CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. TDx^{R} Gentamicin

1.1 No. 9512-01, Gentamicin Calibrators

Six vials with accurately measured amounts of gentamicin in normal human serum at following concentrations

Vial	Gentamicin concentration (µg/ml)
A	0
B	0.5
C	1.5
D	3.0
E	6.0
F	10.0

1.2 No. 9512-10, Gentamicin Controls

Three vials of gentamicin in normal human serum should read within the following range

Vial	Gentamicin Concentration (μg/ml)
L	0.85 - 1.15 for 1
M	3.6 - 4.4 for 4
H	7.2 - 8.8 for 8

(Preservative : 0.1% Sodium azide)

1.3 No. 9512-20, Gentamicin Reagent Pack :

There are three vials

Vial	Component
P	Pretreatment solution : Surfactant in buffer containing protein stabilizer (3 ml) Preservative : 0.1% Sodium azide
S	Gentamicin antiserum (sheep) in buffer with protein stabilizer (3 ml) Preservative : 0.1% Sodium azide
T	Gentamicin-fluorescein tracer in buffer containing surfactant and protein stabilizer (3 ml) Preservative : 0.1% Sodium azide

1.4 No. 9512-20, Buffer (for in vitro diagnostic use)

It was bovine gamma globulin in phosphate buffer, had 0.1% sodium azide as preservative.
(Store at 15-30^{o}C)

2. **Other Reagent**

2.1 Working Standards. Gentamicin Sulfate Powder, USP Reference. Containing 651 ug of anhydrous gentamicin base per mg of solid (Sigma, U.S.A.)
Lot no. 67F-0171

2.2 Methanol Absolute HPLC grade (J.T. Baker Inc., Phillpsburg, U.S.A.)
Lot no. B 30087

2.3 Ethanol Absolute HPLC grade (E. Merck, Germany)
Lot no. 929 K12503484

2.4 Tripotassium Ethylenediaminetetraacetate (Fluka Chemic AG, Germany) Fluka chemika 03665
Lot no. 284198 389

2.5 *O*-Phathalaldehyde (OPA) (Sigma, U.S.A.)
Lot no. 5218868

2.6 Boric Acid (BDH Analar, England)
Lot no. 5875080B

2.7 Trichloroacetic Acid GR (TCA)(E. Merck, Germany)
Lot no. 731 K295807

2.8 2-Mercaptoethanol (Merck-schuchardt, Germany) Lot no. 5218868

2.9 Potassium Hydroxide (KOH) (BDH Analar, England) Lot no. 9548750E

2.10 Tripotassium Ethylenediaminetetraacetic acid (Fluka chemic AG, Germany) Fluka chemika 03620 Lot no. 280231 1088

3. **Apparatus**

3.1 HPLC Column (μ-Bondapak-C_{18}, Waters Associates, Inc., Milford, MA., U.S.A.)

3.2 HPLC Pump (Model 510, Waters Associates, Inc., Milford, MA., U.S.A.)

3.3 Injector (Model U6K, Waters Associates, Inc., Waters Associates, Inc., Milford, MA., U.S.A.) Milford, MA., U.S.A.)

3.4 Spectrofluorometer (Model FP-210, Serial no. 20428, Jasco, Inc., Hachioji, Tokyo, Japan)

3.5 Integrator (Water 740 Data Module, Waters Associates, Inc., Milford, MA., U.S.A.)

3.6 pH Meter (Model 7, Coming serial no. 007/3154, Corning, Inc., Halstead Essex, England)

3.7 Vortex-2 Genie (Model G-560E, Scientific Industries, Inc., Bohemia, N.Y., U.S.A.)

3.8 Centrifuge [Model CS, Internal (ICE) Centrifuge International Equipment, Inc., Needham His, MASS, U.S.A.)

3.9 Analytical Balance (Metler B6, Switzerland)

3.10 Centrifuge (Model GS-100, Clements, Inc., W.G., West Germany)

3.11 Freezer (Forma Bio-Freezer, Forma Scientific, Inc., U.S.A.)

3.12 Automated Fluorescence Polarization Analyzer (Diagnostic Division, Abbott Laboratories, Inc., Irving, Tx, U.S.A.)

METHODS

I Serum Gentamicin Levels and Pharmacokinetic Parameters of Thai Patients

1. Subjects

Thai inpatients of Medical Department of Chulalongkorn Hospital were selected by physicians with appropriate conditions. Patients included in this study were not in critically ill. All patients received gentamicin alone or gentamicin combined with another non-aminoglycoside antibiotic. Patients with normal renal function were included. Age of the patients were ranged

from seventeen to eighty-five. All patients were treated by traditional physician prescribing practices.

