Instructions for the practical lesson on biochemistry

**Topic:** Biochemical examination of diabetes mellitus

**Task 1: Estimation of glycemia and OGTT**

**Reagents:**
Commercially available kit Bio-La-Test Glukosa Liquid 500 S made by PLIVA-Lachema, a.s. is employed for the analysis.

1. **Working solution OGTT:**
   - glucose oxidase $\geq 166.0 \, \mu$kat/l
   - peroxidase $\geq 16.0 \, \mu$kat/l
   - 3-methylphenol 10.0 mmol/l
   - 4-aminoantipyrine 1.0 mmol/l
   - phosphate buffer, pH 8 140.0 mmol/l

2. **OGTT Standard solution of glucose 10 mmol/l**
3. **OGTT serum 1:** fasting serum
   - OGTT serum 2: 60 minutes after glucose load
   - OGTT serum 3: 120 minutes after glucose load

**Procedure:**

<table>
<thead>
<tr>
<th>Volume in ml</th>
<th>Test-tube 1</th>
<th>Test-tube 2</th>
<th>Test-tube 3</th>
<th>Test-tube 4</th>
<th>Test-tube 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 1</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Serum 2</td>
<td>0.01</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Serum 3</td>
<td>–</td>
<td>0.01</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Standard</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.01</td>
<td>–</td>
</tr>
<tr>
<td>Distilled water</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Mix well all the test-tubes and incubate for 30 min at room temperature in dark.
Measure absorbances at 500 nm against blank within 30 minutes after the end of incubation.

**Calculation:**

$$\text{Serum glucose (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}}$$

$C_{\text{standard}} = 10 \, \text{mmol/l}$

**Evaluation:**

1. From the three data points obtained, draw a glycemic profile
2. Compare the fasting value and the 2-hour value with the physiological limits, and conclude whether your patient is healthy, displays an impaired glucose tolerance, or even diabetes mellitus.
Task 2: Estimation of glycated serum proteins (fructosamine)

Reagents:
1. Working solution for glycated proteins (‘reagent’):
   - Carbonate buffer, pH 10.3 – 10.4
     - $\text{Na}_2\text{CO}_3$ 75.0 mmol/l
     - $\text{NaHCO}_3$ 25.0 mmol/l
     - Nitroblue tetrazolium (NBT) 0.48 mmol/l
2. Standard solution of glycated protein 250 µmol/l
3. Bovine serum
4. Glucose 0.2 mmol/l in 100 mM carbonate buffer, pH 10.3 – 10.4
5. Sample of glycated proteins: 0.5 ml serum combined with 1 ml glucose solution, allowed to stand at least 5 days at room temperature

Procedure:
Concentration of glycated proteins in the serum sample freshly mixed with glucose will be compared to the same mixture of serum and glucose that has been incubated several days at room temperature.
1. Prepare the mixture of serum and glucose: combine 100 µl of glucose solution with 50 µl serum.
2. Pre-warm the photometric cuvettes to 37 °C. Then mix directly in the cuvettes:

<table>
<thead>
<tr>
<th>Measure in ml:</th>
<th>Serum freshly mixed with glucose</th>
<th>Serum glycated for several days</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum freshly mixed with glucose</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Serum glycated for several days</td>
<td>–</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>Standard</td>
<td>–</td>
<td>–</td>
<td>0.1</td>
</tr>
<tr>
<td>Reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mix and incubate exactly 10 minutes at 37 °C.
Measure absorbances (A1) at 530 nm against distilled water.

