Quantitative Estimation of Cholesterol

- It is the major sterol in animal tissues
- It is amphipathic with a polar head group (the OH^- group at C-3) and a non-polar hydrocarbon body (the steroid nucleus and the hydrocarbon side chain at C-17) which can be as long as a 16-Carbon fatty acid in its extended form
- It is the constituent of plasma membrane and also serves as a precursor of several important biological compounds, like, bile salt, hormones etc.
- Free cholesterol is equally distributed among the cells and plasma while the esterified form occurs only in plasma

Zak's Method

- In this method, ferric chloride, concentrated sulfuric acid & glacial acetic acid are used as reagents for cholesterol estimation
- In presence of conc. H_2SO_4, cholesterol is dehydrated to form cholesterol 3,5 – diene
- In presence of excess conc. H_2SO_4 a red coloured complex is formed by the catalytic action of ferric ions
- The intensity of the red colour is measured at 560nm

Reagents Required

- Cholesterol stock solution – %10mg
- Glacial Acetic acid
- Ferric chloride solution
- Concentrated Sulfuric acid

Protocol

Take 6 clean & dried test tubes

↓

Mark them as per concentration of cholesterol (e.g. Blank, 25µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, & unknown)

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Pour stock cholesterol solution into all the tubes except blank & unknown (as per protocol)

↓

Pour unknown cholesterol solution into the ‘unknown’ marked test tube

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Add glacial acetic acid to make up the volume in each test tube to 2ml

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Now add 2ml ferric chloride solution in each of the 6 test tubes

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Mix well

↓

Then carefully add 2ml concentrated sulfuric acid in each of the 6 test tubes

↓

Mix well

↓

Measure absorbance of each tube at 560 nm (Blank set ‘zero’)

- Record the observations in a tabular form against different cholesterol concentrations
- Prepare a standard curve by plotting different cholesterol concentrations in X-axis and corresponding absorbance values at 560nm in Y-axis
- Determine unknown cholesterol concentration from the standard curve

Precautions

- Care should be taken during addition of concentrated H_2SO_4 and glacial acetic acid
- Accurate volume of stock cholesterol solution should be pipetted for making different concentrations of cholesterol standard solution
- All the test tubes should be cooled to room temperature before taking absorbance values as O.D. values are sensitive to temperature

Liebermann-Burchard Method

- In the presence of acetic anhydride and concentrated H_2SO_4, sterols develop colours ranging from blue to green
- Under the anhydrous conditions, a number of molecular rearrengements take place
- The nature of chromophoric groups are not known
- This is called Liebermann – Burchard test and is used for the quantitative estimation of cholesterol

Reagents Required

- Cholesterol stock solution – 100 µg/ml – prepared in chloroform or glacial Acetic acid
- Glacial Acetic acid
- Chloroform
- Concentrated Sulfuric acid
- Acetic anhydride
- Reagent preparation –

  Mix 2ml concentrated H_2SO_4 with 40ml acetic anhydride to prepare a colourless reagent

  The reagent should be prepared freshly before use and kept in cold (in ice bucket) all the time

Protocol

Take 6 clean & dried test tubes

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Mark them as per concentration of cholesterol (e.g. Blank, any four conc. ranging from 50 to 500 µg/tube, & unknown)

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Pour stock cholesterol solution into all the tubes except blank & unknown (as per protocol)

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Pour unknown cholesterol solution into the ‘unknown’ marked test tube

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Add chloroform or glacial acetic acid to make up the volume in each test tube to 1ml

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Now add 5ml freshly prepared reagent in each of the 6 test tubes

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Mix well

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Incubate the tubes in dark for 15 minutes

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Measure absorbance of each tube at 640 nm (Blank set ‘zero’)

- Record the observations in a tabular form against different cholesterol concentrations
- Prepare a standard curve by plotting different cholesterol concentrations in X-axis and corresponding absorbance values at 640nm in Y-axis
- Determine unknown cholesterol concentration from the standard curve

Precautions

- Care should be taken during addition of concentrated H_2SO_4 and glacial acetic acid
- Accurate volume of stock cholesterol solution should be pipetted for making different concentrations of cholesterol standard solution
- All the test tubes should be cooled to room temperature before taking absorbance values as O.D. values are sensitive to temperature

References

- Practical manual provided by University of Calcutta
- Laboratory Manual in Biochemistry by J. Jayaraman
- Libermann-Burchard's test: Part 1 (Qualitative test) https://youtu.be/UdRE2AdlT8w
- Libermann-Burchard's test: Part 2 (Qualitative test) https://youtu.be/hc7cauZHNK8
- Liebermann-Burchard's Reagent (Quantitative test) https://youtu.be/4VZr0k1Dd8M
- Estimation of Cholesterol Liebermann-Burchard Reaction (Quantitative test) https://youtu.be/acA309vvFNE