Available patient data included age, sex, weight, height, medical history, diagnosis, accurate record of gentamicin doses administered, gentamicin serum concentrations, duration of gentamicin therapy, result from treatment and results of pertinent laboratory data such as serum creatinine; creatinine clearance; complete blood count (CBC); culture and sensitivity of clinical specimens.

2. **Dosage Regimen**

Dosage regimen of gentamicin was recommended by physicians prescribing in general practice of Department of medicine Chulalongkorn Hospital.

3. **Drug Administration**

The calculated amount of dose was diluted with 50-100 ml of intravenous solution then the drug was infused at a constant rate for 30 minutes. Subsequent doses were administered at constant dosing interval.

4. **Sample Collection**

After the patients had been on a fixed dosage regimen of gentamicin for at least 2 days; and were therefore considered to be at steady-state. On the third day, blood samples (5 ml) were drawn prior to the infusion

of the first dose and at 30 minutes and two hours after the infusion was finished. When samples were not drawn precisely at these times, the actual time of sampling was known and utilized. Peak values were not extrapolated to the end of the infusion. These blood samples were withdrawn from the arm contralateral to that used for infusion.

Serum was separated as soon as clotting had occurred by centrifugation (3000 rpm for 10-15 minutes at room temperature). Serum samples were assayed immediately by. fluorescence polarization immunoassay (TDx^{R} Analyzer System).

5. Analytical Method

Gentamicin levels in serum samples were determined by Immunoassay method (Fluorescent Polarization Techniques, TDx^{R} Analyzer system, Abbott Laboratories, North Chicago, IL 60064).

5.1 Perform an assay calibration

Items required are Calibration Carousel, Cuvettes, Sample Cartridge, Reagent Pack, Calibrators and Controls.

5.1.1 Prepare the carousel

- Load the carousel with 15 cuvettes in positions # 1 to # 15

- Load the carousel with 15 sample cartridges in positions # 1 to # 15
- Pipette at least 50 ul of calibrators in the sample wells as follows :

  Calibrators A in wells 1 and 2,

  Calibrators B in wells 3 and 4,

  Calibrators C in wells 5 and 6,

  Calibrators E in wells 7 and 8,

  Calibrators F in wells 9 and 10,

  Control L in well 13,

  Control M in well 14 and

  Control H in well 15

(Note : Gently invert the reagent pack, calibrator pack and control pack 3 times before use.)

5.1.2 Load the carousel in the instrument

5.1.3 Load the reagent pack in the instrument

5.1.4 Close the door

5.1.5 Press RUN

5.1.6 The instrument commences operation

5.1.7 Wait for run to complete

5.1.8 Keep the printout for later discussion

5.2 Perform an assay run

Items required are Assay carousel, Cuvettes, Sample Cartridges and Reagent Pack.

5.2.1 Prepare the carousel

- Load the carousel with 3 cuvettes in positions # 1 to # 3 (For 3 specimens).

- Load the carousel with 3 sample cartridges in positions # 1 to # 3.

- Pipette at least 50 ul of specimens in the sample wells as follows :

  specimen # 1 in well # 1,

  specimen # 2 in well # 2 and

  specimen # 3 in well # 3.

5.2.2 Load the carousel in the instrument

5.2.3 Load the reagent pack in the instrument

5.2.4 Close the door

5.2.5 Press RUN

5.2.6 The instrument commences operation

5.2.7 Wait for run to complete

5.2.8 Keep the printout for later discussion

5.3 Data analysis

5.3.1 Percentage of patients whose serum gentamicin levels were within therapeutic range

Gentamicin concentration of the serum sample was drawn just prior to the infusion of the first dose on the third day of gentamicin therapy was considered as the minimum or the trough concentration at steady-state while the concentration of the serum sample drawn at 30 minutes after the infusion was considered to be the maximum or the peak concentration at steady-state (19). Acceptable gentamicin peak levels were within the range of 4 to 8 µg/ml while acceptable range for trough concentrations were 0.5 to less than 2 µg/ml (35,17,19).

