CHAPTER III
PATIENTS AND METHOD

Patients and Sample collection

1. Patients

The subjects included in this study were pediatric patients whose age less than 15 years-old with epilepsy and have been diagnosed and followed at neurological clinic, Department of Pediatrics, Ramathibodi Hospital. All must be treated with valproic acid as a single antiepileptic drug with normal renal and liver functions as diagnosed by physicians and laboratory tests, which are confirm by blood test. There must be no associated critical illness. Information regarding the purpose and method of this study was in formed and written consent was obtained prior to the initiation of the study.

Estimation of sample size with significant $p<0.05$ is calculated from this formula(22)

$$n = \frac{[Z_{\alpha/2}+Z_{\beta}]^2 \sigma_{\alpha}^2}{(\mu_1-\mu_o)^2}$$

$Z_{\alpha/2}$: 0.05 (two-tailed) = 1.96

σ_{α} : standard deviation of elimination constant from related literature = 0.021 hr^{-1} (9)

Z_{β} : 20% (two-tailed) = 0.84

$\mu_1-\mu_o$ = 20% of mean of elimination constant = 0.2x0.066 hr^{-1} (9)

$$n = \frac{(1.96+0.84)^2 (0.021)^2}{(0.2x0.066)^2} = \text{approximate 20 patients}$$

