CHARPTER III
PATIENTS AND METHOD

This study was conducted from February to September 2010 at Prasat Neurological Institute, Bangkok, Thailand.

1. Study design

A retro-prospective descriptive method was used. Demographic data and measured drugs serum concentrations from patients were collected, *CYP3A5* genes were genotyped, and the data were then analyzed.

2. Patients

2.1 Population and samples

2.1.1 Population is patients with epilepsy or neurological disease who used CBZ as monotherapy or coadministration with PHT, PB or VPA.

2.1.2 Samples are patients with epilepsy or neurological disease who were outpatients at Prasat Neurological Institute during February to September 2010 and met the inclusion criteria.

2.2 Inclusion criteria

2.2.1 Age not less than 13 years old.

2.2.2 Patients who were diagnosed to have epilepsy or neurological disease.

2.2.3 Patients who were treated with CBZ monotherapy or comedication with one of the other classical AEDs, PHT, PB or VPA.

2.2.4 Patients who received stable dose of CBZ in control released dosage form not less than 1 month before blood sampling.

Patients in comedication groups should used the coadministration drug not less than 1 month before blood sampling.

Patients who co administered with VPA should receive controlled released dosage form only.

2.2.5 All patients consented to enroll in this study.

2.3 Exclusion criteria

2.3.1 Patients with acute or chronic hepatic disease.

2.3.2 Patients with acute or chronic kidney disease.

2.3.3 Patients with drug non-compliance detected from interviewing by the investigator.

2.3.4 Patients who treated chronic diseases with drugs that reported to have some effects on pharmacokinetics of CBZ, such as, verapamil, diltiazem, gemfibrozil, isotretrinion, isoniazid, haloperidol, theophylline, ticlopidine, cimetidine, omeprazole, trazodone, fluoxetine, risperidone, clarithromycin, erythromycin, rifampicin.

2.3.5 Patients whose medical records were not complete or whose required data could not be revealed or were missing.

2.4 Sample size determination

2.4.1 CBZ monotherapy [46]

The purpose of this study was to determine whether patients with difference allele of *CYP3A5*, *CYP3A5*1* and *CYP3A5*3*, would show difference in their CBZ clearance which was a hypothesis testing about the difference of the means of two independent groups of population.

A study in Thailand found that the frequency of *CYP3A5*1* allele in Thai population was 34% and *CYP3A5*3* allele was 66%, that is, the ratio of *CYP3A5*1*: *CYP3A5*3* was 1:2. [17]

To assign:

N was the sample size of CBZ monotherapy patients

N_1 was the sample size of CBZ monotherapy patients with *CYP3A5*1*

N_2 was the sample size of CBZ monotherapy patients with *CYP3A5*3*

$N = N_1 + N_2, N_2 = 2N_1$

$$\frac{N_1 N_2}{N_1 + N_2} = \frac{(Z_\alpha + Z_\beta)^2 S_p^2}{D^2}$$

$$\frac{2N_1^2}{3N_1} = \frac{(Z_\alpha + Z_\beta)^2 S_p^2}{D^2}$$

$$N_1 = 3\frac{(Z_\alpha + Z_\beta)^2 S_p^2}{2D^2}$$

$$S_p^2 \text{ (pooled variance)} = \frac{S_1^2 + S_2^2}{2}$$

Previous study by Park PW. et al. reported that the polymorphism of *CYP3A5* effects on CBZ clearance. Patients with *CYP3A5*1* have higher CBZ clearance than *CYP3A5*3* (0.056 ± 0.017 L/hr/kg VS 0.040 ± 0.014 L/hr/kg, $p<0.05$).

To assign:

$\alpha = 0.05, Z_\alpha = 1.64$

$\beta = 0.20, Z_\beta = 0.84$

$$S_p^2 \text{ (pooled variance)} = \frac{(0.017)^2 + (0.014)^2}{2}$$

$$= 0.0002425$$

Park PW. et al. found that the difference of CBZ clearance between patients with *CYP3A5*1* VS *CYP3A5*3* was 29%, so, this study set the difference of CBZ clearance to detect to be 25%.

D (mean difference) = 0.01379

$$N_1 = 3\,\frac{(1.64+0.84)^2\,(0.0002425)}{2(0.01379)^2}$$

$$= 11.76 \approx 12$$

$N_2 = 24$, $N = 36$

The sample size of CBZ monotherapy patients was 36.

