CHAPTER II

EXPERIMENTAL

Development of Chromatographic Techniques for the Determination of Ketoconazole

2.1 Development of High Performance Thin Layer Chromatographic Method for the Determination of Ketoconazole

2.1.1 Reagents and Chemicals

Deionized water was used throughout all experiments. All chemicals used are of analytical reagent grade which are listed as follows:

- Ketoconazole, Reference standard, Sigma, Switzerland
- Ethanol, BDH laboratory supplies, England
- Acetone, BDH laboratory supplies, England
- Ethyl acetate, BDH laboratory supplies, England
- Glacial acetic acid, Farmitalia Carlo Erba, Italy
- Hydrochloric acid, Farmitalia Carlo Erba, Italy
- Sulfuric acid, Farmitalia Carlo Erba, Italy
- Methanol (HPLC grade), BDH laboratory supplies, England

2.1.2 Apparatus and Instruments

The instruments and apparatus used were as follows:

2.1.2.1 High Performance Thin Layer Chromatograph

- Linomat IV, CAMAG, Switzerland
- Automated Multiple Development (AMD system), CAMAG, Switzerland
- TLC Scanner III, CAMAG, Switzerland
- Computer Aided Testing Software (CATS)

2.1.2.2 HPTLC precoated plates silica gel 60 F_{254}, 20 × 10 cm (layer thickness 0.25 mm), Merck.

2.1.2.3 UV-Visible Spectrophotometer, model SPECKOL 1200, Jena Analytic, Germany.

2.1.2.4 pH meter, model pH 900, Precisa, Switzerland

2.1.2.5 UV viewer, model Chromato-VUE C-70G, USA.

2.1.2.6 Micro centrifuge, ABBOTT, model 3531, USA.

2.1.2.7 Syringe filter membrane, Nylon, pore size 0.45 μm, 13 mm, Chrom Tech, USA.

2.1.3 Procedure

Three ketoconazole shampoo samples including: Nora, Kenalyn and Nizoral were taken analysis. Each sample contains 2% of ketoconazole in an aqueous suspension consisting of coconut fatty acid diethanolamide, disodium monolauryl ether sulfosuccinate, F.D & C Red No. 40, hydrochloric acid, imidurea, laurdimonium

hydrolyzed animal collagen, macrogol 120 methyl glucose dioleate, perfume bouquet, sodium chloride, sodium hydroxide, sodium lauryl ether sulfate and purified water.

Three ketoconazole creams including: Nizoral, Fungasin and Ketazon were taken for analysis. Each gram of white, odorless cream contains ketoconazole 20 mg (2%). Nonmedicinal ingredients are cetyl alcohol, isopropyl myristate, polysorbate, propylene glycol, purified water, sodium bisulfite, sorbitan monostearate and stearyl alcohol.

All samples were purchased from Drug Stores in Chiang Mai Province, Thailand.

2.1.3.1 Preparation of Standard Solutions

The required quantities of the ketoconazole standard were accurately weighed and dissolved in ethanol to a final concentration of 1000 mg L^{-1}. Working standard solutions (3-20 mg L^{-1}) were obtained by appropriate dilution of the ketoconazole stock solution in ethanol.

2.1.3.2 Preparation of Sample Solutions

Shampoo and ketoconazole cream samples containing ketoconazole were transferred to a tarred 50 mL screw-capped centrifuge tube and accurately weighed. A 50 mL of ethanol was added (ca 2%). The tube was shaking for 10 min and centrifuged at 7,600 rpm for 10 min. The clear supernatant was collected in a stopper test tube and used for spotting on the TLC plate. Commercially available pre-coated silica gel 60 F_{254} (Merck) plates (20 × 10 cm, 0.25 mm thickness) were employed. Plates were activated for 30 min at 120 ^{o}C prior to use.

2.1.3.3 High Performance Thin Layer Chromatographic Determination of Ketoconazole

2.1.3.3.1 Maximum Absorption of Ketoconazole Standard Solution

The maximum absorption spectrum of ketoconazole standard was performed by dissolving standard ketoconazole in the selected mobile phase. Then the maximum absorption of the drug solution was measured spectrophotometrically. Result showed that the drug gave maximum absorption at 298 nm.

2.1.3.3.2 TLC Developing Solvent

Several developing solvents were tested. Three systems of developing solvents were investigated. They are as follows;

(1) Ethanol-Acetone (90:10, v/v)

(2) Ethanol-Ethyl acetate (50:50, v/v)

(3) Ethanol-acetone-1.0 mol L^{-1} H_2SO_4 (80:10:10, v/v)

In this investigation, the developing solvent with the composition of ethanol-acetone-1.0 mol L^{-1} H_2SO_4 (80:10:10, v/v/v) gave higher resolution for ketoconazole and was used for TLC development.