2. Data collection

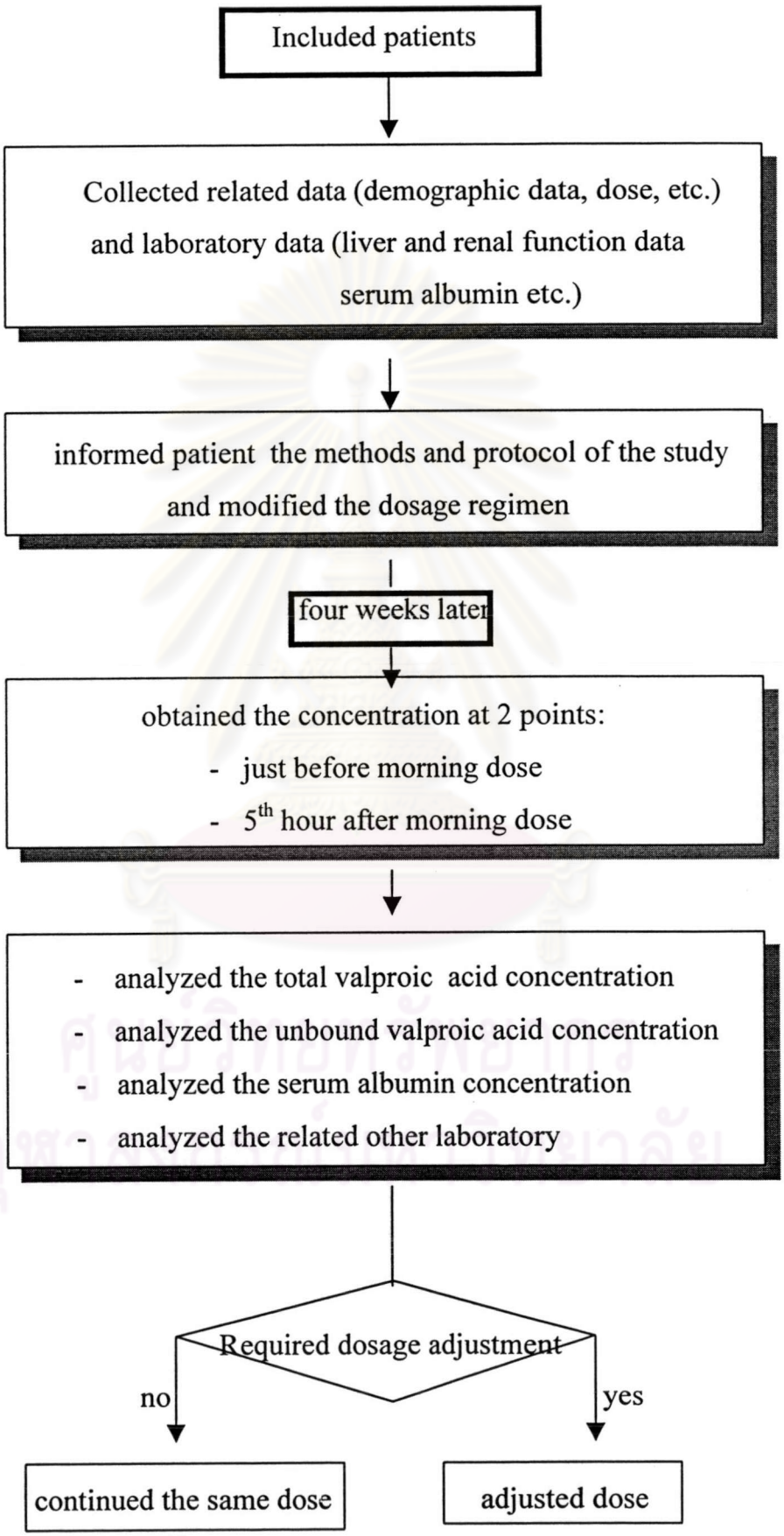
2.1 Patients information

All patients data related to the study; includes demographic data, medical histories, diagnosis, administered drug, dosage regimens, related laboratory data such as EEG and brain image, clinical response and any adverse effect were recorded. Each patients must have good compliances which was determined by interviewing the parents and/or patients.

2.2 Blood sample

Blood samples were obtained after valproic acid was given at same dose for at least one month, which reached steady state. Each patient's usual valproic acid regimen were modified from either bid or tid schedules to 12 hour or 8 hour regimens at least 5 days prior to evaluate in order to standardize dosage interval between patients. The blood was obtained just before the morning dose of valproic acid (trough) and at 5^{th} hour after administration. Each sample was allowed to clot and was centrifuged immediately at 5,000 rpm for 5 minutes at room temperature. The serum was then separated and frozen at –20°C until the time of assay. Serum level of total and unbound valproic acid were measured by fluorescence polarization immunoassay (TDxFLx Abbott Laboratories) ; unbound valproic acid levels were determined after ultrafiltration (25 °C ,1000 g ,35 fixed angle x 20 min, Centrifree TM micropartition Sytem). Serum albumin, other serum chemistry and hematology (CBC) were obtained from the same serum samples. (23)

Research protocol



Analysis of valproic acid concentration(23)

The Valproic Acid assay utilizes Fluorescence Polarization Immunoassay (FPIA)

Fluorescense polariztion immunoassay (FPIA) and TDX®

The Abbott TDX® system (Abbott Laboratories, North Chicago, IL) is based on FPIA technique. This method combines competitive protein binding with fluorescence polarization to give a direct measurement without the need for a separation procedure. All competitive binding immunoassays for measuring therapeutic drugs are based on competition between the drug in the patient sample and a labeled drug, called tracer. Sampled drug and tracer compete for a limited number of binding sites on antibodies specific to the drug being measured. The determination of concentration of unlabeled drug in each sample is considered by the amount of the bound to the specific antibody. In the TDX® system, the label on the tracer drug is the fluorescent dye-fluorescein. The change of polarization and concentration of the unlabeled drug is established by measuring the polarization valves of calibrators with known concentrations of the drug. A calibration curve stored in system memory is used to automatically determine the concentrations of unknown patient samples.

1. **TDx Valproic acid**

1.1 No. 9514-01, Valproic acid calibrators:

Six vials (2.5 mL in each vial) with accurately measured amounts of valproic acid in human serum at the following concentrations:

Vial	Valproic Acid Concentration (μg/mL)
A	0.0
B	12.5
C	25.0
D	50.0
E	100.0
F	150.0

Preservative : 0.1%Sodium Azide.

1.2 No. 9514-10, Valproic acid controls:

Three vials(2.5 mL in each vial) of valproic acid in human serum should read within the following ranges:

Vials	Valproic Acid Concentration (μg/mL)
L	33.75 - 41.25
M	67.50 - 82.50
H	112.50 -137.50

Preservative: 0.1%Sodium Azide

1.3 No. , Valproic acid Reagent pack

The valproic acid reagent consist of the following:

Vial	Component
S	<25% Valproic Acid Antiserum (Sheep) in buffer with protein stabilizer (3 mL)
T	<0.01% Valproic Acid fluorescein tracer in buffer containing surfactant and protein stabilizer (3 mL)
P	Pretreatment Solution. Surfactant in buffer containing protein stabilizer (3 mL)

Preservative: 0.1% Sodium Azid

2. TDx Free Valproic Acid

2.1 No.9537-01, Free Valproic Acid Calibrators:

Six vials with accurately measured amounts of valproic acid in phosphate buffer with stabilizer at the following concentrations:

Vial	Valproic Acid Concentration (μg/mL)
A	0.0
B	2.0
C	5.0
D	9.0
E	15.0
F	25.0

Preservative: 0.1% Sodium Azide

2.2 No. 9537-10, Free Valproic Acid Controls:

Three vials of valproic acid in phosphate buffer with stabilizer should read within the following ranges:

Vials	Valproic Acid Concentration (μg/mL)
L	3.6 – 4.4
M	10.8 –13.2
H	18.0 –22.0

Preservative: 0.1% Sodium Azide.

