INSTITUTE OF MEDICAL BIOCHEMISTRY AND LABORATORY MEDICINE

Diabetes mellitus and disorders of saccharide metabolism

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. Bovine serum
3. Glucose 0.2 mmol/l in 100 mM carbonate buffer, pH 10.3 – 10.4
4. 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 I (freshly mixed with glucose)
- Serum sample II (glycated with glucose for several days - 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 bovine serum and glucose (serum I): combine 100 µl of glucose solution with 50 µl of bovine serum. Serum II is already prepared.
2. Pre-warm the photometric cuvettes to 37 °C. Then mix directly in the cuvettes:

Measure in ml:	Serum I (freshly mixed with glucose)	Serum II (glycated for several days)
Serum I)freshly mixed with glucose)	0.1	–
Serum II (glycated for several days)	–	0.1
NBT	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		

The extent of glycation can be expressed as $\Delta A = A_2 - A_1$. Calculate ΔA for both serum samples and compare.

Task 3: Detection of glucose and fructose in urine

Reagents:

1. Benedict reagent:

   $CuSO_4 .5\ H_2O$ 17.3 g is dissolved in about 100 ml of distilled water.
   Na_2CO_3 100 g & sodium citrate 173 g is dissolved in about 700 ml of distilled water.
   Both solutions are combined and the volume is adjusted to 1 L with distilled water.

   $CuSO_4$ 

2. Selivanov reagent: *Solution of resorcinol 5 g/l in HCl 200 g/l* 

3. Test strips for urinary glucose (glukoPHAN or some of the polyfunctional strips made by PLIVA-Lachema a.s.)

Urine samples: Urine with glucose
Urine with glucose and ascorbic acid (AA)
Urine with fructose
Physiological urine
Two unknown samples of urine A and B

Procedure:

Benedict reaction:

	Test tube 1 Urine with glucose	Test tube 2 Urine with glucose +AA	Test tube 3 Urine with fructose	Test tube 4 Physiological urine	Test tube 5 Unknown sample
Benedict reagent	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Urine with glucose	cca 4 drops	-	-	-	-
Urine with glucose +AA	-	cca 4 drops	-	-	-
Urine with fructose	-	-	cca 4 drops	-	-
Physiological urine	-	-	-	cca 4 drops	-
Unknown sample	-	-	-	-	cca 4 drops
Shake briefly with the mixture in each tube and place the tubes to a boiling water bath. Read the result after 3-4 minutes. Evaluate both color change and occurrence of precipitate.					

Selivanov reaction:

	Test tube 1 Urine with glucose	Test tube 2 Urine with glucose +AA	Test tube 3 Urine with fructose	Test tube 4 Physiological urine	Test tube 5 Unknown sample
Urine with glucose	cca 0.5 ml	-	-	-	-
Urine with glucose +AA	-	cca 0.5 ml	-	-	-
Urine with fructose	-	-	cca 0.5 ml	-	-
Physiological urine	-	-	-	cca 0.5 ml	-
Unknown sample	-	-	-	-	cca 0.5 ml
Selivanov reagent	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml
Shake briefly with the mixture in each tube, place the tubes to a boiling water bath, and keep observing the color changes. Read the result after 1-2 minutes.					

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.

Tasks:

With all four known samples of urine and one unknown samples perform 1) Benedict test (for reducing substances), 2) Selivanov test (for ketoses) and 3) diagnostic strip test (for glucose).

Record not only which tests yield positive results, but also how quickly they develop. Summarize all observations in the table in your lab report. Compare the results obtained with various tests and try to explain the differences.

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.

Urine samples: Urine with ketone bodies
Physiological urine
Unknown sample of urine

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.

Tasks:

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.