BIOCHEMICAL EXAMINATION OF LIVER FUNCTIONS

Warning: In order to successfully finish this practical training, it is necessary for each group to have calculator. This practical training follows the lecture “Biochemistry of liver”. The practical training manual itself is on pages 4-8.

1. LIVER AND ITS FUNCTION

Liver is one of the most metabolically active organs in the body. It plays the crucial role in the energy and intermediary metabolism. The metabolism of saccharides, lipids and proteins is carried out in liver. Hepatocyte tissue is active in the biosynthesis of heme or bile acids. Moreover, the liver is active in biodegradation and detoxification of both, endogenous (bilirubin, cholesterol, ammonia), and exogenous (toxic compounds, drugs ...) substances. Liver plays a partial role in digestion and maintaining of the homeostasis. Reticuloendothelial system of the liver is active in immunity and in retaining the exogenous antigens (Kupffer cells). This multilateral participation of liver in the vital function of the organism makes this organ indispensable.

1.1 Biochemical examination of liver

Laboratory examination of liver functions is, sometimes, commonly called “liver tests”. These tests are usually carried out in order to discover/prove the liver disease. The diagnosis of liver disorders is based on the activity of intracellular and membrane bound enzymes. However, none of the tests can completely describe the functionality of the liver and none is entirely specific for liver. Biochemical liver examination is focused on the tests elucidating:

- permeability and integrity of hepatocyte membranes.
- bile production and excretion.
- changes in the hepatocyte proteosynthesis.
- detoxification.
- disorders of intermediary metabolism.
- disorders of energy metabolism.

In this practical training we will focus on the first two items.

1.2 Tests of the permeability and integrity of hepatocyte membranes

Important place in the detection of liver parenchyma damage take the aminotransferases. Increase activity/concentration of the aminotransferases in blood is the most sensitive and most common indicator of the hepatocyte membrane damage. Increased hepatocyte membrane permeability for alanine-aminotransferase (ALT) and cytoplasmic isoenzyme of aspartate-aminotransferase (cAST) can be caused by the decreased energy intake. However, mitochondrial AST leaves the cell only after more severe cell damage (necrosis), and, therefore, de Ritis coefficient or more exactly ratio AST/ALT is one of the prognosis indicators (values higher than 1.0 indicate worse prognosis). AST and ALT still remain to be quite sensitive indicators of the hepatobiliar disorders. The ALT is more specific for liver disorders than AST. Diagnostic sensitivity of ALT is referred as 83 % with diagnostic specificity 98 % with relation
to healthy individuals, and 84 % with relation to individuals with non-hepatopathy disorders. Thus, the observation of ALT in physiological interval, with high probability, excludes the hepatobiliary disorder. Diagnostic sensitivity of AST, with relation to hepatopathy is 71 %, specificity is even lower. Higher increase of AST, following increased ALT indicates the presence of mitochondrial AST, and, therefore, more extensive damage of hepatocytes. Increased activity of aminotransferases in blood serum is caused by pathological hepatopathies, such as, for instance, acute virus hepatitis, infectious mononucleosis, toxic liver damage, liver cirrhosis, etc. On the other hand, decreased activities can indicate vitamin B₆ deficiency.

### 1.3 Tests of the disorder of bile acids production and excretion

The mostly used tests of this group are tests of the activities of two enzymes, alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT).

#### 1.3.1 Alkaline phosphatase

Enzyme ALP activity in plasma is caused by the combination of the activities of several multimolecular forms originating in posttranslational modifications. Its role in organism is not fully observed, nevertheless, participates in the bone mineralization. ALP is a mixture of more than 17 isoenzymes. The basic ones are three tissue specific isoenzymes – intestinal, placental and placental resembling, and tissue-nonspecific, which includes three isofoms – liver, bone and kidney. Increased ALP activity in serum indicates liver and bile duct disorders (extra- and intrahepatic cholestase, biliary cirrhosis) and bone disorder (osteomalacia).

