CHAPTER III

MATERIALS AND METHODS

Materials

1. Test Product.

Methotrexate sodium parenteral 50 mg vials were provided by Lederle, USA, whose control number was 42183.

2. Reagents.

Acetic acid, glacial

Acetonitrile (HPLC grade)

p-Aminoacetophenone

4-Aminoantipyrine

p-Aminosalicylic acid

Ammonia water

Ammonium sulfate

n-Butanol

Chloroform

8-Chlorotheophylline

Disodium hydrogen phosphate

Ethyl acetate

Ethyl ether, anhydrous

1-Hexanesulfonic acid

Hydrochloric acid

Isopropanol

Methanol

Methotrexate, standard (RX Company Ltd.)

Methylparaben

Perchloric acid

Phenacetin

Potassium dihydrogen phosphate

Potassium hydroxide

Sodium acetate

Sodium aminosalicylate

Sodium carbonate

Sodium dihydrogen phosphate

Sodium hydroxide

Sulfadiazine

Thiopentone sodium

Trichloroacetic acid

Trimethoprim

Tris (hydroxymethyl) aminomethane

3. Equipments

Analysis column (ODS, 0.4 X 30 cm, particle size 5 μm)

Analysis column (radial μ Bondapak C18, 10 cm)

Guard column (RP C18, 5 cm)

High performance liquid chromatography (Waters, model 510)

Sep pak C18

Methods

1. In Vitro Studies.

High performance liquid chromatography was used to determine serum methotrexate.

1.1 Determination of Suitable Internal Standard and Condition for HPLC. A Widely used technique of quantitation on HPLC involved the addition of an internal standard in serum samples to compensation for various analytical errors such as losses of the interest compound during sample workup. With this approach a known compound at a fixed concentration is added to the sample to give a separate peak in the chromatogram. Internal standard should have a resolved peak, no interferences, and be eluted close to methotrexate. It must have equivalently to compound of interest for analyses involving pretreatments. It must be stable, unreactive with sample components, column packing, or mobile phase. It was desirable to be commercially available in high purity, and not be present in serum sample (Snyder, and Kirkland, 1979). Nine chemical reagents (phenacetin, trimethoprim, 4-aminoantipyrine, p-aminoacetophenone, sodium aminosalicylate, p-aminosalicylic acid, sulfadiazine, methylparaben, and 8-chlorotheophylline) were tried to use as internal standard in various conditions. These conditions were applied from the Canfell, Chen, Cohen,

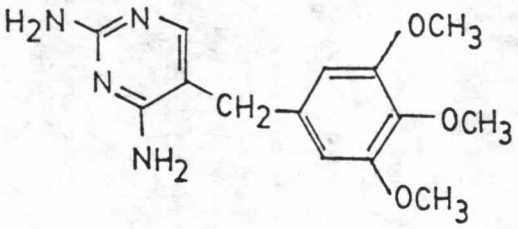
Collier, Howell, Lawson, and Watson (Canfell, and Sadee, 1980; Chen, and Chiou, 1981; Cohen, Hisayasu, Barrientos, Nayar, and Chan, 1980; Collier, MacLeod and Soldin, 1982; Howell, Wang, Hosoya, and Sutow, 1980; Lawson, and Dixon, 1981; Watson, Cohen, and Chen, 1978) [Appendix D]. The reagents were dissolve in water and injected into HPLC, then they were evaluated which one was suitable to be internal standard for each condition. Chemical structure of the reagents were shown in Figure 4.

1.2 Determination of Procedure to Measure Serum Methotrexate Concentration by HPLC. Method to find serum methotrexate level was determined by having regard to the resolved peaks of methotrexate and internal standard, percentage of recovery of methotrexate, and the sensitivity for methotrexate should be high enough to detect serum methotrexate concentration of 0.2 mcg/ml. Methods to determine serum methotrexate level using HPLC were applied from the methods of Canfell, Chen, Cohen, Collier, Howell, Lawson, and Watson (Canfell et al., 1980; Chen et al., 1981; Cohen et al., 1980; Collier et al., 1982; Howell et al., 1980; Lawson et al., 1981; Watson et al., 1978) [Appendix D].

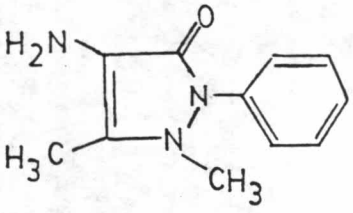
2. In Vivo Studies.

