CHAPTER III

MATERIALS AND METHODS

Materials

1. Fluconazole, Lot No. 7Flu009, Euresian, India.
2. Prednisolone, Lot No. S183011, Asean referance substance.
3. Anhydrous sodium dihydrogen phosphate, Lot No. 348694/1 795, Fluka, Switzerland.
4. Sodium monohydrogen phosphate, Lot No. 338 A714386, Merch, Germany.
5. Acetonitrile, Lot No. 98080060, Lab - scan, Ireland.
6. Methanol, Lot No. 98100114, Lab - scan, Ireland.
7. Sodium bisulfite, Lot No. 7448 KDEJ, Malinckridt, USA.
8. Disodium edetate, Lot No. 359064/1 54396, Fluka, Switzerland.
9. Propyl gallate, Lot No. 40113813, Merck, Germany.
10. Sucrose, Mitrphol, Thaidland.
11. Saccharin Sodium, Lot No. JC 05/1 Srichand united dispensary CO., LTD., Thailand.
12. Ethanol absolute, Lot No. K25846283 844, Merck, Germany.
13. Propylene glycol U.S.P., Lot No. PL 79/729, Srichand united dispensary CO., LTD., Thailand.
14. Polyethylene glycol 400, Lot No. PID 19/586, Srichand united dispensary CO., LTD., Thailand.
15. Polyethylene glycol 4000, Lot No. 494429, BASF, Germany.

16. Sorbitol 70% w/v, Lot No. SFA 101/551, Srichand united dispensary CO., LTD., Thailand.
17. Methyl paraben, Lot No. 360539/1 50797, Fluka, Switzerland.
18. Propyl paraben, Lot No. 358569/1 53597, Fluka, Switzerland.
19. Sodium chloride, Lot No. K91013804, Merck, Germany.
20. Peppermint oil, Lot No. OAT 15/740, Srichand united dispensary CO., LTD., Thailand.

Equipments

1. Analytical balance, Sartorius GMPH, Germany.
2. pH meter, Model SA 520, Orion, USA.
3. Hot air oven, TY. ULM 700 Schutzort Din 40050-IP20, Memmert®, Germany.
4. Hot air oven, D 06057 Modell 100 Memmert.®, Germany.
5. Cool daylight lamps, 25 W, Osram, Germany.
6. Digital iluminance meter, Model TES 1332, TES Electrical Electronic, Taiwan.
7. Ultrasonic bath, Bransonic 221, Branson, Smith-Kline company, USA.
8. A holder fiter, Part No. 80357, Water, USA.
9. High performance liquid chromatography (HPLC) instrument equipped with :

    a tunable absorbance detector (Model 484, Waters, USA)

    a constant flow pump (Model 600E, Waters, USA)

    an integrator (Model 746, Waters, USA)

an autoinjector (Model 712 WISP, Waters, USA)

a Spherisorb C_{18} ODS2 (250 × 4.6 mm), 5 micron,

serial No 102826322058, Water, USA.

10. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)

11. Vertical rotator

Methods

1. Method of quantitative analysis of fluconazole

1.1 Validation for the quantitative determination of fluconazole in solvents and mixed solvents by UV Spectroscopy

The parameters essential to ensure the acceptability of the performance of an analytical method are accuracy, precision, sensitivity, specificity, and linearity (USP 23)

1.1.1 Preparation of standard solutions

A stock solution of fluconazole was prepared by dissolving 50 mg of fluconazole in ethanol. The solution volume was adjusted to 50 ml in a volumetric flask.

Standard solutions were prepared by pipetting 1, 1.5, 2, 3 , 4 ml of fluconazole stock solution and transfering each aliquot to each one of 10-ml volumetric flasks. Another 3 and 6 ml aliquots of stock solution were also pipetted and transfered each aliquot to each one of 25-ml volumetric flasks. The solutions

were adjusted to volume with ethanol so that the concentrations of the standard solutions were 100, 120, 150, 200, 240, 300 and 400 mcg/ml, respectively.

1.1.2 Accuracy

a) Analysis of fluconazole in solution.

Three sets of standard solutions of fluconazole were prepared . Each individual sample was analyzed by UV Spectrophotometry at 260 nm. Percent analytical recovery of each sample was calculated.

