16.0 VITAMIN ANALYSIS

16.1 Determination of vitamin A – Using HPLC

Principle

The sample is saponified with ethanolic potassium hydroxide solution and the vitamin A is extracted into light petroleum. The light petroleum is removed by evaporation and the residue is dissolved in 2-propanol. The vitamin A concentration in the 2-propanol extract is determined by reverse-phase liquid chromatography using conditions that give a single peak for all retinol isomers.

Reagents

- Water, HPLC grade
- Potassium hydroxide solution (Dissolve 500 g of KOH in water and dilute to 1 litre)
- Ethanol- 95%
- 2-Propanol
- Light petroleum (40-60ºC)
- Vitamin A standard
- All-trans-retinyl acetate, vitamin A acetate ($C_{22}H_{32}O_2$), 328.5 g/mol, with a purity of at least 90%.
- All-trans-retinol, vitamin A alcohol ($C_{20}H_{30}O$), 286.5 g/mol, with a purity of at least 90%
- Methanol, HPLC grade
- Mobile phase for liquid chromatography: Mix together methanol and water (770 + 30 v/v)
- Sodium sulfate (Na_2SO_4) anhydrous
- Sodium ascorbate solution, p = 100 g/l.

Apparatus

- High-performance liquid chromatograph (Column: length 250 mm, ID 4.6 mm, packed with a stationary phase consisting of octadea (C_{18}) groups bonded to silica).
- UV-Visible spectrometer
- Boiling water bath
- Rotary vacuum evaporator
- Extraction apparatus
- Membrane filter-0.45 µm pore size

Procedure

Saponification

- Weigh about 0.1 g sample in 1 litre conical flask.
- Add 200 ml of ethanol. Swirl the flask.
- Add 2 ml of sodium ascorbate and 50 ml of potassium hydroxide solution and mix by swirling.
- Fit a reflux condenser to the flask and immerse the flask in the boiling water bath.
- Allow the contents of the flask to reflux for 60 min.
- Remove and cool the flask to room temperature as rapidly as possible under a stream of cold water.

Extraction of vitamin A (retinol)

- Transfer the contents of the flask to extraction cylinder.
- Rinse the flask with two 25 ml portions of ethanol and transfer the rinsing to cylinder.
- Repeat the rinsing of the flask with two 125 ml of petroleum and one 250 ml of water, each time transferring the rinsing to the cylinder.
- Stopper the cylinder and shake well for 1 min.
- Cool under a stream of cold water while waiting for the two liquid phases to separate, before removing the stopper.

  When the layers have separated, remove the stopper, wash the sides of the stopper with a few ml of light petroleum and insert the adjustable tube, positioning the lower open end so that it is just above the level of the interface. By application of a slight pressure of inert gas to the side-arm tube, transfer the upper, light petroleum layer to a 1 litre separating funnel.
- Add 125 ml petroleum to cylinder and shake for 1 min.
- Allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube. Repeat above step again.
- Wash the combined light petroleum extracts with four 100 ml portions of water using at first only gentle inversion then only gentle shaking in order to keep emulsion formation to a minimum.
- Transfer the washed extract through a medium/fast filter paper containing 60 g of anhydrous sodium sulfate, into a flask suitable for vacuum evaporation.
- Rinse the separating funnel with two 20 ml of light petroleum and add rinsing, through the filter to the evaporation flask.
- Wash the filter further with two 25 ml of light petroleum and collect the washings in the evaporation flask.
- Evaporate the petroleum extract to dryness under vacuum at a temperature not exceeding 40ºC.
- Restore atmospheric pressure by admitting inert gas.

High performance liquid chromatography

- Dissolve the residue in the minimum volume of 2 propanol and transfer quantitatively to a 20 ml volumetric flask.
- Rinse the evaporation flask with three small portions of 2-propanol, transferring the rinsing to the volumetric flask. Dilute to volume with 2 propanol and mix and inject 10 µl of the sample extract into HPLC.
- Calculate the peak area.

