CHAPTER IV

PATIENT AND METHOD

Patient and Method

This study is a pharmacokinetic study of 5-FU in colorectal cancer patients at Rajavithi hospital, Bangkok, Thailand.

Patient

Study Population

Colorectal cancer patients who were out-patients of Oncology unit, department of medicine, Rajavithi hospital were eligible to participate in this study. Blood samples were collected in the first day of one of chemotherapy cycles. Before being performed, this study was submitted to the ethical committee according to Rajavithi hospital. Written informed consent was obtained from all patients after they were informed about this study purpose and methodology. Inclusion and exclusion criteria were as followed:

Inclusion criteria

The patients who had all of these characteristics were included in this study

1. Colorectal cancer patients age over 18 years old who received chemotherapy for colorectal cancer treatment since October 2002.
2. The patients had received intravenous folinic acid 20 mg/m^2/day in 15 min followed by 5-FU 425 mg/m^2/day in 15 min consequently for 5 days every 28 days.
3. The patients had a performance status 2 or less according to Eastern Cooperative Oncology Group classification.
4. The patients were willing to participate in this study.

Exclusion criteria

The patients who had one of the following characteristics were excluded from this study

1. The patients had abnormal hematological parameters. (leukocyte $\leq$ 3000 μg/L, absolute neutrophil $\leq$ 1500 μg/L, platelet $\leq$ 100000 /μL, hemoglobin $\leq$ 10g/dL, hematocrit $\leq$ 32 g/dL,)
2. The patients had total bilirubin $\geq$ 1.5 mg/dL or AST or ALT $\geq$ 100 iu or serum creatinine $\geq$ 1.5 mg/dL))
3. The patients received any concomitant chemotherapeutic agent during this study.
4. The patients had cerebral metastases or history of any other malignancy.
5. The patients received other medications that might interact with 5-FU such as allopurinol, methotrexate.
6. The patients who could not be followed up throughout the study period.
7. The patients were diagnosed from physicians to be not safe to enroll in this study.

Method

Plasma disposition of 5-FU was evaluated in patients at day 1 of the 5 days cycle of 5-FU. Five ml of venous blood samples were collected at 0, 5, 10, 15, 20, 30, 60 and 90 minutes after the beginning of 5-FU short-term infusion into heparinized tube. Blood samples were immediately centrifuged at 4000 rpm, 4 ^{o}c for 10 min and then stored at -80^{o}c until analysis by HPLC method. Pharmacokinetic parameters including Tmax, Cmax, t1/2, Ke, CL and Vd were then determined. Cmax and Tmax were directly observed from plasma concentration-time curves. T1/2, Ke, Vd, Cl and AUC were calculated by winnonlin software version 4.1 and showed in appendices. Patients were monitored for adverse effects including hematological effect, diarrhea, hand-foot syndrome and oral mucositis during the period of blood sample collection and the followed cycle of chemotherapy.[6,9,69]

Materials

1. Chemical and Reagents

- Fluorouracil injection 250 mg/ 5ml (A.B.I.C pharmaceutical)
- Leucovorin injection 50 mg/ 5ml (A.B.I.C pharmaceutical)
- 5-Fluorouracil HPLC grade (Sigma Aldrich- U.S.A)
- 5-Bromouracil HPLC grade (Sigma Aldrich- U.S.A)
- Ethyl acetate AR grade (Merck-Germany)
- Methanol HPLC grade (Merck-Germany)
- Acetonitrile HPLC grade (Labscan- Thailand)
- Orthophosphoric acid AR grade (Labscan- Thailand)
- Potassium dihydrogenphosphate (Merck-Germany)

2. Instrumentation

1. high performance liquid chromatograph , composed of (Variance, U.S.A)
   - Liquid chromatograph
   - UV detector
   - Autosampler
   - Solvent delivery system
   - C18 column
2. Vortex mixer, genie 2 (Scientific industries- U.S.A)
3. Water bath 60°c
4. Nylon membrane filter
5. filter set
6. Refrigerator -70°c (Forma Scientific- U.S.A)
7. Dynac Centrifuge (Clay Adams- U.S.A)
8. GS-6KR Centrifuge (Beckman- U.S.A)

9. Blood sampling set such as syringe 5 ml, 21G needle, 23G scalp vein, injection plug, normal saline, heparinized tube, plasma tube, gauze, micropore and alcohol.

