CHAPTER III

MATERIALS AND METHODS

Laboratory Evaluations of Norfloxacin Against Bacterial Enteropathogens

The pathogens used were the Salmonella, Shigella, Edwardsiella, Aeromonas, Plesiomonas and Vibrio species obtained from the stool swabs or rectal swabs of the patients who were studied at the Department of medicine, Bamrasnaradura Hospital, Nondhaburi.

1. Antimicrobial Susceptibility Tests

Using the disc agar diffusion method (80-84). The principle of the method involves the use of a constant concentration of an antibiotic within a reservoir in the agar that is used to culture the pathogen in question. The susceptibility of the pathogen to the antibiotic is indicated by the diameter of the clear zone of inhibition around the reservoir which is proportional to the susceptibility of the pathogen tested.

1.1 Medium and Preparation of Plates

Medium : Mueller Hinton Agar was used. The following shows the ingredients per litre.

Beef, Infusion form	300 gm
Casamino Acids, Technical	17.5 gm
Starch	1.5 gm
Bacto-Agar	17 gm

To rehydrate the medium, suspend 38 gm in 1,000 ml cold Purified Water USP, distilled or deionized water, and heat to boiling to dissolve the medium completely. Dispense into flasks and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 ^{o}C). Avoid excessive heat during rehydration of sterilization. Final pH is 7.3$\pm$0.1

In the preparation of plates, 25 ml of steriled Mueller Hinton Agar was dispensed into each steriled glass petridish 90 mm diameter. The agar was allowed to harden on a flat level surface. After solidifying, the plates may be used on the same day or refrigerated at 2-8 ^{o}C for no longer than 7 days, unless some method was used to minimize water loss from evaporation. Just prior to use, the plates should be placed in a 35 ^{o}C incubator with lids partly ajar, until excess surface moisture was evaporated.

1.2 Discs

The antibiotic discs tested were as follows:

- Ampicillin 10 µg/disc
- Chloramphenicol 30 µg/disc
- Tetracycline 30 µg/disc
- Trimethoprim/Sulfamethoxazole (TMP/SMX, SXT) 1.25 µg/23.75 µg per disc
- Norfloxacin (Lexinor®) 10 µg/disc

1.3 Preparation of Inoculum

With a steriled loop, the tops of 4 or 5 isolated colonies of a similar morphologic type were transfered to a tube containing 4 to 5 ml of steriled broth and incubated at 37 °C for 3-5 hours. Then standardized the inoculum to match a 0.5 turbidity standard of Mac Farland when comparing the tubes against a white background with a contrasting black line.

Mac Farland 0.5 turbidity standard was prepared by adding 0.5 ml of 1.175 % w/v barium chloride hydrate ($BaCl_2.2H_2O$) to 99.5 ml of 1 % w/v (0.36 Normal) sulfuric acid. The standard was kept in tightly sealed screw-capped tube at room temperature in the dark for not more than 6 months.

1.4 Preparation of Test Plates

Within 15 minutes of adjusting the density of the inoculum, a steriled cotton swab on a wooden applicator stick was dipped into the standardized inoculum. The excess fluid was removed by rotating the swab with firm pressure against the inside of the tube above the fluid level. The swab was then used to streak the dried surface of a Mueller Hinton agar plate in a three different planes to ensure an even distribution of the inoculum.

Replace the plate lids and allowed the inoculated plates to remain on a flat level surface undisturbed for 3 to 5 minutes (no longer than 15 minutes) to allow for absorption of excess moisture.

After that the discs were distributed evenly to be no closer than 15 mm from the edge of the petridish and so that no two discs were closer than 24 mm from center to center. The plates were then inverted and left at room temperature for 30 minutes, then incubated at 37 ^{o}C for 16-18 hours.

1.5 Interpretation of the Test Results

The diameters of the inhibition zones of the antimicrobials were measured with a sliding calipers with an accuracy of nearest 0.1 mm. The end points were taken as the area showing no visible growth that

could be detected with the unaided eye. Faint growth or tiny colonies near the edge of the inhibition zones were ignored if they were presented. The zone-size interpretative chart of Kirby-Bauer was used as shown in table 1.

Table 1. Zone-size Interpretative Chart (85-87)

Antimicrobial agents	Disc potency	Diameter of zone of inhibition (mm)		
		Resistance	Intermediate	Susceptible
Ampicillin, when testing Enterobacteriaceae	10 µg	≤ 11	12 - 13	≥ 14
Chloramphenicol	30 µg	≤ 12	13 - 17	≥ 18
Tetracycline	30 µg	≤ 14	15 - 18	≥ 19
Trimethoprim/ Sulfamethoxazole	1.25 µg/ 23.75 µg	≤ 10	11 - 15	≥ 16
Norfloxacin	10 µg	≤ 12	13 - 16	≥ 17

1.6 Quality Control Procedure

Using Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 or Pseudomonas aeruginosa ATCC 27853 as the tested pathogens. The susceptibility test results for norfloxacin 10 µg/disc must be between 17-28, 28-36 or 22-29 mm respectively (86).