Note: 1 IU of vitamin A is equal to 0.300 µg of all-trans-retinol.
1 IU of vitamin A is equal to 0.344 µg of all-trans-retinyl acetate.

Hydrolysis of all-trans-retinyl acetate for calibration

- Prepare a solution of all-trans-retinyl acetate in ethanol, so that 1 ml contains approximately 15000 IU of vitamin A.
- Transfer 2.5 ml of this solution to a 150 ml flask.
- Add 20 ml of ethanol, 1 ml of potassium hydroxide and 5 ml of sodium ascorbate solution.
- Fit a condenser to the flask. Immerse the flask in the boiling water bath and allow to reflux for 60 min.
- Cool the flask to room temperature under a stream of cold water and transfer the contents to a separating funnel.
- Rinse the flask with 50 ml of water, followed by 25 ml of ethanol, adding the rinsing to the separating funnel.

- Extract the aqueous phase with one 80 ml of petroleum and then with two 50 ml portions of light petroleum.
- Combine petroleum extracts, and then wash with two 50 ml portions of water. Add 2 g of anhydrous sodium sulfate.
- Transfer petroleum extract to 250 ml volumetric flask and dilute to volume. The retinol concentration of this solution (solution 1) is approximately 150 IU/ml.

Standardization of retinol solution for calibration

- Pipette 5 ml solution 1 into a 50 ml volumetric flask and remove the solvent, at ambient temperature, with a stream of inert gas.
- Dissolve the residue in 2-propanol, and then dilute to volume with 2-propanol.
- Measure the absorbance (A) of the solution, using 2-propanol as reference, at wavelengths of 310 nm, 325 nm and 334 nm. The absorbance values will be approximately 0.7 to 0.8. If necessary, an intermediate dilution may be used.

Using the following equation, calculate the corrected absorbance at 325 nm:

$$A_{325,corr} = 6.815 \times A_{325} - 2.555 \times A_{310} - 4.26 \times A_{334}$$

If $A_{325,corr} / A_{325}$ is less than 0.97, use the value of A_{325},corr for the standardization; otherwise use A_{325}

The retinol concentration of solution 1 is given by :

Concentration (IU/ml) = A_{325} x 183 IU/ml, or
Concentration (IU/ml) = A_{325},corr x 183 IU/ml.

Preparation of a retinol standard for chromatography

- Prepare a retinol solution in 2-propanol. For each 1000 IU of vitamin A/kg of sample, a retinol concentration of 2.5 IU/ml is expected in the extract.
- Evaporate an aliquot volume of solution 1 to dryness at ambient temperature with a stream of inert gas. Dissolve the residue in the appropriate volume of 2-propanol to provide the required retinol concentration and mix it.
- If necessary, filter the standard solution through a membrane filter.
- For calibration purposes, a standard solution of vitamin A (retinol) in 2-propanol prepared by diluting a stock standard solution of all-trans-retinol made by dissolving an appropriate quantity of all-trans-retinol standard substance directly in 2-propanol (5.4) may be used.
- In this case, check the vitamin A standard by measuring the absorbance of the standard solution in quartz cells at wavelengths of 300 nm, 325 nm, 350 nm and 370 nm against 2-propanol as reference. Determine the A/A325 ratio at each of the wavelengths for all-trans-retinol. If the ratio does not exceed 0.602, 0.432 and 0.093 at 300 nm, 350 nm and 370 nm respectively, the standard substance is suitable for use.

Calculation

Calculate the numerical value of the vitamin A content of the test sample by the equation.

$$wA = 20{,}000 \times \frac{c}{m}$$

where,

wA is the numerical value of the vitamin A content of the test sample, in IU/kg.
c is the numerical value of the retinol concentration of the extract, in IU/ml.
m is the numerical value of the mass of the test sample, in grams.

Reference: Indian Standards, IS 15120 : 2002.