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Vitamin A determination by HPLC		Replaces: New Date of revision: September 2008
Prepared by:	Approved by:	Put into force by:
Dennis Eriksen	Dennis Eriksen	Dennis Eriksen

1. Purpose

The purpose of this SOP is to update method 1.01.03.03

2. Index

3. Enclosures

1. Dokumentation

2. Examples of chromatograms

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4. Principle

Vitamin A (Retinol) is determined by high performance liquid chromatography with UV-detection after saponification and extraction. The method is in particular usable for tablets containing large amounts of betacaroten and for products containing a little amount of vitamin A.

Minimum concentration of the sample: About 0.3 µg of vitamin A/g.

NB: Make two single determinations at two different days.

5. Apparatus

Shimadzu High Pressure Liquid Chromatograph

Autoinjector SIL 10 A XL
CBM box 10 A
UV- Vis detector SPD 10A
Pump LC 10 AT
FVC 10 AL

or use similar HPLC equipment

Grinding mill, Krups 75 or similar

Perkin Elmer model Lambda 20 UV/VIS Spectrophotometer or similar.

6. Reagents

Potassium hydroxide e.g. Merck art. 5021

Ascorbic acid e.g. Merck art. 127

Sodium sulphate e.g. Merck art. 106649

Butylhydroxytoluen (BHT), e.g. Fluka art. 34750

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Ether e.g. Superfos Kemi, art. 1416064

Ethanol (99%), e.g. DDSF

Nitrogen, e.g. D.I.B

1-pentanol = n-amylalcohol, e.g. Merck art. 975

Heptane e.g. Ratburn art. 1004

Milli-Q Water

Isopropanol = 2-propanol-R1, e.g. Merck art. 101040

Vitamin A e.g. Fe standard (= 160.000 mcg/g)

Diluted Sodium Hydroxide solution (2M) e.g. Baker art. 7067

Phenolphthalein solution R1 e.g. Merck art. 7233

Potassium hydroxide solution (17M / 90%):

Dissolve 180 g of potassium hydroxide in 126 ml of water.
Store for three month

Sodium sulphate solution (3%):

Dissolve 30 g of anhydrous sodium sulphate in water and dilute with water to 1000 ml.
Store for three month

BHT-solution (0.1%), alcoholic:

Dissolve 1.0 g of butylhydroxytoluen in ethanol and dilute with ethanol to 1000 ml.
Store for three month

Sodium ascorbate solution (2%):

Dissolve 3.5 g of ascorbic acid in 10 ml of diluted sodium hydroxide solution and dilute with water to 200 ml.

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Do not store

7. Chromatographic conditions

Column:	C_{18}, 15 cm x 3.9 mm, YMC 120 Å, 5 µm, OdDMeSi - B- 564, or similar.
Mobile phase:	Heptane : 1-propanol (99:1)
Flow rate:	2 ml/minute
Detection:	UV-absorption 325 nm
Injection volume:	100 µl
Attenuation:	App. 2^8 (8)
Chart Speed:	App. 3 mm/minute
Run time:	App. 12 minutes
Retention time:	App. 7 minutes for Retinol

8. Method

8.1 Solutions

8.1.1 Standard solution:

Weigh out, in duplicate, about 0.30 g of the standard in a conical flask. Use A-acetate concentration, Fe standard (= 160.000 mcg/g).

Add 10 ml of sodium ascorbate solution (2%) and heat in a steam bath for 5 minutes.

Add 30 ml of BTH-solution (0.1%) and 3 ml of potassium hydroxide (17M).

Connect the flask of an air condenser and reflux for 30 minutes on a steam bath (shake frequently).
After cooling, transfer the solution by 30 ml of sodium sulphate 3% and 100 ml of ether to at separating funnel containing 100 ml of ether.

Continue from the extraction step.

8.1.2 Test solution

1. Liquid, anhydrous solutions:
Mix the sample and weigh out accurately the sample (= p g) (see table below) into an Erlenmeyer flask. Dilute to 10 ml with sodium ascorbate solution (2%).