1.1.3 Precision of the method

1.1.3.1 Within run precision

Three sets of the seven standard solutions of fluconazole were determined within one day. The percent coefficient of variation (%CV) for each concentration was calculated.

1.1.3.2 Between run precision

Three sets of the seven standard solutions of fluconazole were prepared and determined on different days. The percent coefficient of variation (% CV) for each concentration was calculated.

1.1.4 Linearity correlation

Three sets of seven fluconazole standard solutions in the concentration range of 100-400 mcg/ml were prepared and analyzed. Linear regression analysis of absorbances versus their concentrations was performed.

1.1.5 Specificity

Under the same condition selected, the peaks scan by UV spectrophotometer of various solvents and mixed solvents (cosolvents) must not interfere with the peak of fluconazole in ethanol at 260 nm.

1.2 Validation for the quantitative determination of fluconazole in fluconazole syrup by HPLC

1.2.1 HPLC conditions

The high pressure liquid chromatographic technique was used for analysis of fluconazole concentrations in the preparation. The technique was modified from Yamreudeewong, Lopezanaya and Rappaport (Yamreudeewong, Lopezanaya and Rappaport,1993) the system was as follows :

Analytical Column	:	Spherisorb C18 ODS2 (250 × 4.6 mm), 5 micron.
Mobile phase	:	a mixture of 23% v/v acetonitrile and 77% v/v water.
Detector wavelength	:	260 nm.
Flow rate	:	1.3 ml/min.
Attenuation	:	16
Chart speed	:	0.1 cm/min.
Injection volume	:	50 microliters.

Internal Standard : prednisolone 20 mcg/ml.

Retention time : fluconazole, 6.50-7.50 min.

prednisolone, 23.00-24.00 min.

1.2.2 Preparation of standard solutions

A stock solution of internal standard was prepared by completely dissolving 20 mg of prednisolone in ethanol in a 100-ml volumetric flask. The solution was adjusted to volume, giving the final concentration of 200 μg/ml.

A stock solution of fluconazole was prepared by dissolving 250 mg of fluconazole in ethanol. The solution volume was adjusted to 25 ml in a volumetric flask.

Standard solutions were prepared by pipetting 250, 275, 300, 500, 700 and 900 μl of fluconazole stock solution and transfering each aliquot to each one of seven 10-ml volumetric flasks, 1 ml of prednisolone stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with purified water so that the concentrations of the standard solution were 250, 275, 300, 500, 700 and 900 μg/ml, respectively.

1.2.3 Accuracy

a) Analysis of fluconazole in solution.

Three sets of standard solutions of fluconazole were prepared and injected. Each individual sample was analyzed by HPLC. Percent analytical recovery of each sample was calculated.

b) Analysis of fluconazole in syrup.

Three sets of fluconazole syrups (50mg/5ml) were prepared and injected. Each individual sample was analyzed by HPLC. Percent analytical recovery of each sample was calculated.

1.2.4 Precision of the method

1.2.4.1 Within run precision

Three sets of the six standard solutions of fluconazole were injected within one day. The coefficients of variation, %CV, of fluconazole to its internal standard peak area ratio from the three sets of standard solutions having the same concentration were calculated.

1.2.4.2 Between run precision

Three sets of the six standard solutions of fluconazole were prepared and injected on different days. The coefficients of variation, % CV, of fluconazole

to its internal standard peak area ratios from the three sets of standard solutions having the same concentration were calculated.

1.2.5. Linearity correlation

Three sets of fluconazole standard solutions in the concentration range of 250-900 μg/ml were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed.

1.2.6 Specificity

Under the chromatographic conditions selected, the peaks of other pharmaceutical components in the syrups must not interfere with the peak of fluconazole. Chromatograms of these solutions were evaluated by comparing with chromatogram of the standard solution of fluconazole.

1.2.7 Stability Indication of fluconazole and other pharmaceutical components in the formula

The solution containing fluconazole, syrup containing fluconazole, and the solution containing each of pharmaceutical component, i.e., propyl gallate, sodium bisulfite disodium edetate, sorbitol, PBS, paraben concentration, mixed solvent (water-ethanol-PEG4000), sodium saccharin and syrup USP were exposed to the 25 w artificial daylight lamp of which the luminescence was 1000 lux at 60 $^{\circ}$ C for 2 months. Three hundrad microliters of these solutions was pipetted and transfer to each of 10-ml volumetric flasks. The solutions were adjusted to volume with purified water. The drug and the degradation products were determinded by HPLC under the same conditions as in 1.2.1.

