CHAPTER 2

EXPERIMENTALS

2.1 Apparatus and instruments

Reverse phase high performance liquid chromatographic system (HPLC) consisting of a pump (Waters 600E, USA), autosampler (Waters 717, USA), UV detector (Waters 486, USA) and C8 column (BDS Hypersil®, 5µm, 150x4.6 mm, Thermo Hypersil–Keystone, USA) were used for analysis of isoniazid and pyrazinamide. The HPLC system for rifampicin consisted of a pump (Jasco, Japan) and C8 column (BDS Hypersil®, 5µm, 150x4.6 mm, Thermo Hypersil–Keystone, USA). UV–visible spectrophotometer (HP 8452 A, USA) was used in ethambutol study.

2.2 Chemicals

The drug reference standard of isoniazid was obtained from the Division of Medical Science (DMSC) reference standard (in–house DMSC standard), while those of rifampicin and ethambutol were from the Asian Reference Substances, and that of pyrazinamide was from the Analytical reference standard of Aventis Pharma (working standard). Acetonitrile was HPLC grade obtained from Lab–Scan (Thailand). Other chemicals such as methanol from Lab–Scan (Thailand), potassium dihydrogen phosphate, sodium perchlorate, disodium hydrogenphosphate, phosphoric acid, citric acid, copper sulphate and sodium chloride from Merck (Germany), potassium bromide from Carlo erba (France), sodium bromide and potassium fluoride from Unilab (Australia) were laboratory reagent grade.
2.3 Samples

Rifampicin capsules in blister pack, isoniazid tablets, pyrazinamide tablets and ethambutol film coated tablets in aluminum foil (antituberculosis drugs) in the same lot manufactured by the government pharmaceutical organization were supplied by the Tuberculosis Institution, Yala (Thailand).

2.4 Analytical method

The assay method for rifampicin was modified from the assay method for rifampicin capsules in the United Stated Pharmacopoeia 24 (USP 24) by using suitable high performance liquid chromatographic system (HPLC). Mobile phase was a mixture of water, acetonitrile, 1.0 M phosphate buffer, 1.0 M citric acid and 0.5 M sodium perchlorate (510:350:100:20:20) and indomethacin was used as an internal standard, which chosen from a chromatogram having capacity factor ($k'$) $\geq 2$ and resolution $> 2$.

Pyrazinamide and isoniazid were determined using the same assay method, reverse phase high performance liquid chromatographic which was modified from the assay method for pyrazinamide in the United Stated Pharmacopoeia 24 (USP 24). The mobile phase was 0.05 M KH$_2$PO$_4$ buffer.

The assay method for ethambutol was modified by using the identification method for ethambutol tablets in British Pharmacopoeia 2001 (BP 2001) and validated quantitatively by using spectrophotometric method. Ethambutol reacted with 1% CuSO$_4$ in 1.0 M NaOH. The blue color was produced and quantified by UV spectrophotometry.

2.4.1 Method validation

The analytical methods were modified from the original method, USP 24 and BP 2001. Therefore, partial analytical method validation was performed as the follows.
2.4.1.1 Linearity

Linearity was determined by building five calibration curves at 5 concentration levels of rifampicin, isoniazid, pyrazinamide and ethambutol. The first three drugs were prepared at 2.5, 5, 7.5, 10 and 12.5 µg/ml from each stock standard solution. Ethambutol was prepared at 19.2, 30.0, 36.0, 48.0 and 60.0 µg/ml. Signals; peak area ratios of drugs to internal standard/peak area for HPLC method or absorbance for spectrophotometric method were plotted versus their concentrations. Linearity was determined from the coefficient of determination ($R^2$) which should be more than 0.999.

2.4.1.2 Specificity

Specificity was performed to ensure that their degradation products did not interfered the detection of drugs effectively. Each drug solution was stored at 80°C for 12 and 24 hours and analyzed by the assay methods compared with the pure compound.

2.4.1.3 Accuracy

Three concentration levels of rifampicin, isoniazid, pyrazinamide at 2.5, 7.5 and 12.5 µg/ml and ethambutol at 16.2, 36.0 and 60.0 µg/ml were prepared at from each stock standard solution for 5 replications. The percentage of the experimental concentrations was calculated as a proportion of the spiked concentrations.