2. Determination of Minimal Inhibitory Concentration (MIC)

The agar plate dilution method was used (81,82, 84,88). To determine the minimal inhibitory concentration (MIC), many isolates of pathogen were spot-inoculated simultaneously on to a series of petridishes containing various concentrations of norfloxacin, after incubation then the results were read. All the materials and methods were described as follows.

2.1 The Test Medium

Using Mueller Hinton agar prepared as described in title 1.1 (page 21).

2.2 The Antibiotic Diluent

Norfloxacin was initially solubilized in 0.1 Normal sodium hydroxide solution and subsequently diluted in sterile distilled water or sterile Mueller-Hinton broth. Fresh dilution was prepared daily.

2.3 Preparation of the Antimicrobial Dilution

They were prepared at the concentration ten times that desired in the final test as follows.

2.3.1 0.20120 gm of norfloxacin working standard, equivalent to 0.20000 gm of norfloxacin reference standard, was solubilized in a small amount of 0.1 Normal sodium hydroxide solution (1 ml is usually sufficient to solubilized 10 mg) then dissolved in

sterile distilled water to make 100.0 ml solution in a volumetric flask, this would obtain a 2,000 mg/L solution.

2.3.2 12.8 ml of the above solution was diluted with 7.2 ml of sterile distilled water to make 1,280 mg/L solution. Then it was further diluted to make a series of two fold dilutions of norfloxacin containing 0.039, 0.078, 0.1563, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 mg/L.

2.4 Preparation of Test Plates

The agar medium was melted and allowed to cool to 45 ^{o}C to 50 ^{o}C in a water bath. Then 18 ml of it was transferred to each sterile flask which contains 2 ml of each norfloxacin dilution.

The flasks were mixed thoroughly but gently and the agar was poured into the sterile 90 mm plates and allowed to harden on a flat level surface.

The agar plates would give the final norfloxacin concentrations of 0.004, 0.008, 0.016, 0.031, 0.062, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128.0 mg/L.

At least one control plate, containing Mueller-Hinton agar without any antimicrobial, was prepared for every series of dilutions.

The plates were freshly prepared on the day that the experiment was done.

2.5 Preparation of the Inoculum

The inoculum was prepared and standardized in the same manner as described in title number 1.3 (page 22).

The control strain of Escherichia coli ATCC 25922 was prepared in the same way.

2.6 Inoculation of Agar Plates

An inoculum-replicating apparatus was used, the portion of the standardized cell suspensions of 30 tested pathogens and 1 control strain and a portion of sterile broth were transferred to the 32 appropriate wells in the seed plate and then the inocula were picked up and gently transferred to the agar surface, being careful to avoid splashing. Control plates without antimicrobial should be inoculated last to insure that viable microorganisms were present throughout the procedure. Inoculation of the test plates should be completed within 30 minutes of adjustment of the turbidity of the first strain. The plates then were incubated at 37 ^{o}C for 16 to 20 hours.

2.7 Reading of Test Results

The agar dilution plates were examined for

the presence of growth. The lowest concentration of drug producing complete inhibition of growth was taken as the end point. A very fine growth or one or two visible colonies may be disregarded in the reading of the test. The control strain end point was checked in each set of tests to confirm the reproducibility of the tests. Control cultures on drug-free media should always show confluent growth.

Clinical Evaluations of Norfloxacin in Acute Bacterial Diarrhoea

1. Study Design

The study was a single centre study of randomized, comparative and double-blind placebo controlled design.

2. Criteria for Selecting the Patients

2.1 Number, Age range and Sex

Number: A total of 154 hospitalized patients with bacteriologically verified gastroenteritis attending to Bamrasnaradura Hospital were evaluated. To compensate for patients in whom no bacterial pathogens may be found initially, 260 patients were included.

Sex: Males and females

Age: 16-65 years

2.2 Inclusion Criteria

- Patients suffering from acute diarrhoea defined clinically as loose or watery stools for more than 4 times within the previous 24 hours.
- Patients with severe enough illness to require hospitalization for at least 3 days.
- Patients giving consent to participate after having been informed about the study.

2.3 Exclusion Criteria

- Patients with known hypersensitivity to quinolones (nalidixic acid, pipemidic acid, etc), trimethoprim and/or sulphonamides.
- Patients with known impaired kidney function (creatinine clearance less than 30 ml/min), known significantly decreased liver function and/or blood disorders.
- Pregnant women.
- Patients on treatment with theophylline, coumarin derivatives, phenytoin or methotrexate. The effect of these drugs may be increased due to metabolic interaction with quinolones.
- Patients suffering from any other major illness.
- Patients with severe illness with or without status of shock and patients requiring parenteral antibiotics.

3. Study Drugs

3.1 Investigational Drug

Norfloxacin tablets of 400 mg (Lexinor®, Astra)

3.2 References

Co-trimoxazole tablets of 160/800 mg (Bactrim® Forte, Roche)

Placebo tablets

3.3 Randomization Procedure

The treatments were randomized in blocks. When entering the study the patient was given a sequential patient number and received either drugs in NFX group or SXT group or placebo group (see title 4.1 page 31).

3.4 Blinding Procedure

A double-blind, double-dummy technique was used and the code would not be broken before this study had been completed.

