INSTITUTE OF MEDICAL BIOCHEMISTRY AND LABORATORY MEDICINE

Biochemical examination of diabetes mellitus

Practical in Medical Biochemistry

General Medicine

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Task 1: Estimation of glycemia and OGTT

Reagents:
Commercially available kit GLU 1000 BLT 00027 made by Erba-Lachema, a.s. is employed for the analysis.

1. Working solution OGTT:

glucose oxidase	≥ 166.0 µkat/l
peroxidase	≥ 16.0 µkat/l
3-methylphenol	10.0 mmol/l
4-aminoantipyrine	1.0 mmol/l
phosphate buffer, pH 8	140.0 mmol/l

2. 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:

Three samples from the same patient are available, taken during the oral glucose tolerance test (OGTT) in time 0, 60, and 120 minutes (marked as serum 1, 2, and 3, respectively).

Volume in ml:	Test-tube 1	Test-tube 2	Test-tube 3	Test-tube 4	Test-tube 5
	Serum 1 (0 min.)	Serum 2 (60 min.)	Serum 3 (120 min.)	Standard	Blank
Working solution	1.5	1.5	1.5	1.5	1.5
Serum 1 (0 min.)	0.01	–	–	–	–
Serum 2 (60 min.)	–	0.01	–	–	–
Serum 3 (120 min.)	–	–	0.01	–	–
Standard	–	–	–	0.01	–
Distilled water	–	–	–	–	0.01
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:

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

$C_{standard}$ = 10 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 ('NBT'):
   Carbonate buffer, pH 10.3 – 10.4
   Na_2CO_3 75.0 mmol/l
   $NaHCO_3$ 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

Principle:

Concentration of glycated proteins is compared in two samples:

- Serum sample freshly mixed with glucose
- Serum sample glycated with glucose - the same mixture of serum and glucose as above, which has been incubated several days at room temperature.

The estimation of concentration of glycated proteins is based on their reduction properties: they slowly reduce the nitroblue tetrazolium (NBT) to a colored product called formazan. The biological sample is allowed to react in two steps. First some of the NBT is quickly reduced by rapidly reacting reductants, such as glucose, ascorbate, etc., that are commonly present in a serum sample. Then the initial absorbance of the NBT reaction product is measured, and the rate of its further slow increase is determined. This slow increase mostly corresponds to the reduction of NBT by glycated proteins.

Procedure:

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:

Measure in ml:	Serum freshly mixed with glucose	Serum glycated for several days	Standard
Serum freshly mixed with glucose	0.1	–	–
Sample (serum glycated for several days)	–	0.1	–
Standard	–	–	0.1
NBT	1.0	1.0	1.0
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:

$$\text{Glycated serum proteins (µmol/l)} = \frac{A2_{serum} - A1_{serum}}{A2_{standard} - A1_{standard}} \times c_{standard}$$

$C_{standard}$ = 250 µmol/l

Task 3: Detection of glucose in urine

Reagents:

1. Fehling solution I: Copper(II) sulfate 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
   Unknown sample of urine

Procedure:

Fehling test

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).

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.

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 about one second. 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 each of the five urine samples.

Record not only which tests yield positive results, but also how quickly they develop. Summarize all observations in the table in your lab report.

Evaluation:

Carefully consider all the 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. Lestradet´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.

Lestradet´s test:

Place a circle of filter paper onto a watch glass and wet it with distilled water. Put a small amount of solid Lestradet´s reagent on the filter paper and wet with 1 – 2 drops of urine. A purple color that develops 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 positive result manifests as a color change from cream white to violet. The color scale is calibrated for the concentration of acetoacetic acid.

Task:

Perform the Legal test, Lestradet test and the diagnostic strip test with:

- Urine containing ketone bodies
- Physiological urine
- Unknown sample of urine

Summarize all results in the table in your lab report, and interpret them.

Task 5: Estimation of glycemia with personal glucometer

The estimation of glycemia with personal glucometer will be performed according to the instructions of your teacher.