3. **Stock solutions and standards**

Stock solution of 5-FU were prepared by dissolving 100 mg 5-FU in 100 mL water, resulting in a solution containing 1.00 mg/mL. Working solution were prepared by stock solution diluted to concentration 0.5,2,10, 50,100,250 and 500 mg/L and adjusted with purified water. Spiked plasma samples were prepared by 40μL of serial dilutions of working solution of 5-FU adjusted with 360 μL human plasma, resulting in calibration standards of 0.05, 0.2, 1, 5, 10, 25 and 50 mg/L. Quality control samples were prepared in human plasma at concentration of 0.2,5 and 50 mg/L. A solution of 5-Bromouracil (5-BU) as internal standard was prepared in purified water to stock solution 1.00 mg/mL and diluted to working solution 300 mg/L, resulting in final concentration of 30 mg/L.[69]

4. **Sample Preparation**

Blood sample were collected in heparinized tube and centrifuged at 4000 rpm 4°c for 10 min. Plasma were separated and stored at -80°c until analysis. Each sample was thawed at room temperature and 40 μL of internal standard was added to 400 μL of plasma. The tube was vortex mixed for 10 sec and 7 mL of ethyl acetate was added as precipitating and extraction solvent. After vortex mixing for 30 sec, the mixture was centrifuged at ambient temperature 4000 rpm for 10 min. The organic layer was separated and evaporated to dryness in water bath 60°c under steam of nitrogen. The residue was dissolved in 150 μL of mobile phase and 20 μL was injected into the HPLC system.[69-70]

Standard was prepared from normal human plasma spiked with different concentrations of 5-FU and analyzed as patients samples.

5. **HPLC conditions**

The mobile phase was composed of potassium dihydrogenphosphate 0.05 M (adjusted to pH 5 with 1 M sodium bicarbonate): acetonitrile (99.3: 0.7, v/v) at flow rate 0.75 mL/min. The detector was set at wavelength 266 nm.

6. **Validation study**[71]

Linearity

Linearity of detection was assessed from plasma samples spiked with 5-FU and 5-BU working solution. The peak ratio of 5-FU versus 5-BU were calculated and plotting the peak area ratio over the range 0.05-50 mg/L.

Precision and accuracy

Precision refers to the reproducibility of the assay, and was assessed at four concentrations in 0.05, 0.2, 5 and 50 mg/L. The coefficient of variation (%CV) at each concentration level should not exceed 15% except 20% for LLOQ.

Accuracy refers to the ratio of measured compound concentration to the known concentration of analysis. The mean value of percent difference between the measured mean concentrations should not deviate more than 15% in 0.2, 5 and 50 mg/L, whereas not more than 20% in 0.05 μg/mL of LLOQ.

Recovery

Recovery was determined by comparing the resulting peak areas between the peak areas from spiked plasma with pure standard solution containing the same concentration. The extraction recovery was also determined for internal standard. The recovery acceptance should be more than 60 percent.

Stability

The stability of 5-FU in human plasma was established with the low and high concentration samples during three consecutive freeze and thaw cycles. The samples were frozen at -70°c for 24 hours under the same conditions repeating two more times, then analyzed on the third cycles. The stability was also tested at room temperature by incubation of samples for 12 hours.

Statistical Analysis

All patient characteristics and AUC were analyzed by SPSS for window version 12.0. Mean, minimum, and maximum values were used to summarize data. 95% confidence intervals were used to report number of patients having appropriate AUC.