#### 1.3.2 γ-glutamyl transferase

GGT is bound in high concentration in prostate cells, which explains its higher level in male serum compared to female serum. GGT does not increase during pregnancy or in childhood (growth period), like in the case of ALP. Thus, simultaneous estimation of ALP and GGT can distinguish bone or hepatobiliary origin of the ALP increase. GGT can also build up multimolecular isoforms, which can be formed during posttranslational modification. Pathological increase of GGT activity in blood plasma suggests mainly hepatobiliary disorder (toxic lesion, cirrhosis, steatosis, alcohol hepatitis).

#### 1.3.3 Bilirubin

Bilirubin is formed by the haemoglobin degradation of old erythrocytes in the reticuloendothelium of spleen, liver and bone marrow from the haemoglobin, erythrocytes precursors and other haemoproteins, (catalase, cytochroms). Bilirubin is water insoluble, but can be dissolved in phospholipids. Its presence in the cell is toxic – participates in blocking of oxidative phosphorylation. Bilirubin excretion starts with its transport via blood bound non-specifically to albumin. Bilirubin-albumin complex is captured by the hepatocyte membrane, and the released bilirubin is actively transported to cytosol, where it is bound to transport proteins. Bilirubin is then conjugated with glucuronic acid, producing non-toxic, water soluble mono- and di- conjugates (glucuronides), which are excreted via bile. Increased bilirubin degradation, decreased capturing by hepatocytes, and insufficient conjugated haemoglobin formation can be the reasons of increased bilirubinemia. Among the pathological changes of bilirubinemia we consider virus hepatitis, extra- and intra-hepatic cholestasis, liver cirrhosis, metastasis, abscesses, and other.
1.4 Biochemical examination of nutritional state

Important effect on the disease is the state of nutrition. Starving patient exhibits higher risk of septic comorbidities than correctly nutritionally provided one. Estimation of biochemical criteria of the nutritional state is based on the plasma proteins assessment (albumin, cholinesterase, transferrin, prealbumin), homeostasis assessment (ions, osmolality, ABB), and check of micronutrients level. Further, the states of nutrition can be classified as malnourishment (malnutrition) or undernourishment, and obesity (overnourishment).

1.4.1 Malnutrition

By definition, this is the state of global or partial nutrition impairment. Protein malnutrition is determined by the decrease of albumin in plasma, and worsened cell immunity parameters. According to the mechanism of origin and metabolic consequences, two groups of malnutrition classification can be defined:

- **common** starvation, which leads to **marantic** type of malnutrition, caused by low intake of both, protein and non-protein nutrients, leading gradual degradation of muscle proteins,
- **stress** starvation, which leads to **kwashiorkor** type of malnutrition, caused by low protein intake with normal intake of non-protein nutritional elements.

1.4.2 Obesity

This state is defined as long-term positive nutrition balance with excessive energy input with higher percentage of adipose tissue in body mass.

1.4.3 Plasma proteins

- **albumin** – longest biological half-time, informs about the protein balance in lest 3-4 weeks, used for the estimation of „default state”.
- **cholinesterase** – shorter biological half-time (about 10 days), estimates liver proteosynthesis.
- **transferrin** – similar to the cholinesterase, however, the concentration is influenced by iron metabolism as well.
- **prealbumin** – the most sensitive marker of liver proteosynthesis, commonly used for monitoring the nutritional state.
- **retinol binding protein (RBP)** – protein with the shortest half-time, its concentration is dependent on the level of vitamin A supplies, and renal function.
- **coagulation factors (prothrombin time)** – very short half-time of soma coagulation factors makes it ideal marker of liver failure.
2. DETERMINATION OF CATALYTIC ACTIVITY OF LIVER ENZYMES

2.1 Alanine aminotransferase

The amino group is enzymatically transferred by Alanine aminotransferase (EC 2.6.1.2, ALT) present in the sample from L-alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate. Pyruvate is reduced to lactate by LDH present in the reagent with the simultaneous oxidation of NADH to NAD\(^+\). The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH.