2.4.2 CBZ coadministration with PHT, PB or VPA. [47]

The study of the correlations between CBZ clearance and V_{max}, PB clearance and VPA clearance estimated sample size from this formula

$$N = \frac{(Z_\alpha+Z_\beta)^2}{Z_0^2} + 3$$

$Z_0 = (0.5)\ \ln\ (1+r/\ 1-r)$

$\alpha = 0.05$, $Z_\alpha = 1.64$

$\beta = 0.20$, $Z_\beta = 0.84$

r = correlation coefficient

To assign correlation coefficient = 0.60

$Z_0 = (0.5)\ \ln\ [(1+0.6)\ /\ (1-0.6)] = 0.693$

$$N = \frac{(1.645+0.84)^2}{0.693^2} + 3 = 15.86 \approx 16$$

The sample size of each combination therapy groups (CBZ+PHT, CBZ+PB and CBZ+VAP) was at least 16.

3. Study protocol

3.1 Study protocol was approved by the ethical committee of Prasat Neurological Institute.

3.2 Patients were selected following inclusion and exclusion criterias.

3.3 The investigator explained the objective and study protocol to the selected patients or their legal representatives. Patients or their legal representatives signed in the informed consent form.

3.4 Demographic data were collected from medical records.

3.5 Made an appointment for patient to have his/her blood sample collected at the next visited time. [Before a visit date the investigator called to remind the patient to bring his/her morning antiepileptic drug(s) along on the visit date and had his/her blood sample drawn before taking antiepileptic drug(s), blood samples for CBZ, PHT, PB and VPA levels monitoring were drawn at steady state, at trough level that was, before the administration of the next dose in the morning.]

3.6 Coordinated the doctor to order blood samples drawing for antiepileptic drug levels measurement and *CYP3A5* genotyping.

3.7 Coordinated the medical technologist for blood sample drawing to measure antiepileptic drug levels and *CYP3A5* genotyping.

3.8 Measured antiepileptic drug levels and *CYP3A5* genotyping.

3.9 Collected all the required data and analyzed.

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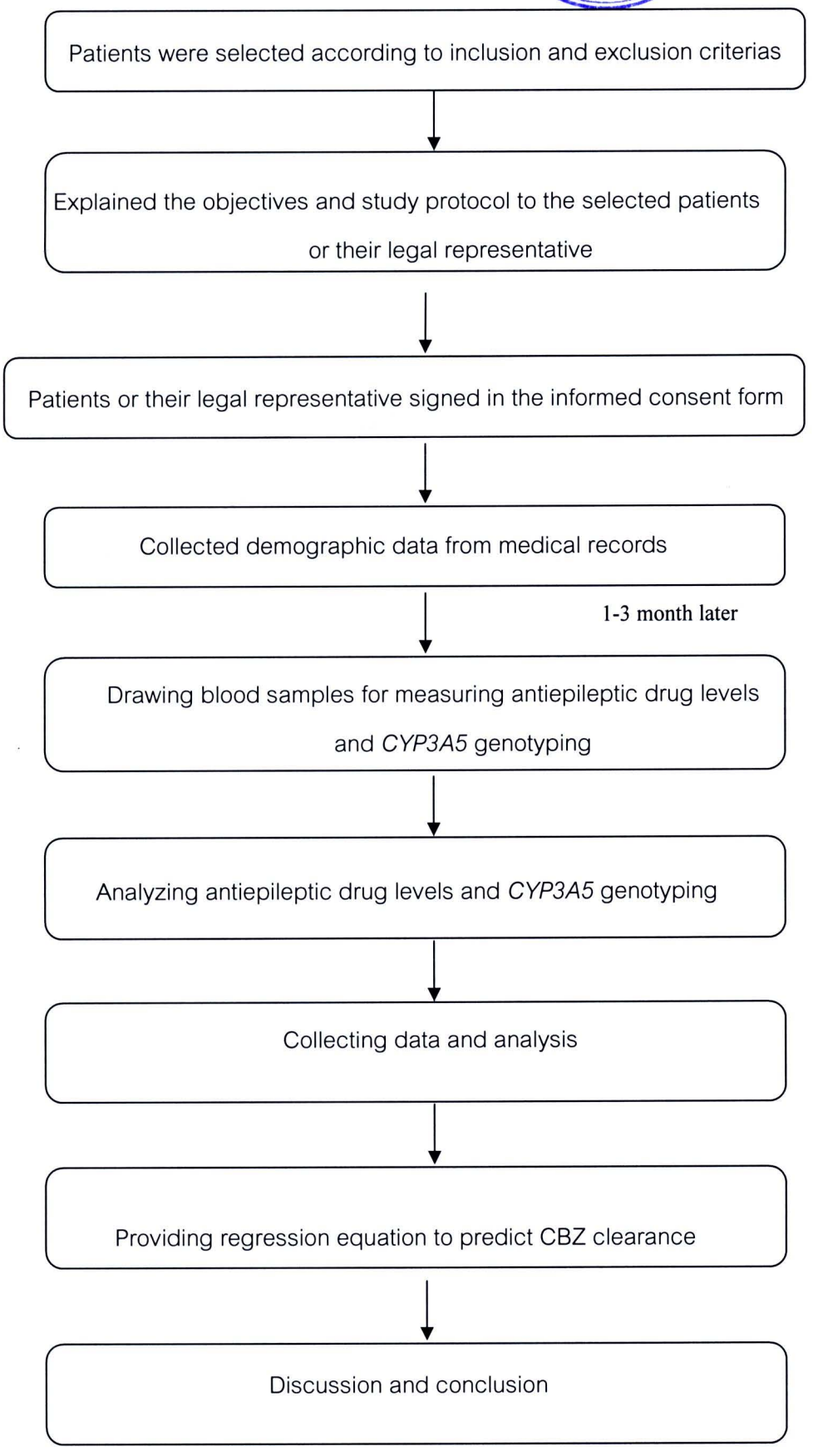
Figure 5: Study protocol



