabilizer. (Preservative : 0.1% Sodium Azide)

2. Apparatus

2.1 Automated Fluorescence Polarization Analyzer (Diagnostic Division, Abbott Laboratories, Inc., Irving, TX, USA.)

2.2 Centrifuge (Diagnostic Division, Abbott Laboratories, Inc., Irving, TX, USA.)

2.3 Centrifuge (Model CS, Internal (ICE) Centrifuge International Equipment, Inc. Needham His, Mass, USA.)

2.4 Vortex-Genie (Model K-550 GE, Scientific Industries, Inc., Bohemia, NY, USA.)

2.5 Freezer (Forma Bio-Freezer, Forma Scientific, Inc., USA.)

Methods

1. Subjects

Thai patients with heart failure (New York Heart Association [NYHA] functional class 2 and 3) admitted to Medical Department of Rajvithi Hospital with appropriate conditions were selected by physicians. Patients included in this study were not in critically ill. All patients were treated with digoxin or digoxin together with ACEI (captopril or enalapril) by traditional physician prescribing practices.

All of available patient data related to the study were recorded ; including age, sex, weight, height, medical history, diagnosis, drugs administered, dosage regimens, duration of digoxin and ACE inhibitor therapy, serum creatinine, and other clinical and laboratory data.

2. Dosage Regimen and Administration

Dosage regimen of digoxin and ACEIs was recommended by physician prescribing in general practice of Medical Department of Rajvithi Hospital. The subjects were divided into three groups:

Group	*Regimens*
1	received a treatment of oral digoxin tablet once a day.
2	received a treatment of oral digoxin tablet once a day. together with 2.5-10 mg/d enalapril.
3	received a treatment of oral digoxin tablet once a day. together with 25-50 mg/d captopril.

3. Sample Collection.

The digoxin serum concentration was considered to achieve steady state after the fixed dosage regimens of the drug were given to the patients for at least 5 days. Venous blood sampling for determination of serum digoxin concentration was drawn (3 ml) from forearm of patient immediately before and at 0.5, 1, 2, 3, 4, 8, 12, and 24 hours after the digoxin dose. Venous blood samples were allowed to clot and centrifuged immediately.(2,000 rpm ; 10 minutes ; at room temperature) Serum was separated and frozen until assayed.

4. Analytical Method

Digoxin concentration was measured by immunoassay using Fluorescence Polarization Technique (TDx® Analyzer System)

4.1 Preparation for Testing Analysis

A pretreatment step was performed on each digoxin sample (calibrators, controls, and patient samples) before testing.

4.1.1 Numbered a centrifuge tube for each sample to be tested and placed in a suitable rack.

4.1.2 Set the precision dispenser to dispense 200 mcl and filled it with Digoxin II precipitation reagent. Dispensed 200 mcl of precipitation reagent into each centrifuge tube.

4.1.3 Accurately pipetted 200 mcl of the serum sample to be assayed into its corresponding centrifuge tube containing precipitation reagent.

4.1.4 After pipetting all the samples, caped each centrifuge tube and mixed each on a vortex mixer for 3-5 seconds to ensure thorough mixing.

4.1.5 Placed the tube into the centrifuge head.

4.1.6 Centrifuged the samples for at least ninety seconds at 9,500 x g, or until a clear supernatant and a hard, compact pellet of denatured protein was obtained.

4.1.7 After centrifuge was complete, verify presence of protein pellet, uncaped each tube and decanted the supernatant into corresponding sample well of a sample cartridge.

4.2 Performing an Assay Calibration.

The required items were calibration carousel, cuvettes, sample cartridge, reagent pack, and calibrators.

4.2.1 Preparation of the carousel

- Loaded the carousel with 6 cuvettes in positions # 1 to # 6.
- Loaded the carousel with 6 cartridges in positions # 1 to # 6
- Transfered the supernatant from centrifuge tube into sample wells as follows :

Calibrators	*Sample wells*
A	1
B	2
C	3
D	4
E	5
F	6

4.2.2 Loaded the carousel in the instrument
4.2.3 Loaded the reagent pack in the instrument
4.2.4 Closed the door of the TDx® analyzer
4.2.5 Pressed run
4.2.6 The instrument commenced operation
4.2.7 Waited for run to complete and kept the printout

4.3 Performing an Assay Controls.

The required item were assay carousel, cuvettes, sample cartridge, reagent pack, and controls

4.3.1 Preparation of the carousel
- Loaded the carousel with 3 cuvettes in positions # 1 to # 3
- Loaded the carousel with 3 cartridge in positions # 1 to # 3
- Transfered the supernatant from centrifuge tube into the sample wells as follows :

Control	*Sample wells*
L	1
M	2
H	3

4.3.2 Loaded the carousel in the instrument
4.3.3 Loaded the reagent pack in the instrument
4.3.4 Closed the door of the TDx®Analyzer
4.3.5 Pressed run
4.3.6 The instrument commenced operation
4.3.7 Waited for run to complete and kept the printout.

4.4 Performing an assay run

Items required were assay carousel, cuvettes sample cartridges and reagent pack.

4.4.1 Preparation of the carousel
- Loaded the carousel with 9 cuvettes in positions # 1 to # 9 (for 9 specimens)
- Loaded the carousel with 9 cartridge in position # 1 to # 9
- Transfered the supernatant from centrifuge tube into sample wells as follows :

Sample number	Sample wells
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9

4.4.2 Loaded the carousel in the instrument
4.4.3 Loaded the reagent pack in the instrument
4.4.4 Closed the door of the TDx® analyzer
4.4.5 Pressed run
4.4.6 The instrument commenced operation
4.4.7 Waited for run to complete and kept the printout

5. Data Analysis

5.1 Calculation of the parameters.

Digoxin pharmacokinetic parameters were calculated from serum digoxin concentrations by using "Feathering method" or suitable computer programs such as CSTRIP and/or RSTRIP and/or PCNONLIN.

5.2 Comparison between groups of patients.

Digoxin pharmacokinetic parameters were compared between patient groups by the unpaired Student t test or by ANOVA.

5.3 Determination of the effect of captopril and enalapril on digoxin absorption, distribution, and elimination.

5.4 Calculation of the parameters from the patients' serum creatinine.

Vd and Ke were calculated using the available data included age, sex, weight, height and serum creatinine according to Equations 1-12 (Appendix A) and compared with those estimated from serum digoxin concentrations by ANOVA.

5.5 Comparison between the predicted and measured blood levels.

The predicted digoxin concentrations were calculated from the available patient data, using Equation 13 and 14 (Appendix A) and compared with the measured values by Student t test.

5.6 Create an equation for estimating digoxin clearance from creatinine clearance.

The digoxin clearance calculated from the serum digoxin concentration was plotted against the patient creatinine clearance. An equation described the relationship between these parameters could be used to estimate digoxin clearance from the patient's creatinine clearance.