5.3.2 Comparison between the predicted and measured blood levels.

The predicted gentamicin blood levels were calculated using the available patient data included age, sex, weight, height and serum creatinine according to Equations 1-4, 6, 7, 11 and 12 (Appendix C).

The calculated peak concentration ($Cpmax_{ss}$) and trough concentration ($Cpmin_{ss}$) were compared with those measured values.

5.3.3 The effect of the number of samples and the sampling time on the calculation of some pharmacokinetic parameters.

Three serum samples had been collected in each patient. The first sample (C_1) was collected just before the study dose (the first dose on the third day), the second sample (C_2) was collected 30 minutes after a 30 minutes infusion of the dose and the third sample ($Cpost_{ss}$, C_3) was collected two hours after the infusion was finished . Different elimination rate constant (Kel) would be obtained when one or two sample points were used in the calculation as compared to the value obtained from three sample points. Moreover, among the values obtained from using two sample points, when different pair of samples was used, the elimination rate constant obtained would be different.

In the same way, the volume of distribution (Vd) and half-life ($t_{1/2}$) which obtained from substitute would be different elimination rate constants as forementioned into equations, would be different.

5.3.4 Calculation of dosage regimen by using individual pharmacokinetic parameters of patient.

Dosage regimen was calculated by using individual pharmacokinetic parameters of the patient. Selected desirable peak (6 ug/ml) and trough (1 ug/ml) concentrations were used in calculated dosing interval

from Equation 8 (Appendix C). The calculated dosing interval was rounded up and adjusted to be a figure that was convenient for dosage administration.

Maintenance dose was calculated by using the adjusted dosing interval and Equation 9 or 10 (Appendix C). Rounded up the maintenance dose to its nearest ten. Different dosage regimen would be obtained when different pharmacokinetic parameters (i.e. : Kel) were used in the calculation. These values were compared.

II Prediction of Creatinine Clearance from Serum Creatinine

In another group, eighteen patients from Medical Department in Chulalongkorn Hospital with normal or abnormal kidney function but did not on dialysis were included in this part of the study. The creatinine clearance values were estimated by using urinary creatinine excretion rate of the individual patients. The standard formula for the calculation of creatinine clearance (CrCl, Ccr) was as follow :

$$Ccr\ (ml/min) = (Ucr \times V)/(Scr \times 1440) \quad \ldots (a)$$

Ucr = Urinary creatinine concentration in mg/dl

V = Total volume of urine in 24 hours (ml)

Scr = Serum creatinine measured at midpoint of the urinary collection period (mg/dl)

1440 = Number of minutes in 24 hours

Compared creatinine clearance values calculated from equation (a) to creatinine clearance values calculated by using serum creatinine (Equations 3 and 4; Appendix C) and nomogram (Figure A; Appendix D). The percent of coefficient of variation was used to analyze the difference of the data.

III Comparison between the Fluorescence Polarization Immunoassay Technology (TDx ^{R} Analyzer) and High Performance Liquid Chromatography (HPLC) method

