

**PLASMA LIPOPROTEINS AND LIPIDS**  
**DETERMINATION OF PLASMA CHOLESTEROL AND TRIGLICERIDE LEVEL**

Lipids are characterized by low polarity and limited solubility in water. Their plasma concentration is about 500-600 mg/100 ml, strongly associated with specific proteins. Lipoproteins are classified on the basis of their electrophoretic properties into:  $\alpha$ - (HDL), pre- $\beta$ - (VLDL),  $\beta$ - (LDL) lipoproteins and chylomicrons. The lipid components of lipoproteins are (i) cholesterol (about 70 % of total amount of cholesterol is esterified with unsaturated fatty acids) (ii) triacylglycerols and (iii) phospholipids.

Lipids are transported in the blood as lipoproteins, containing a core of lipids (triacylglycerols, cholesteryl esters) surrounded by a sphere of polar lipids and apoproteins.

Lipoproteins have two major functions: (i) solubilization of lipids, and (ii) regulation of lipid transport of lipids in and out of specific target cells and tissues.

The cholesterol ( $C_{27}H_{46}O$ , MW: 386.65) is an amphipatic lipid, component of the lipoprotein particles of plasma and structural component of plasma membranes of the cells. About half of the cholesterol in the body is synthesized, while the other half is absorbed from food. A part of the cholesterol is excreted in the bile fluid. The conversion of the cholesterol to bile salts helps to solubilize and digest lipids. The transport of cholesterol by LDL in the circulation to the tissues plays an important role in the membrane formation of cells and in the biosynthesis of steroid hormones.

The rate of biosynthesis of cholesterol depends on the interaction between the amount of the dietary cholesterol and its endogenous cholesterol biosynthesis. Exogenous (dietary) fat is hydrolyzed in the intestine, absorbed, then resynthesized in the mucosal cells. The lipids are transported as chylomicrons. Endogenous triglycerides are produced by the liver and adipose tissue cells. Their transport is in association with VLDL.

Statistical data show increased occurrence of heart and vascular diseases. There is a relationship between the elevation of concentration of some plasma lipid parameters and atherosclerosis. Hyper- and dyslipoproteinemias play an essential role as a risk factor. The data show that (i) in 70 % of patients with arterial occlusion hyperlipoproteinemia is detectable, (ii) lipids deposited in the connective tissue of the arterial wall show plasma lipoprotein origin (iii) about 70 % of patients with primary hyperlipoproteinemia show coronary sclerosis and/or myocardial infarction, (iv) there is a correlation between the frequency of myocardial infarction and the reduced plasma lipid concentration during the therapy of patients with hyperlipoproteinemia.

200-300 or >300 mg/ml of cholesterol level was determined at 60 % or 25 % of patients who died in myocardial infarction, respectively. The prognosis of myocardial infarction also needs the evaluation of both HDL- and LDL-cholesterol level. The lower the HDL-cholesterol level the higher is the risk of myocardial infarction. The determination of HDL-cholesterol is important for patients who have hypercholesterolemia, as well as hypertriglyceridemia. Simultaneously elevated levels of serum triglycerides and decreased levels of HDL-cholesterol indicate high coronary risk.

Table 1 summarizes the data of lipids and lipoproteins influencing the atherosclerotic risk.

The atherosclerotic risk is						
	normal	some risk	high risk	normal	some risk	high risk
Total cholesterol	<200	200-240	>240	<5,16	5,16-6,20	>6,20
Triglyceride	<150	150-199	>200	<1,70	1,70-2,26	>2,27
LDL-cholesterol	<100	100-190	>190	<2,58	2,58-4,9	>4,9
HDL- cholesterol	> 60	60 - 40	<40	> 1,55	1,55-1,03	< 1,03
	[mg/dL]			[mmol/L]		

**Table 1.** : Data of lipid and lipoprotein components of plasma influencing atherosclerotic risk at normal slightly enhanced and enhanced atherosclerotic risk.

DETERMINATION OF THE CONCENTRATION OF SERUM CHOLESTEROL:

The concentration of total cholesterol, HDL-cholesterol and triglycerol of both normal and "ill" patients will be determined. VLDL and LDL fraction of plasma in the presence of Ca/Mg/Mn ions and polyanions/sulfated polyanions precipitates. After centrifugation HDL-cholesterol content of the supernatant will be determined.