3. Centrifree and MPS micropartition Devices(24)

Centrifree and MPS micropartition devices rapidlly and efficiently separate free from protein-bound microsolute in small volumes (0.15-1.0 ml) of serum, plasma, and other biological samples using a method called ultrafiltration. Accurate partition occurs in minutes without dilution, change physiologic pH, ion composition or unbound microsolute concentraation. These devices are constructed with low-adsorptive hydrophilic membranes and O-

rings without plasticizers to assure excellent recovery. Hold-up volume is 10 μL or less.

In contrast to dialysis, gel filtration, or charcoal adsorption, ultrafiltration provides improved accuracy when measuring free ligand concentration, binding capacity, or affinity constants. It eliminates time-consuming methodology, the need for specialized equipment, dilution errors, and shifts in binding equilibrium.

Ultrafiltration dose not change free microsolute ligand volume or concentration. Protein becomes selectively partitioned into a fraction of the sample volume (retentate), while free ligand passes essentially unhindered through the membrane along with solvent. The laws of mass action and conservation of mass for ideal protein binding predict that free ligand concentration in the ultrafiltrate will remain constant, provided molar binding capacity and affinity are dependent of total protein concentration. Results for several systems showing constant free ligand concentration in successive ultrafiltrate fractions support those predictions.

Changing free ligand concentration not caused by membrane rejection or adsorption is evidence of altered capacity or affinity of binding proteins due to aggregation or other nonideal protein-protein interactions. Depending upon the application, analysis of the resulting filtrate for free ligand concentration either quantitatively or qualitatively.

4. Apparatus

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

4.2 Centrifuge

4.3 Freezer

Drug Analysis

Analysis of total and free valproic acid concentration (23,24)

Sample collection and preparation for testing analysis: serum or nonheparinized plasma samples are suitable for assay by the free valproic acid assay. Samples should be ultrafiltratered immediately, if possible. However, samples may be stored at room temperature for up to eight hours prior to ultrafiltration without alteration of the drug levels. Free drug levels are stable in frozen condition (-20 °C) for up to two weeks. Frozen samples should be warmed to room temperature and mixed completely before ultrafitration.

Also samples should be securely capped with minimized opening prior to filtration to prevent pH changes.

Sample preparation for the free valproic acid assay requires the separation of the free valproic acid fraction from the protein-bound fraction prior to analysis. Methods to isolate the free fraction include equilibrium dialysis and ultrafiltration. If ultrafiltration is used, for optimal results the filter must be extremely hydrophilic, exhibit no nonspecific binding to the membrane surface and provide essentially 100% separation of free from protein-bound drug without changing the concentration of the free fraction.

Data Analysis

1 Pharmacokinetic analysis

1.1 Pharmacokinetic parameter analysis

Pharmacokinetic parameters were calculated from the data using standard method. The equations used were (26-28)

$$Cp_t = [(S)(F)(Dose)/V_d]\frac{e^{-kt}}{1-e^{-k\tau}}$$

$$Cpss_{ave} = \frac{(S)(F)(Dose/\tau)}{Cl}$$

$$Cl = K_e \times V_d$$

$$t_{1/2} = \frac{0.693}{K_e}$$

Cp_t : drug concentration at that time

$Cpss_{ave}$: mean drug concentration at steady state

S : fraction of active drug accounting for salt form

F : Bioavailability

V_d : volume of distribution

K_e : elimination constant

τ : dosing interval

Cl : clearance

$t_{1/2}$: half life

1.2 Free fraction calculate (29,30)

$ff = [D_F]/[D_T]$

ff = free fraction

$[D_F]$ = free (unbound) valproic acid level

$[D_T]$ = total (free + bound) valproic acid level

2. Statistic analysis

The first step, all data were analyzed by SPSS. Demographic data were analyzed and presented as percentage and mean±SD. The pharmacokinetic parameters were presented as mean±SD and compared by t-test. Age was subdivided into two groups which are those whose age between 2 to 10 and those whose an older than 10 years for comparison clearance and volume of distribution comparing. A significant is determined at 0.05 (two-sided). Pharmacokinetic parameters were compared by sex and dosage form too.

In the second step, covariates were incorporated stepwise, either additively or multiplicatively, into the basic regression model to develop the immediate and full models. Quantitative covariates (age, TBW, daily dose, and serum albumin) were included in a linear way.