The plate first was cleaned up by development with acetone in a saturated tank. After, the plate was dried at room temperature. Standard ketoconazole in ethanol solutions containing 3, 5, 7, 10 and 20 mg L^{-1} of ketoconazole and the sample solutions (shampoo or ketoconazole cream samples) were applied respectively, on a precoated silica gel 60 F_{254} aluminium plate. A Linomat IV was employed with a constant rate of 3 s μL^{-1}, a 3 mm band width was applied and the space between two bands was 5 mm. The plate was inserted into an automated multiple development

(AMD) system containing ethanol-acetone-1.0 mol L^{-1} H_2SO_4 (80:10:10, v/v/v) as mobile phase which was saturated with solvent vapor for 15 min prior to use. The plate was developed to a height of 9 cm. The chromatogram was dried automatically in the chamber and then viewed under short wavelength UV light (254 nm) to mark the scanning area. The peak area of each band was quantitatively measured by means of a TLC scanner III at wavelength of 298 nm, under the following parameters: - Photo mode: reflection, Scan mode: Zigzag. Other parameters were set according to the Camag TLC scanner's instruction manual.

Calibration curve of ketoconazole was constructed by plotting peak areas versus various concentrations of ketoconazole.

2.1.4 Method Validation

The following parameters have been used to validate the developed HPTLC methods for the estimation of ketoconazole in shampoo and cream samples.

2.1.4.1 Sensitivity

The sensitivity of the assay was determined in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity range and correlation coefficient. The detection limit of the method was investigated by analyzing various concentrations of ketoconazole standard solutions using the proposed HPTLC method. The limit of detection and quantitation were calculated from the standard deviation (S.D) of responses and slope of curve (S) using the equations:

$$LOD = 3 (S.D / S)$$

$$LOQ = 10 (S.D / S)$$

2.1.4.2 Robustness

Robustness of the proposed method was carried out by making little changes in the composition of the mobile phase or developing solvent. The changes in the R_f value of the spot or retention time of the chromatogram, difference in area of the peak was observed with respect to mobile phase composition. The %R.S.D was calculated. A low value of %R.S.D indicated that method was with standing small changes and so the method was robust.

2.1.4.3 Linearity

The linear range of the standard curve was also studied. The plate was developed in acetone for elimination of impurity. Then the standard ketoconazole solutions containing 3, 5, 7, 10 and 20 mg L^{-1} of ketoconazole were applied on a silica gel 60 F_{254} plate and determined by using the procedure as mentioned in the experimental section.

2.1.4.4 Precision and Accuracy

Precision of the method was established by using solutions of two different concentrations 5 mg L^{-1} and 10 mg L^{-1} of ketoconazole standard solution. Each solution was analyzed three times (n=3) on the same day and relative standard deviation (R.S.D) was calculated to ascertain intra-day precision. The studies were also repeated on three different days to establish inter-day precision.

2.1.5 Application

The proposed HPTLC method was applied to the determination of ketoconazole in the three commercially available shampoos and ketoconazole creams. The contents of ketoconazole in shampoos and creams were analyzed using the procedure described in the procedure section.

2.2 Development of an Ion Pair Liquid Chromatographic Method for the Determination of Ketoconazole

2.2.1 Reagents and Chemicals

Deionized water was used throughout all experiments. All chemicals used are of analytical reagent grade which are listed as follows:

- Ketoconazole, Reference standard, Sigma, Switzerland
- Sodium dodecyl sulfate (SDS), Sigma, Switzerland
- CTAB (Cetyl trimethylammonium bromide), Sigma, Switzerland
- Brij 35 (polyoxyethylene(23) lauryl ether), Sigma, Switzerland
- Glacial acetic acid, Farmitalia Carlo Erba, Italy
- Hydrochloric acid, Farmitalia Carlo Erba, Italy
- Sulfuric acid, Farmitalia Carlo Erba, Italy
- Acetonitrile (HPLC grade), BDH laboratory supplies, England
- Methanol (HPLC grade), BDH laboratory supplies, England

2.2.2 Apparatus and Instruments

The instruments and apparatus used were as follows:

2.2.2.1 UV-Visible Spectrophotometer, model SPECKOL 1200, Jena Analytic, Germany.

2.2.2.2 High Performance Liquid Chromatograph, Hewllet Packard, Germany

- Auto injector, HP 1100, Germany
- Binary pump, HP 1100, Germany
- Degasser, HP 1100, Germany
- UV-Visible absorbance detector, HP 1100, Germany

2.2.2.3 pH meter, model pH 900, Precisa, Switzerland

2.2.2.4 Micro centrifuge, ABBOTT, model 3531, USA.

2.2.2.5 Syringe filter membrane, Nylon, pore size 0.45 μm, 13 mm, Chrom Tech, USA.

2.2.2.6 Chromolith® Flash RP-18e column (25 × 4.6 mm), Merck KGaA (Darmstadt, Germany)

2.2.2.7 ODS® C_{18} column (125 × 4.6 mm), Cole-Parmer Instrument Company Ltd. United Kingdom

2.2.2.8 RESTEK® C_{18} column (100 × 4.6 mm), Restek, USA

2.2.3 Procedure

2.2.3.1 Preparation of Standard Solutions

A 1000 mg L^{-1} stock solution of ketoconazole was prepared daily by dissolving 25.0 mg of the standard in methanol and making up the volume to 25 mL with

methanol. Working standard solutions were obtained by appropriate dilution of the ketoconazole stock solution in methanol.