## Principle of the determination of cholesterol

Cholesterol esters will be hydrolyzed by cholesterol esterase. Cholesterol will be oxidized into cholest-4-en-3-on and H<sub>2</sub>O<sub>2</sub> by bacterial cholesterol oxidase. H<sub>2</sub>O<sub>2</sub> in the presence of phenol and amino-4- antipyrine forms a complex of red color showing absorption maximum between 505 nm.

### Cholesterol esterase

cholesterol ester  $\longrightarrow$  cholesterol + fatty acids

### Cholesterol oxidase

cholesterol  $\longrightarrow$  cholest-4-en-3-on + H<sub>2</sub>O<sub>2</sub>

### peroxidase

H<sub>2</sub>O<sub>2</sub> + phenol + amino-4-antipyrin  $\longrightarrow$  quinoneimine + 2 H<sub>2</sub>O

## Determination of the serum cholesterol level (total)

### Solutions:

- 1.) Reagent #1: 50 mmol/L PIPES buffer pH = 6.9  
24 mmol/L phenol
- 2.) Reagent #2: 250 U/L cholesterol esterase  
250 U/L cholesterol oxidase  
1000 U/L peroxidase  
0.5 mmol/L amino-4-antipyrin  
2.5 mmol/L NaCl
- 3.) Reagent#3: standard solution of cholesterol  
(5.17 mmol/L = 200 mg/100 ml)
- 4.) Serum sample - normal level of cholesterol  
- elevated level of cholesterol

Dissolve Reagent #2 in Reagent #1 = Reagent1+2

### Experiment:

Prepare the following solutions according to the table below:

Solution / #	1	2	3	4
Reagent 1+2.	1 ml	1 ml	1 ml	1 ml
Cholesterol St <sub>total</sub>	-	20 µl	-	-
Sample ( normal )	-	-	20 µl	-
Sample( ill )	-	-	-	20 µl
Distilled water	20 µl	-	-	-

Shake the tubes well and incubate them for 5 min at 37°C. Read the **absorbance of the samples (A)** at 505 nm. Tube #1 serves as blank

### Calculation:

The concentration of cholesterol present in the samples can be calculated from the measured absorbance of the samples and of the standard using the following equation:

$$[\text{Cholesterol, total}] = \frac{A_{\text{serum}}}{A_{\text{standard}}} * n$$

n = 5.17  $\rightarrow$  mmol/L, n = 200  $\rightarrow$  mg/100 ml, n = 2  $\rightarrow$  g/l

## Determination of the serum HDL cholesterol level

### Separation of HDL fraction

HDL fraction will be separated from the other lipoproteins, which are precipitated in the presence of phosphotungstic acid-MgCl<sub>2</sub>. After the centrifugation, cholesterol content of the supernatants will be determined according to the method described above for the total cholesterol content.

### Reagents:

2 M MgCl<sub>2</sub> solution

Precipitating reagent: 32 g/l phosphotungstic acid:0.4 mmol/L MgCl<sub>2</sub>

Plasma or serum of normal and "ill" patients.

### Separation procedude:

Add 500 µl plasma of normal and "ill" patient, resp.,  
50 µl precipitating reagent into marked Eppendorf tubes.

Mix the samples thoroughly and centrifuge them at 10 000 RPM for 15 min at 4°C. After centrifugation the supernatant must be "clear". Pipette supernatants into test tubes and determine concentration of HDL-cholesterol.

### Solutuons:

Reagent#1: 50 mmol/L PIPES buffer pH=6.9  
24 mmol/L phenol

Reagent#2: 250 U/L cholesterol-esterase  
250 U/L cholesterol-oxidase  
1000 U/L peroxidase  
0.5 mmol/L amino-4-antipyrin  
2.5 mmol/L NaCl

Reagens#3: 1.3 mmol/L = 50 mg/dL HDL-cholesterol standard

Serum samples: (normal level of cholesterol, elevated level of cholesterol) pretreated with precipitating reagent (see separation of HDL fraction)

### Experiment:

<b>Solution / #</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
Reagent 1+2.	1 ml	1 ml	1 ml	1 ml
Cholesterol St <sub>HDL</sub> )	-	20 µl	-	-
Sample ( normal )	-	-	20 µl	-
Sample( ill )	-	-	-	20 µl
Distilled water	20 µl	-	-	-