2. Determination of solubility of fluconazole

2.1 Preparation of solvents

Water, ethanol, propylene glycol and polyethylene glycol 400 were used as a pure solvent for solubility study.

The binary system of solvents selected to dissolve fluconazole were the mixture of water-ethanol, water-propylene glycol and water-polyethylene glycol 400. The concentrations of these solvents and water were varied from 10 to 50 percent v/v.

Another binary system used in this investigation was polyethylene glycol 4000 and water. The concentration of polyethylene glycol 4000 were varied at concentrations of 1, 2, 3, 4 and 7 % w/v.

Mixed solvents containing polyethylene glycol 4000, ethanol, propylene glycol and water were prepared by varying their concentrations as follows:1-2-2-95 %, 1-4-4-91 %, 1-7-7-85 %, 2-2-2-94 %, 2-4-4-90 %, 2-7-7-84 %, 3-2-2-93%, 3-4-4-89 %, 3-7-7-83 %, 4-2-2-92 %, 4-4-4-88 %, 4-7-7-82 %, respectively.

2.2 The solubility of fluconazole in solvents and mixed solvents

Excess of fluconazole was placed into parafilm-capped test tubes containing 5 ml of each solvents and mixed solvents. The test tubes were vortexed well and placed in a vertical rotator at 30^{0}C for 24 and 48 hours to ensure saturation. The solutions were filtered using filtered papers to remove excess

insoluble drug. The clear solutions obtained were determined for fluconazole concentrations.

Aliquots of fluconazole solutions were transferred to appropriate volumetic flasks and brought up to the final volume with water. Solutions were then analyzed using a UV spectrophotometer at 260 nm. The concentrations of fluconazole were quantified utilizing a standard curve.

3. Preparation of fluconazole syrup

Mixed solvent from 2. gave an approximate solubility equal to 10 mg/ml, was selected for dissolving fluconazole. The composition of the formulations are shown in Table 4. Fluconazole syrups were prepared by dissolving 1 g fluconazole in the mixed solvent (polyethlene glycol 4000 4 g, ethanol 7 ml and propylene glycol 7 ml). Sodium saccharin (0.2 g), sodium hydrogen phosphate (4.482 g), disodium hydrogen phosphate (0.327 g) were dissolved in distilled water. Then antioxidants (0.001 g ,0.005 g , 0.01 g propyl gallate or 0.05 g , 0.075 g or 0.100 g sodium bisulfite or 0.005 g ,0.01 g , 0.05 g disodium edetate) paraben concentrate (20 %w/v methyl paraben , 2 %w/v propyl paraben in propylene glycol), sorbitol and syrup USP were added. Flavor was added and the solution was mixed. The final volumes were adjusted to 100 ml with distilled water. Formulation 1 was the control furmulation without antioxidant.

Table 4 . Fluconazole syrup formulations designed for investigation of antioxidant effect.

Formulation	1	2	3	4	5	6	7	8	9	10
Fluconazole (g)	1	1	1	1	1	1	1	1	1	1
Mixed solvent (ml) (ETOH 7 ml+ PG 7 ml + PEG 4000 4 g)	16	16	16	16	16	16	16	16	16	16
Sodium saccharin (g)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sodium hydrogen phosphate (g)	4.482	4.482	4.482	4.482	4.482	4.482	4.482	4.482	4.482	4.482
Disodium hydrogen phosphate (g)	0.327	0.327	0.327	0.327	0.327	0.327	0.327	0.327	0.327	0.327
Paraben concentration (ml)	1	1	1	1	1	1	1	1	1	1
Syrup USP (ml)	40	40	40	40	40	40	40	40	40	40
Sorbitol 70% w/v (ml)	10	10	10	10	10	10	10	10	10	10
Peppermint (ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Propyl gallate (g)	-	0.001	0.005	0.01	-	-	-	-	-	-
Sodium bisulfite (g)	-	-	-	-	0.05	0.075	0.100	-	-	-
Disodium edetate (g)	-	-	-	-	-	-	-	0.005	0.010	0.050
Distilled water to (ml)	100	100	100	100	100	100	100	100	100	100

4. Stability testing

4.1 Physical stability of fluconazole syrups

4.1.1 Heating cooling cycle

The samples of all preparations of fluconazole syrups were stored in a incubator at a controlled temperature of 45 $^{\circ}$ C for 24 hours. Then the syrups were transferred to a refrigerator at temperature of 4 $^{\circ}$ C for 24 hours. The process was repeated for 6 cycles. Physical appearances such as color, clarity were inspected visually.