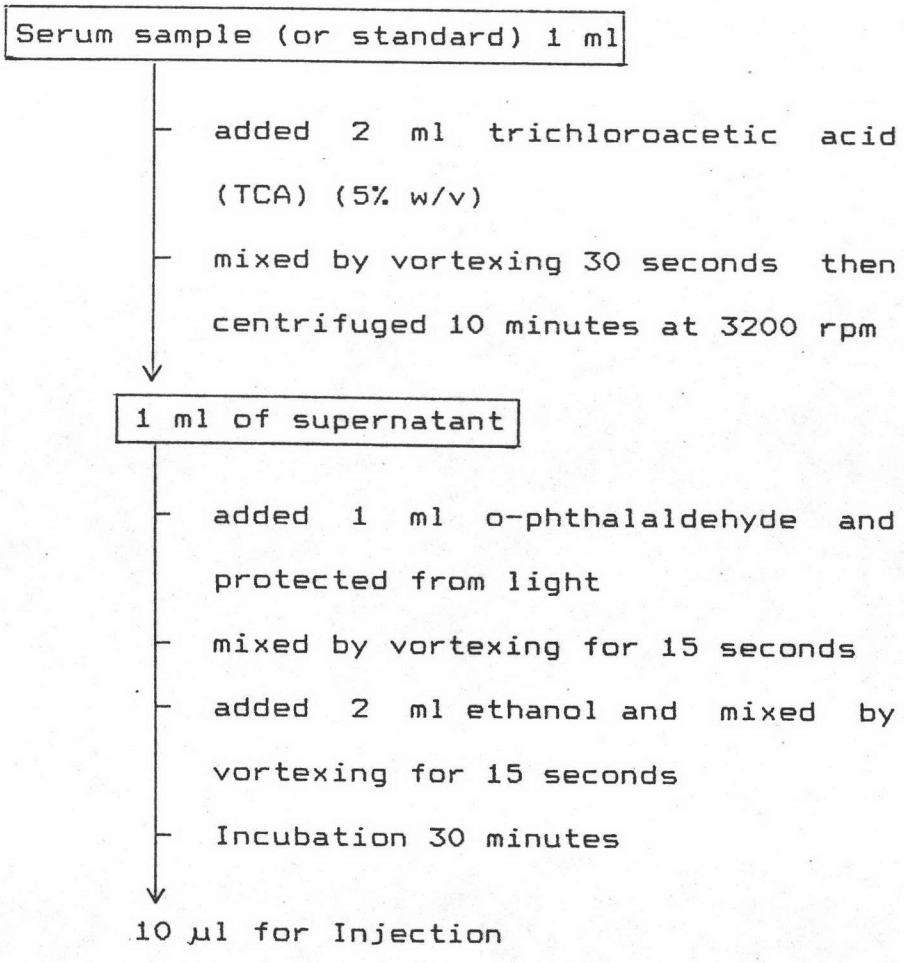
Thirty serum samples were determined by both the Fluorescence Polarization Immunoassay (FPIA) Technology and the High Performance Liquid Chromatography (HPLC) method. The purpose was to compare and to confirm the analytical results obtained from these two methods.

These thirty serum samples were randomly selected from those samples previously mentioned in I.4 (under the topic "Sample Collection"). The first portion of these thirty samples were assayed immediately by Fluorescence Polarization Immunoassay (TDX^{R} Analyzer) as formention while the second portion of the serum samples (approximately 2 milliliters) were stored in the glass tubes (Pyrex^{R}) at -70^{o}C until assayed by HPLC. Time of storage ranged from one to twenty-one days. The HPLC procedure was modified from the method developed in the

Pharmacology Laboratory, Faculty of Medicine, Chulalongkorn Hospital. The detail of the procedure was as follow :