Shake the tubes well and incubate them for 5 min at 37°C. Read the **absorbance of the samples (A)** at 505 nm. Tube #1 serves as blank

### Calculation:

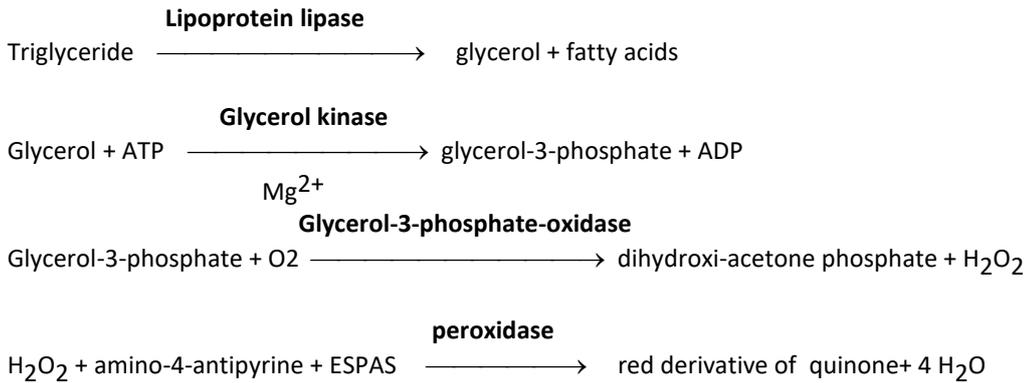
The concentration of cholesterol present in the samples can be calculated from the measured absorbance of the samples and of the standard using the following equation:

$$[\text{HDL}] = \frac{A_{\text{serum}}}{A_{\text{standard}}} * n$$

n = 1.3 → mmol/L or n = 50 → mg/100 ml

## DETERMINATION OF SERUM TRIGLYCERIDES:

### Principle of determination



### Solutions:

- **Reagent #1** 50 mmol/L PIPES buffer pH = 7.5; 1 mmol/L N-ethyl-N-sulfopropyl-m-anizidine (ESPAS)
- **Reagent #2** 1100 U/L lipoprotein lipase; 800 U/L glycerol kinase; 3000 U/L glycerol-phosphate oxidase; 350 U/L peroxidase; 0.7 mmol/L 4 amino-antipyrine; 0.3 mmol/L ATP
- **Reagent #3** standard solution of glycerol (2.28 mmol/L = 200 mg/100 ml)
- **Serum samples** - one with normal level of triglyceride; - one with elevated level of triglyceride

Dissolve Reagent #2 in 10 ml of Reagent #1 = Reagent1+2

### Experiment:

Prepare the following tubes:

Solution / #	1	2	3	4
Reagent 1+2.	1 ml	1 ml	1 ml	1 ml
TAG Standard (	-	20 µl	-	-
Sample ( normal )	-	-	20 µl	-
Sample( ill )	-	-	-	20 µl
Distilled water	20 µl	-	-	-

Shake the tubes well; incubate them for 5 min. at 37°C. Read **the absorbance of the samples (A)** at 505 nm. Tube#1 serves as blank.

### Calculation:

The concentration of triglycerides present in the samples can be calculated from the measured absorbance of the samples and of the standard using the following equation:

$$[\text{Triglycerides}] = \frac{A_{\text{serum}}}{A_{\text{standard}}} * n$$

$n = 2.28 \rightarrow \text{mmol/L}$ ,  $n = 200 \rightarrow \text{mg/100 ml}$ ,  $n = 2 \rightarrow \text{g/l}$

### Calculation of LDL-cholesterol concentration of samples:

Calculate the LDL-cholesterol content of samples according to the following equation:

$$\text{LDL-cholesterol} = \text{total cholesterol} - (\text{HDL-cholesterol} + \text{triglyceride} / 5)$$

Do not use this equation at 400 mg/ml or higher concentration of triglycerides or for data of samples of patients with hyperlipoproteinemia type I and III! LDL-cholesterol determination also can be carried out at pH 5.12 (isoelectric precipitation with heparin)

Determination of lipid constituents in plasma or serum is normally done on blood drawn from patients fasting for 12 or 16 hours. The "normal" value of serum cholesterol and triglycerides varies individually and with ages.

**Questions:**

- 1.) Explain the data of "ill" sample (triglyceride, total-, HDL- and LDL-cholesterol)
- 2.) How can the lipid content of hyperlipidemic plasma be decreased?