2.1. Subjects. Eleven Thai patients suffering from head and neck cancer whose ages were more

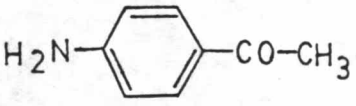
Phenacetin



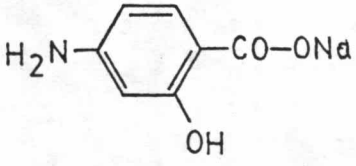
Trimethoprim



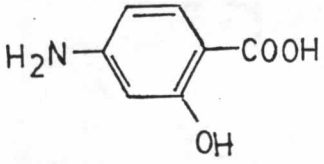
4-Aminoantipyrine



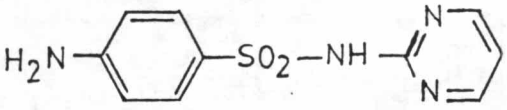
p-Aminoacetophenone



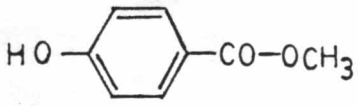
Sodium aminosalicylate



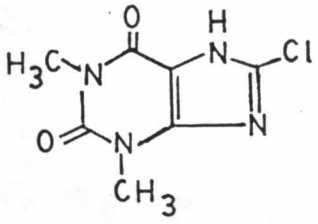
p-Aminosalicylic acid



Sulfadiazine



Methylparaben



8-Chlorotheophylline

Figure 4. Chemical structure of internal standards

than 18 years participated in this study. A medical history, physical examination and standard laboratory screen for individual subject were performed prior to the study to ensure the absence of any significant hepatic disturbances, renal disturbances and/or vascular disorders. The method of the study was fully explained to all subjects and all gave their written consents before entering the study. They were permitted to take no medication for at least one week preceding the study and during the experimental period.

2.2. Drug Administration. Twenty milliliters of normal saline solution were added to methotrexate sodium parenteral 50 mg and given bolus intravenous injection of methotrexate 1 mg/kg of body weight to the patients. For the next 6 weeks, each patient gradually had radiotherapy of 6,500 radians of Co-60.

2.3. Sample Collection. Blood samples (3 - 5 ml) were drawn from the antecubital vein prior to dosing and at 10, and 30 minutes, 1, 2, 4, 6, 8, and 12 hours after drug administration. They were allowed to clot at room temperature for 1 - 2 hours. After centrifugation (3000 rpm for 30 minutes), the serum samples were collected and kept at -20°C until subsequent analysis.

2.4. Standard Curve. Internal-standard calibrations were constructed by chromatographing appropriate volumes of calibration mixtures containing methotrexate with a constant concentrations of the internal-standard compound. Known amounts (0.1768, 0.442, 0.884, 2.652, 4.420, 6.188, and 8.840 mcg) of standard methotrexate were added to 1 ml of pooled human serum. These samples were analyzed following the procedure for determining serum methotrexate concentration. Peak heights of methotrexate and internal standard were determined and methotrexate to internal-standard peak-height ratios were plotted versus the known concentration of methotrexate. The ratio of peak height of methotrexate and internal standard obtained versus the methotrexate concentration were fitted to a straight line using linear regression (Neter, and Wasserman, 1974) [Appendix F].

2.5 Pharmacokinetic Analysis (Gibaldi, and Perrier, 1982a). Individual serum methotrexate profile was analyzed using PCNONLIN nonlinear estimation program (Metzler, and Weiner, 1984). In this study, it was proposed that the time course of methotrexate in serum for each subject could be well described by a two-compartment model. The equation used to predict the serum concentrations of a drug is as follow:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad (1)$$

where C_t is the serum concentration at time t. α and β are the rate constants for distribution and elimination, respectively. A and B are the serum methotrexate concentrations that are extrapolated from α line and β line respectively. The term α, β, A, and B are generally obtained from the nonlinear least-squares fit of serum methotrexate concentration versus time data to Eq.1 using PCNONLIN nonlinear estimation program (Appendix H).

The peak height concentration (C_0) is calculated using the equation:

$$C_0 = A + B \qquad (2)$$

The apparent volume of distribution (Vd_{area}) is calculated using the following equation:

$$Vd_{area} = \frac{D}{\beta\,(AUC_0^{\infty})} \qquad (3)$$

where D is the dose which is administered and AUC is area under the serum concentration-time curve.

The total clearance (Cl_T) is calculated from the following equations:

$$Cl_T = \beta . Vd_{area} \qquad (4)$$

2.6 Evaluation of Clinical Study. The tumor size of each patient were compared before and after treatment with methotrexate subsequent by

radiotherapy. Reduction of tumor size of patients are evaluated using the tumor response criteria (Miller, Hoogstraten, Staquet, and Winkler, 1981). Complete response is no measurable or palpable tumor on gross inspection. Partial response is more than 50% decreasing in the longest diameter of the product and its perpendicular diameter, but no increase in size of any other tumors. For stable response the difference in diameter of the product is less than 50% but not more than 25% increasing of the diameter in the second product without new lesions. Progressive response is increased in diameter of the product and the cancer will distribute to other parts of the body.