2. Oily solutions:
Weigh out accurately the amount of sample (=p g) (see table below) into a 100 ml Erlenmeyer flask.

3. Tablets
Pulverise 20 tablets in a grinding mill.
Weigh out accurately the powder (=p g) (see table below) into a 100 ml Erlenmeyer flask. Add 10 ml of sodium ascorbate solution (2%) and heat in a steam bath for 5 minutes with frequently shaking.

	Vitamin A conc. in the sample (µg/ml, µg/g or µg/tabl.)	Weigh an amount of the sample containing about:
A	≥ 10 and < 80	80 µg of vitamin A
B	≥ 80 and < 400	400 µg of vitamin A
C	≥ 400 and < 2000	2000 µg of vitamin A

8.2 Saponification

Add 30 ml of alcoholic BTH-solution (0.1%) and 3 ml of potassium hydroxide (17M).

Connect the flask of an air condenser and reflux for 30 minutes on a steam bath (shake frequently).

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Cool and transfer with 30 ml of sodium sulphate 3% and 100 ml of ether to a 500 ml separating funnel containing 100 ml of ether.

8.3 Extraction

Shake for 2 minutes.

Let stand until the layers are clearly separated (about 30 minutes), and discharge the lower aqueous layer (if an emulsion is formed, add some drops of ethanol 99%). Wash the ether extract with 4 x 50 ml of water; shake carefully in the beginning in order to avoid emulsification. Afterwards pour a couple of ml of water phase into a centrifuge tube containing a few drops of phenolphthalein-R1.

If it is red - continue washing until the washings are no longer coloured red. Afterwards, transfer the ether layer to a 250 ml volumetric flask or a round-bottom flask.

8.4 Final preparation

8.4.1 Tablets and solutions

A+B: Transfer through a cotton plug covered with anhydrous sodium sulphate by ether to a round-bottom flask. Evaporate in vacuum and dilute with n-heptane until a final concentration of 3-4 mcg/ml is found.

C: Transfer to a 250 ml volumetric flask and fill up to volume with ether. 20.00 ml in a 50 ml flask is evaporated under a steam of nitrogen until a rest of 2 ml is left. Dilute with n-heptane to a final volume of 50 ml.

8.5 Standard

Transfer by ether into a 250 ml volumetric flask (= STD A) and fill to volume.

8.5.1 Standard assay concentration determination

Dilute 2.00 ml of STD A with 2-propanol to a final volume of 100 ml.
Measure the absorbances at 310, 325 and 334 nm according to SOP QAM-10101.
Calculate the assay determination.

8.5.2 Standard to HPLC

Dilute 2.00 ml of STD A with n-heptane to a final volume of 100 ml.

8.6 Chromatography

Transfer sample and standard to vials and inject 100 µl of the solutions (double).
Record the area or height of the A-vitamin peaks.
NB: Keep water away from the apparatus.

9. Calculations

Tablets

$$\frac{At \cdot tw \cdot c \cdot a \cdot f \cdot S \cdot 1000 \cdot 1000}{As \cdot p \cdot 100 \cdot 250 \cdot F} \quad [\mu g \text{ of vitamin A / tabl}]$$

Oily solutions / Solubilized aqueous solutions

$$\frac{At \cdot d \cdot c \cdot a \cdot f \cdot S \cdot 1000 \cdot 1000}{As \cdot p \cdot 100 \cdot 250 \cdot F} \quad [\mu g \text{ of vitamin A / ml}]$$

a diluted in amount of ml of ether for test

As, At the areas or heights of the A-vitamin peaks in the chromatogram of the standard and the test solutions respectively

f dilution factor for test

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tw average tablet weigh in g

d uniformity of mass in g/ml

c standard amount in g

S standard assay in % (spectrophotometrically determination)

F dilution factor for standard

10.Reference

Ferrosan methods of analysis 1.01.03.01 and 1.01.01, USP 24 p. 1890.