Endogenous sample pyruvate is rapidly and completely reduced by LDH during initial incubation period to avoid interference during the assay.

\[
\begin{align*}
\text{H}_3\text{C}-\text{C}^\text{CH}_2\text{NH}_2 & \quad \text{+} \quad \text{HO-CH}^\text{CH_2}-\text{CO}^\text{OH} \quad \xrightarrow{\text{ALT}} \quad \text{HO-CH}^\text{CH_2}-\text{CO}^\text{OH} \quad \text{+} \quad \text{H}_3\text{C}^\text{CH}^\text{CO}^\text{OH} \\
\end{align*}
\]

Procedure:
1. Pipette 1 mL of working solution (ALT) into the test tube (mixture of Tris buffer+L-alanine+LDH with 2-oxoglutarate+NADH in proportion 4:1) and incubate this tube in water bath at 37 °C for exactly 5 min.
   - Prepare the spectrophotometer – set the wavelength, measure the blank (distilled water).
2. Then add 0.1 mL of serum into the tube.
3. Mix it well and incubate at 37 °C for exactly 1 min.
4. Immediately measure the initial absorbance against blank. Measure the absorbance change exactly after 1, 2 and 3 minutes.

Calculate 1 minute absorbance change (\(\Delta A/\text{min}\)) and calculate the catalytic concentration of ALT in the sample according to the following equations.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Absorbance</th>
<th>(\Delta A)</th>
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<tbody>
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<td>average (\Delta A/\text{min})</td>
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</table>

Tab.I

Calculation: ALT (µkat/L) = \(\Delta A/\text{min} \times 29.08 \times F\)

The catalytic concentration of ALT in the sample: .................. µkat/L

\(^1\) F is a dilution factor. It determines how many times you diluted the sample.
2.2 Aspartate aminotransferase

The amino group is enzymatically transferred by Aspartate aminotransferase (EC 2.6.1.1, AST) present in the sample from L-aspartate to the carbon atom of 2-Oxoglutarate yielding oxaloacetate and L-glutamate. Oxaloacetate is reduced to malate by MDH present in the reagent with the simultaneous oxidation of NADH to NAD⁺. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due the oxidation of NADH.

![Chemical structures of the reaction](image)

Procedure:

1. Pipette 1 mL of working solution (AST) into the test tube (mixture of Tris buffer+L-aspartate+MDH with 2-oxoglutarate+NADH in proportion 4:1) and incubate this tube in water bath at 37 °C for exactly 5 min.
   + Prepare the spectrophotometer – set the wavelength, measure the blank (distilled water).
2. Then add 0.1 mL of serum into the tube.
3. Mix it well and incubate at 37 °C for exactly 1 min.
4. Immediately measure the initial absorbance against blank. Measure the absorbance change exactly after 1, 2 and 3 minutes.

Calculate 1 minute absorbance change (ΔA/min) and calculate the catalytic concentration of AST in the sample according to the following equations.

<table>
<thead>
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<td>average ΔA/min</td>
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**Tab.2**

Calculation: \[\text{AST (\(\mu\text{kat/L}\))} = \Delta A/\text{min} \times 29.08 \times F^2\]

The catalytic concentration of AST in the sample: ……………… \(\mu\text{kat/L}\)

\(^2\) F is a dilution factor. It determines how many times you diluted the sample.
2.3 γ-glutamyltransferase

Gamma-glutamyltransferase (EC 2.3.2.2, GGT, γ-GT) catalyzes the transfer of the γ-glutamyl group from γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine, liberating 3-carboxy-4-nitroaniline. The catalytic concentration is determined from the rate of 3-carboxy-4-nitroaniline formation at 410 nm.