4. Sampling

Eighty five patients who met the inclusion criteria were participated in this study. Blood sampling for CBZ, PHT, PB and VPA concentrations were obtained at steady state. Whole blood was drawn from patients before the administration of the next dose of antiepileptic drugs in the morning. Volume of blood sample was 10 mL for the patients who received CBZ monotherapy and 15 mL for the patients who received CBZ with PHT, PB or VPA. Blood samples were collected in 2 tubes, 5 or 10 mL of clot blood tube (red-stopper) for measured antiepileptic drugs level measurement and 5 ml of Vacutainer® tube (purple-stopper) containing EDTA for *CYP3A5* genotyping.

Whole blood in the EDTA tube was prepared as buffy coat by centrifuge at 2,500 x g for 10 minutes at room temperature. After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes. Pipette 200 mcL of buffy coat into microcentrifuge tube size 1.5 mL and stored in a freezer at -20 °C until extracted for DNA.

5. Bioanalysis

5.1 DNA extraction

Buffy coat were used for DNA extraction by QIAamp® DNA Blood Mini kit.

5.1.1 Materials

Chemical and reagents

1.	Absolute etanol	Carlo erba	Italy
2.	Buffer AL	Qiagen	Germany
3.	Buffer AW1	Qiagen	Germany
4.	Buffer AW2	Qiagen	Germany
5.	Buffer AE	Qiagen	Germany
6.	QIAGEN®protease	Qiagen	Germany
7.	Protease solvent	Qiagen	Germany

Apparatus

1.	Centrifuge (Universal 320)	Hettick	Germany
2.	Vortex mixer (S0100-220)	Labnet	USA
3.	Heating block (Dri-block DB-2D)	Techne	UK
4.	Microcentrifuge (5415R)	Eppendorf	Germany
5.	Spectrophotometer (Smart spec 3000)	Bio-rad ™	USA
6.	Freezer	Sanyo	Japan
7.	Real-Time PCR system (Applied Biosystems 7500)		USA

Supplies

1.	Microcentrifuge tube (1.5 ml)	Treff AG.	Switzerland
2.	Pipette tip (Blue and Yellow)	Scientific Plastics	USA
3.	Micropipette 1,000 mcL	Eppendorf	Germany
4.	Micropipette 200 mcL	Eppendorf	Germany
5.	Micropipette 20 mcL	Eppendorf	Germany
6.	QIAamp Mini spin Column	Qiagen	Germany
7.	Collection tube 2 mL	Qiagen	Germany
8.	Disposable gloves		