Incubate exactly further 10 minutes at 37 °C.
Measure again absorbances (A2) at 530 nm against distilled water

Calculation:

\[
\text{Glycated serum proteins (µmol/l)} = \frac{A_{2\text{serum}} - A_{1\text{serum}}}{A_{2\text{standard}} - A_{1\text{standard}}} \times C_{\text{standard}}
\]

\[
C_{\text{standard}} = 250 \text{ µmol/l}
\]
Task 3: Detection of glucose in urine

Reagents:

1. Fehling solution I: Copper(II) sulphate cryst. 70 g/l
2. Fehling solution II: Sodium hydroxide 250 g/l
   Potassium-sodium tartrate cryst. 350 g/l
3. Sulfosalicylic acid dihydrate 200 g/l
4. Test strips for urinary glucose (glukoPHAN or some of the polyfunctional strips made by PLIVA-Lachema a.s.)
5. Urine samples: Urine with glucose
   Urine with glucose and ascorbic acid
   Urine with fructose
   Physiological urine

Procedure:

Fehling test

a. Proteins in urine sample interfere with this test, therefore, it is advisable to perform the test with sulfosalicylic acid first. If protein is found, it should be removed by precipitation and filtration (add about 0.2 ml of acetate buffer pH 4.7 per 2 ml of urine, mix, boil 1 min. and filter).

b. Prepare a sufficient amount of fresh Fehling reagent shortly before use. In a beaker mix the Fehling solution I (copper sulfate) and II (NaK-tartrate with NaOH) in a ratio approximately 1:1. Heat a small portion (about 1 ml) of the Fehling reagent in a test tube to boiling – it must not change color. By this way a presence of contaminating reducing agents is excluded.

c. To about 1 ml of urine sample in a test tube add approximately the same volume of the Fehling reagent. Boil in water bath. If the glucose or another reducing compound is present, a green-yellow, yellow, or even a brick-red precipitate develops. The color depends on the amount of glucose in the urine sample (green ppt – about 25 mmol/l glucose, brownish-red ppt – about 100 mmol/l, red ppt – over 150 mmol/l).

Diagnostic strip test

Completely immerse the reagent pad in a urine specimen for 1 – 2 seconds. Wipe edge of the strip against rim of the test tube to remove excess urine. Hold the strip in horizontal position. Wait about 60 seconds and then compare the color of the test pad to the scale printed on the tube label. If urine contains ascorbic acid, the color development can be retarded.

Task:

Perform the Fehling test as well as the diagnostic strip test with all four urine samples.
Summarize all observations in the table in your lab report.

Evaluation:

Carefully consider all results, positive or negative, and try to explain them on the basis of theoretical information on the specificity of each test, as well as the possible causes of false positive or false negative results.
Task 4: Detection of ketone bodies in urine

Reagents:

1. Sodium nitroprusside crystalline
2. Sodium hydroxide 100 g/l
3. Glacial (concentrated) acetic acid
4. Lestradeť’s reagent: ammonium sulfate 20 g, sodium carbonate anhydrous 20 g, sodium nitroprusside 0.2 – 1 g.
5. KetoPHAN or some polyfunctional diagnostic strips made by PLIVA-Lachema, a.s.
6. Urine samples

Procedure:

Legal’s nitroprusside test:
Dissolve a small amount of solid sodium nitroprusside in a few ml of distilled water in a test tube. To about 2 ml of urine add 2 – 3 drops of the aqueous solution of sodium nitroprusside and alkalize with 3 drops of NaOH. A red color appears that is caused by creatinine (physiological component of urine). Divide the colored solution into two parts. Add a few drops of glacial acetic acid into one part of solution: if the color changes to yellow it was caused by creatinine. In contrast, in the presence of ketone bodies the red color turns to red-violet upon addition of the acetic acid.

Lestradeť’s test:
Place a circle of filter paper onto a watch glass and wet it with distilled water. Put a small amount of solid Lestradeť’s reagent on the filter paper and wet with 1 – 2 drops of urine. A purple color developing within 1 minute indicates presence of ketone bodies.

Diagnostic strip test:
Immerse the reagent pad into urine specimen for 1 – 2 seconds. Wipe edge of the strip against rim of the test tube to remove excess urine. Hold the strip in horizontal position. After about 60 seconds compare the test pad to the color scale on the tube label. The color scale is calibrated for the concentration of acetoacetic acid.

Task:
Perform the Legal test, Lestradeť test and the diagnostic strip test with both the urine containing ketone bodies and the physiological urine.
Summarize all results in the table in your lab report.

Task 5: Detection of glucose and ketone bodies in unknown sample of urine

Perform the tests for glucose and ketone bodies with the provided unknown sample of urine.