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\begin{align*}
\text{GGT} & \rightarrow \\
\text{O} & \quad \text{O} \quad \text{N} \\
\text{O} & \quad \text{NH} \quad + \\
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{H} \\
\text{N} & \quad \text{H} \quad 2 \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{H} \quad + \\
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{H} \\
\text{N} & \quad \text{H} \\
\text{O} & \quad \text{H} \\
\text{N} & \quad \text{H} \quad 2 \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{H} \quad + \\
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{H} \\
\text{N} & \quad \text{H} \\
\end{align*}
\]

Procedure:
1. Pipette 1 mL of working solution (GGT) into the test tube (mixture of Glycylglycine and γ-Glutamyl-3-carboxy-4-nitroanilide in the proportion 4:1) and incubate this tube in water bath at 37 °C for exactly 10 min.
2. Prepare the spectrophotometer – set the wavelength, measure the blank (1 mL of working solution).
3. Then add 0.1 mL of serum into the tube.
4. Mix it well and immediately measure the initial absorbance against blank. Measure the absorbance change exactly after 1, 2 and 3 minutes.

Calculate 1 minute absorbance change (\(\Delta A/\text{min}\)) and calculate the catalytic concentration of GGT in the sample according to the following equations.

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<tr>
<td>average (\Delta A/\text{min})</td>
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</table>

Tab.3

Calculation: \[\text{GGT (}\mu\text{kat/L}) = \Delta A/\text{min} \times 23.188 \times F \]

The catalytic concentration of GGT in the sample: ................. \(\mu\text{kat/L}\)

\(^3 F\) is a dilution factor. It determines how many times you diluted the sample.
2.4 Alkaline phosphatase

Alkaline phosphatase (alkaline phosphohydrolase of orthophosphoric monoesters, E.C.3.1.3.1., ALP) splits 4-nitrophenylphosphate into 4-nitrophenol and phosphate in N-methyl-D-glucamine buffer. ALP is activated by sodium chloride. The enzyme activity is measured by the amount of the liberated 4-nitrophenol which can be determined by the kinetic method at 420 nm.

![Chemical reaction diagram]

**Procedure:**

1. Pipette 1 mL of buffer (N-methyl-D-glucamine buffer) into the test tube and incubate this tube in water bath at 37 °C for exactly 5 min.
2. Then add 0.02 mL of serum into the tube.
3. Mix it well and preincubate the tube (with buffer and serum) at 37 °C for exactly 5 min.
   + Prepare the spectrophotometer – set the wavelength, measure the blank (distilled water).
4. Then add 0.2 mL of the ALP substrate.
5. Mix it well and immediately measure the initial absorbance against blank. Measure the absorbance change exactly after 1, 2 and 3 minutes.

Calculate 1 minute absorbance change (ΔA/ min) and calculate the catalytic concentration of ALP in the sample according to the following equations.

<table>
<thead>
<tr>
<th>Time [min]</th>
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<th>ΔA</th>
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<tr>
<td>average ΔA/min</td>
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</tbody>
</table>

Tab.4

Calculation: \[ \text{ALP (\(\mu\text{kat/L}\))} = \Delta A/\text{min} \times 72.796 \times F \]

The catalytic concentration of ALP in the sample: ............... \(\mu\text{kat/L}\)

\(^4\) F is a dilution factor. It determines how many times you diluted the sample.
Discussion:

1. Which other analytes would you include to biochemical tests of liver functionality?
2. Is there a more significant difference between alkaline and acid phosphates than the pH optimum?
3. Why, during growth period in childhood, the concentration of ALP increases?

Conclusion:

Physiological ranges of enzymes:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Range</th>
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<tbody>
<tr>
<td>ALP</td>
<td>0.66 – 2.2 µkat/L</td>
</tr>
<tr>
<td>AST</td>
<td>0.1 – 0.72 µkat/L</td>
</tr>
<tr>
<td>ALT</td>
<td>0.1 – 0.78 µkat/L</td>
</tr>
<tr>
<td>GGT</td>
<td>Women 0.14 – 0.68 µkat/L</td>
</tr>
<tr>
<td></td>
<td>Men    0.14 – 0.84 µkat/L</td>
</tr>
</tbody>
</table>