**Annex -1**

OUTLINE OF METHODThe sample is dissolved in Acetonitrile and the hexaconazole is determined by reverse phase liquid chromatography using Dibutyl phthalate as internal standard and UV detector

REAGENTS

*Acetonitrile* HPLC grade

*Water* - HPLC grade

*Methanol* – HPLC grade

Dibutyl phthalate Internal Standard

Hexaconazole reference standard of known purity. Store under refrigeration

*Mobile Phase* Acetonitrile: Water: Methanol (70: 20: 10 v/v), thoroughly degassed

***Internal Standard Solution*** Weigh about 1.4 gm (to nearest 0.1 mg) of Dibutyl Phthalate, dissolve in Acetonitrile and make up to 100 ml. Shake well or sonicate to dissolve material completely

***Calibration solution***Weigh (to the nearest 0.1 mg) into volumetric flask (100 ml) about 0.100 g Hexaconazole standard, add about 50 ml Acetonitrile and sonicate for 5 minutes. Add 5 ml internal standard solution, shake well and make up the volume to mark with acetonitrile

Pipette out 5 ml in a 50 ml volumetric flask and dilute to mark with mobile phase

**APPARATUS**

***High Performance Liquid Chromatography***equipped with manual or auto injector and variable UV detector capable of measuring at 230 nm

*Chromatographic Column* 250 X 4.6 mm (i.d.) packed with 5 µm octadecyl-silane bonded silica gel (ODS or C-18)

*Data system or electronic integrator*

*Ultrasonic Bath*

*Filter* porosity 0.45 µm, solvent compatible

PROCEDURE

1. *Operating Conditions* (typical)

*Mobile Phase* Acetonitrile: Water: Methanol (70: 20: 10 v/v)

*Column Temperature 20˚C ±5˚C*

*Flow Rate* about 1.0 ml/min

*Injection Volume* 20 µl

*Detector Wavelength* 230 nm

*Retention Time* Hexaconazole: about 4.5 min

 Internal Standard: about 6.5 min

*Run Time* 12 min.

1. *System Suitability Check.* Equilibrate the column by pumping mobile phase through the column for at least 30 min. Adjust the operating parameters, so that the elution time of hexaconazole and Internal Standard peak are within 4.0 – 5.0 min and 6.0 – 7.0 min respectively. Make repetitive injections of calibration solution and calculate response ratios by dividing the hexaconazole peak areas by that of internal standard peak areas. The response ratios for calibration solution injections (*R’*) must agree within ± 1.0 % for two consecutive injections before continuing.
2. *Preparation of Sample solution.* Weigh ( to the nearest 0.1 mg) into volumetric flask (100 ml) enough sample to contain about 0.100 g hexaconazole (*w mg*) add about 50 ml mobile phase and sonicate for 5 minutes. Allow it to attain room temperature and then add 5 ml internal standard solution, shake well and make up the volume to mark with acetonitrile.

Pipette out 5 ml in a 50 ml volumetric flask and dilute to mark with mobile phase

Note 1-Hexaconazole is generally formulated as SC, WDG and EC formulations, for WDG formulations, after transferring required quantity of sample to the 100 ml volumetric flask, add about 5 ml distilled water and sonicate for 5 minutes to disintegrate the granules completely. Add about 50 ml acetonitrile and sonicate further for 5 minutes for effective extraction of active ingredient.

No special instruction for SC formulation sample.

The internal standard method is not suitable for EC formulation due to potential interference of the adjutants hence Hexaconazole content in EC formulation can be determined by external standard method (without internal standard) with same instrument condition and solution concentration.

Note 1 -Formulation sample solutions need to be filtered through Whatman No. 41 filter paper before injecting onto HPLC system.

1. *Determination.* Inject in duplicate 20 µl aliquots of sample solution (no more than 3 samples, i.e. 6 injections) bracketed by duplicate injections of calibration solution CA and CB. Calculate response ratios of sample injections (*R*) by dividing hexaconazole peak areas by internal standard peak areas. The response ratios of sample injections must agree within ± 1.0 % (± 0.5 % of their average). If not, repeat the determination starting with calibration injections. Re-inject the calibration solution. Average the response ratios of calibration solution injections immediately preceding and following the sample solution injections. These must agrees within ± 1.0 % (± 0.5 % of their average) or repeat any portion of the determination that does not meet the criteria.
2. *Calculation.*



 

Where:

*Hs =* area of acephate peak in the calibration solution

*Hw* =area of acephate peak in the sample solution

*Ir* =area of internal standard peak in the calibration solution

*Iq* =area of internal standard peak in the sample solution

*Ra* =average of two response ratios of the calibration solution injections

*R’a =* average of two response ratios of the sample solution injections

*s =* mass of acephate in the calibration solution (mg)

*w* = mass of sample taken (mg)

*P* = purity of hexaconazole standard (g/kg)