1. **Sample preparation**

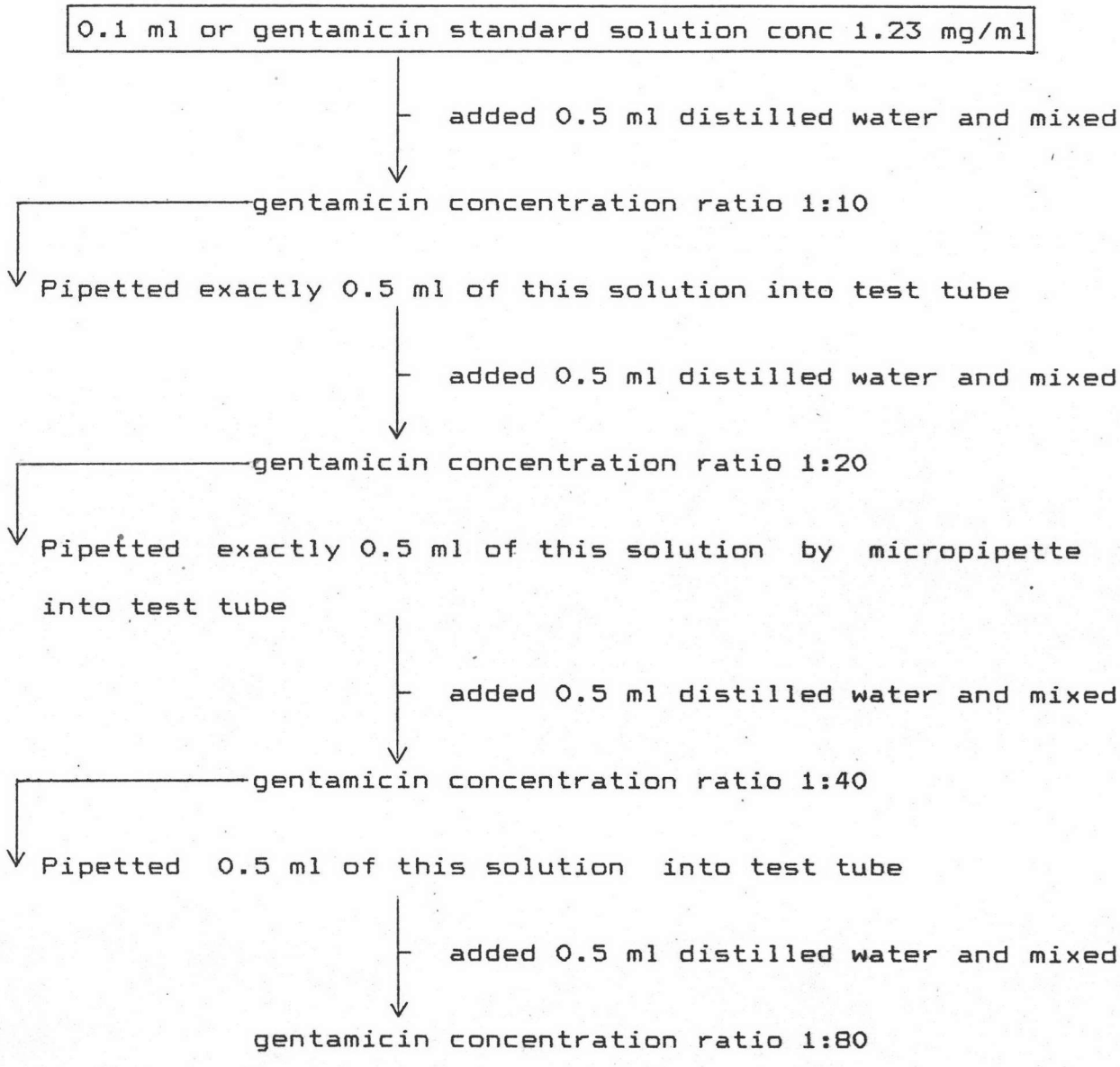
Serum samples were clarify by protein-precipitation with trichloroacetic acid and derivertized by O-phthaladehyde as follow :



The incubated solution was injected into the column and analyzed by HPLC as would be described later under the section of chromatographic conditions.

2. Serum standard preparation

Standard solution of gentamicin were prepared in distilled water to yield the final concentrations of 0, 1, 2, 4 and 8 µg/ml. Micropipette was used for measuring the exact amount of drug.



0.1 ml of each gentamicin concentration ratio was added into each of the five test tubes containing 0.9 ml pool human blank serum, except the first test tube was added with 0.1 ml of distilled water.

Preparing and analyzing these serum standards used the same procedure as serum samples, both serum standards and serum samples were prepared and analyzed concomitantly.

3. **Liquid chromatographic conditions**

Apparatus	:	HPLC Pump, Injector Fluorescent, Absorbance Detector, Integrator
Column	:	μ-Bondapak-C_{18} 30 cm x 3.9 mm id
Mobile Phase	:	methanol - EDTA pH 6.5 (Appendix A)
Flow Rate	:	1.5 ml per minutes
Pressure	:	1500-2000 psi
Temperature	:	ambient
Detector	:	excitation maximum 360 nm and emission 430 nm
Integrator	:	attenuation 16 chart feed 5.00 minimum area 2000
Injection volume	:	10 μl

A 10 µl volume of solution, prepared from serum samples or serum standards as previously described, was injected into the column and analyzed by HPLC. Area under the peak of three major components of gentamicin C_1, C_{1A} and C_2 were calculated by the integrator.

Concentration of gentamicin in each serum samples was determined by comparing the ratio of area under the peak of gentamicin to that of gentamicin standard curve.

5. **Standard curve**

Areas under the peak of serum-standard gentamicin were plotted against their known gentamicin concentrations to construct the standard gentamicin concentration curve. This curve was analyzed by linear regression. From the linear regression of the standard curve, the concentration of gentamicin in serum samples were determined.

6. **Data analysis**

Of the same serum sample, concentration determined by HPLC method was compared to concentration determined by FPIA (TDx^R Analyzer). Statistical analysis was then performed.