4.2 Effect of light on stability of fluconazole syrup

4.2.1 Storage conditions in the presence of light

One and a half milliliter aliquots of fluconazole syrup formula 1 were pipetted by using a micropipet and transferred to 2 ml transparent borosilicate glass vials. The vials were closed with rubber closures and covered with aluminum caps using a hand crimper. The vials containing fluconazole syrup were placed in a black plastic box in a hot air oven at controlled temperature of 60^{0}C. A 25 w artificial daylight lamp was positioned at the center of the top of each box in the position which yield the light luminescence of 1000 $\pm$ 10 lux. The black plastic boxes were used to avoid disturbance of luminescence from other lamps. Nine black plastic boxes were in the oven.

Physical appearances such as pH, color, and clarity were observed. Percent of drug remaining in the formula was also determined.

4.2.2 Storage conditions in the absence of light

One and a half milliter aliquots of fluconazole syrup formula 1 were piptted by using a micropipet and transferred to 2-ml transparent borosilicate glass vials. The vials were closed with rubber closures and covered with aluminum caps using a hand crimper. Each group of three vials was placed in a small opaque box which stored in the hot air oven at controlled temperature of 60^{0}C. Three samples of fluconazole syrup were assayed.

Physical appearances such as pH, color, and clarity were observed. Percent of drug remaining in the formula was also determined.

4.3 Effect of antioxidants on stability of fluconazole syrup

4.3.1 Effect of free radical inhibitors

Effect of propyl gallate

One and a half milliliter aliquot of fluconazole syrups containing 0.001, 0.005 and 0.01 % w/v propyl gallate in formula 2, 3, 4 were filled in the vials respectively and stored in the hot air oven in the same storage conditions as previously described in 4.2.1 and 4.2.2.

Physical appearances such as pH color and clearity were observed. Percent of drug remaining in the formulas were also determined.

4.3.2 Effect of oxygen scavenger

Effect of sodium bisulfite

One and a half milliliter aliquots of fluconazole syrups containing 0.050, 0.075,and 0.100% w/v sodium bisulfite in formula 5, 6, 7 were filled in the vials, respectively and stored in the hot air oven in the same storage conditions as previously described in 4.2.1 and 4.2.2.

Physical appearances such as pH color and clearity were observed. Percent of drug remaining in the formulas were also determined.

4.3.3 Effect of chelating agents

Effect of disodium edetate

One and a half milliliter aliquots of fluconazole syrups containing 0.005, 0.010 and 0.050% w/v disodium edetate in formulations 8 , 9, 10 were filled in the vials respectively and stored in the hot air oven in the same storage conditions as previously described in 4.2.1 and 4.2.2.

Physical appearance such as pH, color and clarity were observed. Percent of drug remaining in the formulas were also determined.

4.4 Physical stability of each component in fluconazole syrups

For comparison of the physical change of fluconazole in formulation. Fluconazole was dissolved in cosolvent. Sodium hydrogen phosphate, disodium

hydrogen phosphate were dissolved in distilled water, and added to the solution of fluconazole. The final volume was adjusted to 100 ml with distilled water to make the same concentration as formula 1. Each component in formula 1-10 was seperately added at the same concentration into the solution. These separate formulas were stored at the same condition as in 4.2.1 for 90 days.

5. Kinetic study on the stability of fluconazole syrup

Formulation 2 was prepared, filled in the vials and kept in constant temperature incubators at 45^0C, 55^0C, 65^0C, 70^0C and at ambient temperature. The samples were taken at suitable time intervals and analyzed for the content of fluconazole. These data were used to determine the order of reaction and the relationship with Arrhenius Equation and / or Arrhenius plot.