3.5 Packing and Labelling

For each patient, tablets were packed in units marked with the patient number, study code, dosage instruction and name of the investigator.

4. Treatment Plan

4.1 Dosage Regimen

Norfloxacin group: (NFX group)	Norfloxacin 1 tablet twice daily, and placebo 1 tablet twice daily.
Co-trimoxazole group: (SXT group)	Co-trimoxazole 1 tablet twice daily, and placebo 1 tablet twice daily.
Placebo group:	Placebo 2 tablets twice daily.

Tablets were taken twice daily (morning and evening).

4.2 Duration of Treatment

All patients were treated for 72 hours with 6 doses of trial drugs. Each dose consisted of 2 tablets as the above dosage regemen.

4.3 Continuation of Treatment

Patients who after 72 hours still presented signs and symptoms should be treated at the discretion of the attending physician.

4.4 Concurrent Therapy

Other drug treatments for unrelated diseases were allowed with the exception of antimicrobial drugs and the drugs mentioned under " 2.3 Exclusion criteria ".

5. Method of Clinical Evaluation

On admission day, the medical histories were obtained. Weight, age and sex were recorded. The patients were then examined for signs and symptoms as follows:

- Prior to initiation of therapy.
- Daily during treatment, up to at least 12 hours after the last dose.
- At follow-up visit, day 10-15.

5.1 Clinical Assessments

The clinical assessments were made according to the following scheme:

Body temperature (oral)	was judged upon the morning measurements, normal value $37.5\ ^{o}C$ or less.
Stool frequency	number of defecations per 24 hours were recorded.
Stool consistency	were assessed as watery, loose or well formed. The daily worst outcome was recorded.
Presence of blood and mucous	recorded as yes or no for each sign
Skin turgor	recorded as normal or reduced

5.2 Time to Recover (Clinical Survival Analysis)

Time to recover was placed in four classes up to the frequency and consistency of the stools.

Recover on day 1: The stool frequency were $\leq$ 3 and stool consistency were not watery on day 1-3.

(day 0 = prior to treatment initiation, day 1 started at the initiation of the study drug administration)

Recover on day 2: The stool frequency were $\leq$ 3 and stool consistency were not watery on day 2-3.

Recover on day 3: The stool frequency were $\leq$ 3 and stool consistency were not watery on day 3.

Not recover: The stool frequency were > 3 and/or stool consistency were still watery on day 3.

6. Method of Laboratory Evaluation for Clinical Efficacy and Safety

6.1 Determination of Presence of Bacterial Enteropathogens

Stool cultures were obtained from stool swabs or rectal swabs prior to initiation of therapy, and there upon daily during therapy, 12-24 hours after the last dose, and if possible at follow-up, 10-15 days after

starting of treatment, in order to detect the bacterial enteropathogens.

Isolation and identification of bacteria from stool swabs or rectal swabs were carried out according to "The Laboratory Procedure for Isolation and Identification of Enteric Bacterial Pathogens" used in Clinical Microbiological Laboratory of Bamrasnaradura Hospital (89).

6.2 Bacteriological Evaluation

The bateriological effects were evaluated as follows:

Elimination: No bacterial pathogen in stool culture.

Persistence: The same specie as found in the preceeding culture was isolated.

Relapse: A specie previously isolated reappeared after one or more negative cultures.

Reinfection: The original pathogen was eliminated, but a new specie appeared.

6.3 Time to Elimination (Bacteriological Survival Analysis)

The patients were placed in four classes according to the time to eliminate the pathogen(s) with no later recurrence or reinfection within the study period at day 1, day 2, day 3 or no elimination within 6 doses.

6.4 Evaluation of Changes in Hematopoietic Function

The routine haematological test and serum creatinine determination were performed prior to therapy and at 12-24 hours after the completion of trial doses.

The haematological test included haemoglobin, haematocrit, white blood cell, neutrophils (PMN), neutrophils (Band), eosinophils, basophils, monocytes and lymphocytes determination.

7. Adverse Drug Experiences

Patients were asked about adverse drug experiences using a non-specific question: " Did you experience any discomfort after taking the medication?". The outcome of this procedure together with spontaneous reported adverse drug experiences or adverse drug experiences observed by the investigator were recorded. The adverse drug experiences were assessed according to intensity, date of onset, duration, judgement whether due to the study drugs and the outcome.

Evaluations whether the adverse drug experiences were due to the study drugs were done by Dr. Sermjit Pathnacharuen, Department of Medicine, Bamrasnaradura Hospital.

Severity of adverse events were classified as follows:

Mild: Awareness of sign or symptom but this was easily tolerated.

Moderate: Discomfort was enough to cause interference with usual activity.

Severe: The problem was incapacitating with inabiliy to work or to perform usual activity.

Association between adverse events and given drug were classified as follows:

Unlikely: Temporal relationship non-existent or very doubtful, and/or other factors were likely to have been causative.

Possible: Temporal relationship existed, but other factors were also likely to have been causative.

Probable: Temporal relationship existed and improvement on withdrawal of the drug or dose reduction was reported, and/or other factors were less likely to have been causative.