2.2.3.2 Preparation of Sample Solutions

For shampoo and ketoconazole cream samples containing ketoconazole were transferred to a tarred 50 mL screw-capped centrifuge tube and accurately weighed. A 50 mL of methanol was added (ca 2%). The tube with loosened cap was shaking for 10 min and centrifuged at 7,600 rpm for 10 min. The clear supernatant was collected and filtered again with 0.45 μm nylon membrane. Each sample solution was prepared in triplicate and injected into the HPLC system.

2.2.3.3 Ion Pair Liquid Chromatographic Determination

Ion pair liquid chromatographic method was performed under isocratic condition. All experimental parameters were optimized as follows:

2.2.3.3.1 Optimization of the Experimental Parameters

The IPLC conditions for the determination of ketoconazole were optimized by studying the influences of various parameters such as flow rates and concentrations of the mobile phase. The optimal value for each parameter was judging from maximum response of the detector, minimum noise of the baseline and relative standard deviation.

2.2.3.3.1.1 Selection of Wavelength

The absorption spectra of an aliquot of standard solution containing 5 mg L^{-1} of ketoconazole was also observed by spectrophotometer. The detector signals were investigated between 200-400 nm.

2.2.3.3.1.2 Section of the Mobile Phase

During the development of the ion paring LC method six different compositions of mobile phases including methanol-8 mM sodium dodecyl sulfate, acetonitrile-8 mM sodium dodecyl sulfate, methanol-10 mM CTAB, acetonitrile-10 mM CTAB, methanol-10 mM Brij® 35 and acetonitrile-10 mM Brij® 35 were tested.

2.2.3.3.1.3 Effect of SDS Concentration

The effect of SDS concentration for determining ketoconazole was studied by varying at concentration ranges of ketoconazole (1-10 mM) while other experimental conditions were maintained at their constant values such as 45% (*v/v*) of acetonitrile and 1.0 mL min^{-1} flow rate.

2.2.3.3.1.4 Effect of pH

The pH effect of mobile phase for determining ketoconazole was studied by varying pH ranges of mobile phase from pH 4.5-7.5 while other experimental conditions were maintained at their constant values such as 45% (*v/v*) of acetonitrile, 8 mM SDS concentration and 1.0 mL min^{-1} flow rate.

2.2.3.3.1.5 Effect of Mobile Phase Flow Rate

The mobile phase, acetonitrile-8 mM SDS (45-55, v/v) at pH 5.5 was employed for the investigated of the mobile phase flow rate. In this study the flow rate was varied from 0.5-2.0 mL min^{-1}.

2.2.3.3.1.6 Comparing Analytical Column

The comparison of the analytical columns were investigated using the optimum conditions. The standard solution (5 mg L^{-1}) was injected into the IPLC system using three different analytical columns. The analytical columns were 25 × 4.6 mm Chromolith® Flash RP-18e column (1), 125 × 4.6 mm ODS® C_{18} column (2) and 100 × 4.6 mm RESTEK® C_{18} column (3) respectively.

2.2.4 Method Validation

The following parameters have been used to validate the developed IPLC method for the estimation of ketoconazole in shampoo and cream samples.

2.2.4.1 Sensitivity and Linearity

The sensitivity of the assay was determined in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity range and correlation coefficient. The detection limit of the method was investigated by determining various concentrations of ketoconazole standard solutions by means of the proposed ion pair liquid chromatographic method. The limit of detection and quantitation were calculated from the standard deviation (S.D) of responses and slope of curve (S) using the equations:

$$LOD = 3 (S.D / S)$$

$$LOQ = 10 (S.D / S)$$

2.2.4.2 Linearity

The linear range of the standard curve was also studied. Then the standard ketoconazole solutions containing 5-500 mg L^{-1} of ketoconazole were separated on

analytical column and determined by using the procedure as mentioned in the experimental section 2.2.3.3.

2.2.4.3 Precision

Precision of the method was established by using solutions of five different concentrations 1, 5, 10, 15 and 20 mg L^{-1} of ketoconazole standard solution. Each solution was analyzed with three times (n=3) on the same day and relative standard deviation (R.S.D) was calculated to ascertain intra-day precision. The studies were also repeated on three different days to establish inter-day precision.

2.2.4.4 Accuracy

The accuracy of the proposed IPLC method was also investigated. Known quantities of ketoconazole were added to previously analyzed samples of ketoconazole and analyzed by the proposed method.

2.2.5 Application

The proposed IPLC method was applied to the determination of ketoconazole in the three commercially available shampoos and ketoconazole creams. The contents of ketoconazole in shampoos and creams were analyzed using the procedure described in the procedure section.