2.4.1.4 Precision

For intra–day precision, three concentration levels of rifampicin, isoniazid, pyrazinamide at 2.5, 7.5 and 12.5 µg/ml and ethambutol at 16.2, 36.0 and 60.0 µg/ml were prepared at from each stock standard solution for 5 replications. While, inter–day precision of isoniazid, pyrazinamide and ethambutol was assessed by repeating the intra–day precision on three different days. The percent of coefficient of variation ($%CV$) or relative standard deviation ($%RSD$) of the signals was calculated. The inter–day precision for rifampicin was not performed since the analytical method was slightly modified by adding the internal standard.
2.5 Sample preparation

A strip of ten capsules of rifampicin was sampled and had their average weight of the content measured. The homogeneous powder was accurately weighed equivalent to 450 mg of rifampicin, placed in 100 ml volumetric flask, dissolved to the volume with solvent mixture; acetonitrile/methanol (50:50) and then diluted with acetonitrile to 450 µg/ml and protected from light. Four milliliters of this solution and 2ml of the internal standard solution were mixed in a 50 ml volumetric flask and adjusted to the volume with diluent; water/acetonitrile/1.0 M Na₂HPO₄ buffer/KH₂PO₄/1.0 M citric acid (640:250:77:23:10).

Ten tablets of pyrazinamide/isoniazid were sampled and had their average weight of the content measured. The homogenous powder was accurately weighed equivalent to pyrazinamide/isoniazid 500 and 100 mg respectively, placed in 100 ml volumetric flask, dissolved with distilled water then filtered. This solution was diluted with distilled water to 100 µg/ml and then four milliliters of each solution transferred to 50 ml volumetric flask and adjusted to the volume with distilled water.

A strip of ten tablets of ethambutol was sampled and had their average weight of the content measured. The homogenous powder was accurately weighed equivalent to 400 mg of ethambutol, placed in 100 ml volumetric flask and dissolved with distilled water then filtered and diluted to 500 µg/ml. This solution reacted with 2 ml of 1% CuSO₄ and 1 ml of 1.0 M NaOH.

2.6 Drug quality

Before the study, each of the four antituberculosis drugs was inspected in the content uniformity of individual ten tablets/capsules.

2.7 Non-isothermal stability study

Isoniazid, pyrazinamide and ethambutol (in foil strip) tablets and rifampicin (in blister strip) capsules were stored in a chamber, which was controlled for relative
humidity with saturated sodium chloride solution. First step, the chamber was stored at 80°C for 20 days and 1 unit of each drug was taken at 0, 1, 5, 10 and 20 days. Then the temperature was changed to 60°C, 70°C and 50°C for 20 days at each temperature and samples were taken at the same pattern. The samples were quantitatively analyzed by the described analytical methods. The drug concentration data were analyzed by using Non-isothermal analyzing data program version 1.0 (W. Wongpoowarak). Parameters; activation energy and gas constant ratio (Ea/R), Initial concentration (C0) and Arrhenius constant (A) from the program were used to design isothermal stability study using the first rate equation and the Arrhenius equation.

2.8 Program validation

The program; Analyzing non-isothermal stability data version 1.0 by W. Wongpoowarak was validated by using data which were simulated from the Arrhenius equation and the first order rate equation. Data were analyzed with this program and parameters were given. Parameter values from the two methods were compared.

2.9 Isothermal stability study

The relative humidity levels used in the isothermal stability study were 80, 50 and 20% RH for all drugs at every temperature. The temperatures levels used were 50°C, 60°C and 70°C for rifampicin capsules and 60°C, 70°C and 80°C for the others. The saturated potassium bromide (KBr), sodium bromide (NaBr) and potassium fluoride (KF) salt solutions were used to control 80, 50 and 20% RH, respectively (Speight, 2005). All chambers were protected from light. Ten units of samples were withdrawn at 0, 30, 60, 90, 120, 150 and 180 days and analyzed. The rate of degradation (k) and shelf-life (T90) were calculated by using the following equations.

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T_{90} = \frac{-\ln(0.9)}{k}
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2.10 Statistical data analysis

The SPSS program version 11.0 was used to analyze the effect of humidity and temperature on antituberculosis drug stability.