5.1.2 DNA Extraction method

1. Equilibrate samples and reagents to room temperature.
2. Heat a heating block to 56°C.
3. Pipette 20 mcL QIAGEN Protease into a 1.5 mL microcentrifuge tube containing buffy coat 200 mcL.
4. Mix by vortex mixer for 15 seconds.
5. Add 200 mcL buffer AL to the sample. Mix by vortex mixer for 15 seconds.
6. Incubate at 56°C for 10 minutes.
7. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

8. Add absolute ethanol (96–100%) 200 mcL to the sample, and mix again by vortex mixer for 15 seconds. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
9. Carefully apply the mixture to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the tube containing the filtrate.
10. Carefully open the QIAamp Mini spin column and add 500 mcL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate.
11. Carefully open the QIAamp Mini spin column and add 500 mcL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 minutes.
12. Place the QIAamp Mini spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.
13. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 mcL Buffer AE or distilled water. Incubate at room temperature (15 – 25°C) for 1 minute, and then centrifuge at 6000 x g (8000 rpm) for 1 minute.
14. For long-term storage of DNA, eluting in Buffer AE and storing at –20°C.

5.1.2 Optical Density measurement

After DNA isolation should bring a sample to measure the amount and quality of DNA by OD measurement. These steps should be done with spectrophotometer as following.

1. Dilute a sample of DNA isolation in 1:5 concentrations, by using DNA 20 mcL add ddH_2O 80 mcL.
2. Prepare dH_2O 100 mcL for control.
3. Set spectrophotometer measure OD at 260 and 280 nm.
4. Calculate OD 260/280 ratio to observe purity and estimate concentration of DNA following this formula.

DNA concentration in mcg/mL or ng/mcL = OD260 x 50 x dilution factor

5.2 *CYP3A5* genotyping

CYP3A5 genotyping was determined by Allelic discrimination assay using real-time polymerase chain reaction (real-time PCR) technique with specific probe and primer (TaqMan® MGB probes, FAM™ and VIC® dye-labeled). See methods at Appendix D.

5.3 Drugs concentration measurement

CBZ, PHT, PB and VPA concentrations in serum were determined by the biochemistry laboratory of Prasat Neurological Institute using an immuno-turbidimetry assay method with an automate analyzer (Synchron LX® Systems, Beckman Coulter Inc., Fullerton, California). The analytical range of CBZ level was 2.0-20.0 mg/L, while the precision specification was 0.6 mg/L or 5.0%. The analytical range of PHT level was 2.5-40.0 mg/L, while the precision specification was 0.5 mg/L or 4.0%. The analytical range of PB level was 5.0-80.0 mg/L, while the precision specification was 1.0 mg/L or 4.0%. The analytical range of VPA

level was 10.0-150.0 mg/L, while the precision specification was 3.6 mg/L or 6.0%.

6. Statistical analysis

Statistical analyses were determined using the Statistical Package for Social Sciences (SPSS Co., Ltd., Bangkok Thailand) software version 17.0. Both descriptive and inferential statistics were determined. The level of significance was set at an α = 0.05.

Continuous variables was determined for normality of the distribution using Kolmogorov–Smirnov test and determined for homogeneity of variance using Levene's test.

Demographic data were determined and presented as mean ± SD, median, percentage or frequency where appropriate for qualitative or quantitative variables.

Statistical comparisons of CBZ clearance and level-to-dose-ratio between patients with *CYP3A5*1* and *CYP3A5*3* were performed using independent t-test or Mann-Whitney U test. Statistical comparisons of CBZ clearance and level-to-dose-ratio between patients with CBZ monotherapy or coadministration with PHT, PB or VPA were performed using one-way ANOVA, median test or Kruskal-Wallis H test.

The correlation between CBZ clearance and demographic data such as weight, gender, age, CBZ dose, coadministration drugs, *CYP3A5* allele were determined by multiple regression analysis.

The correlation between CBZ clearance and PHT V_{max}, PB clearance and VPA clearance were determined using simple linear regression. The assumptions of linear regression were tested; linearity of the relationship between dependent and independent variables, independence of the errors (no serial correlation), homoscedasticity (constant variance) of the errors versus the predictions (or versus any independent variable) and the normality of the error distribution

Regression equation to predict CBZ clearance from demographic data and polymorphism of *CYP3A5* was provided using regression analysis or multiple regression analysis with forward-inclusion method.