ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK A VFN

Tetrapyrrols, liver, blood clotting. Cardiomarkers

Practical lesson in medical biochemistry

General Medicine

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Task 1: Estimation of total bilirubin in serum

Reagents:
(Commercial kit BioSystems Bilirubin (total) is used)

1. **Reagent AT**: Sulfanilic acid 29.0 mmol/l, HCl 0.2 mol/l, cetrimide[1] 50 mmol/l 
2. **Reagent BT**: Sodium nitrite 11.6 mmol/l 
3. **Working reagent**: 1 ml reagent BT + 4 ml reagent AT 
4. **Serum** – unknown sample (infectious material)

Procedure:

	Test tube No 1 Blank 1	Test tube No 2 Blank 2	Test tube No 3 Serum sample
Deionized water	**100 µl**	**-**	**-**
Serum	**-**	**100 µl**	**100 µl**
Reagent AT	**-**	**1 ml**	**-**
Working reagent	**1 ml**	**-**	**1 ml**
Mix contents of the tubes and allow to stand for 2 minutes at ambient temperature.			
Measure absorbance of the sample (tube No 3) and the Blank 2 (tube No 2) against Blank 1 (tube No 1) at the wavelength 540 nm.			

Evaluation and conclusion:

Subtract the absorbance value of Blank 2 from the absorbance of the sample (including Blank 2 in addition to usual Blank 1 corrects for the color of the serum itself).

Results of bilirubin measurement with several standards of known concentrations are provided (table on p. 4). Use these values for construction of a **calibration graph**; plot a straight line going through the origin. Then read the bilirubin concentration in the unknown sample from the calibration graph.

Alternatively, use the data to calculate the **calibration factor**:

f = concentration/absorbance

$$\text{Average calibration factor} = \frac{f1 + f2 + f3 + f4 + f5}{5}$$

S-Total bilirubin (µmol/l) = A_{sample} × average factor

In the conclusion, assess whether the measured values of total bilirubin is within reference limits.

[1] Tetradecyltrimethylammonium bromide, a quaternary ammonium antiseptic and surfactant (detergent).

Task 2: Estimation of direct bilirubin in serum

Reagents:
(Commercial kit BioSystems Bilirubin (direct) is used)

1. **Reagent AD**: Sulfanilic acid 35.0 mmol/l, HCl 0.24 mol/l 
2. **Reagent BD**: Sodium nitrite 3.5 mmol/l 
3. **Working reagent**: 1 ml reagent BD + 4 ml reagent AD 
4. **Serum** – unknown sample (infectious material)

Procedure:

	Test tube No 1 Blank 1	**Test tube No 2 Blank 2**	**Test tube No 3 Serum sample**
Deionized water	**100 µl**	**-**	**-**
Serum	**-**	**100 µl**	**100 µl**
Reagent AD	**-**	**1 ml**	**-**
Working reagent	**1 ml**	**-**	**1 ml**
Mix contents of the tubes and allow to stand for exactly 5 minutes in thermo block set to 37 °C.			
Measure absorbance of the sample (tube No 3) and the Blank 2 (tube No 2) against Blank 1 (tube No 1) at the wavelength 540 nm.			

Evaluation and conclusion:

Subtract the absorbance value of Blank 2 from the absorbance of the sample (including Blank 2 in addition to usual Blank 1 corrects for the color of the serum itself).

In order to convert the measured absorbances to concentration use again the provided results of bilirubin measurement with several standards of known concentrations (table on p. 4). Either construct a **calibration graph** for reading the concentration of the unknown sample, or use the data to calculate the **calibration factor**.

In the conclusion assess whether the measured value of direct bilirubin is within the reference limits. Then take into account the measurement of total bilirubin as well and **try to decide what type of icterus** (pre-hepatic, post-hepatic or hepatocellular) is found.

Data for construction of calibration graph or calculation of calibration factor for the estimation of total and direct bilirubin in serum

Standard No.	Bilirubin concentration (µmol/l)	Absorbance 540 nm
1	16	0.065
2	32	0.145
3	53	0.240
4	80	0.360
5	110	0.505

Task 3: Fluorescence and spectrophotometry of hematoporphyrin

Reagents:

1. Concentrated sulfuric acid 
   Take care when working with concentrated sulfuric acid!
   Use the plastic shield to protect your eyes!
2. Blood diluted 1:1 (infectious material)
3. Hematoporphyrin solution for spectrophotometry:  0.5 ml of diluted blood (1:9) mixed with 4.5 ml of sulfuric acid 0.5 mol/l.
4. Unknown samples of urine (infectious material)

Procedure:

a) Observation of fluorescence:

Concentrated sulfuric acid converts heme of hemoglobin into hematoporphyrin that displays an intense red fluorescence under UV light. Examine the provided solutions of hematoporphyrin under a UV lamp (366 nm). Compare with the sulfuric acid alone. Similar fluorescence can be seen with the urine samples if they contain porphyrins.

b) Spectrophotometry of porphyrins:

Hematoporphyrin solution (prepared by mixing diluted rat blood with sulfuric acid) is provided in the closed cuvettes at the diode array spectrophotometers. Measure spectrum of this solution in the wavelength range 350-500 nm against diluted sulfuric acid (also prepared at the photometer), and record all the spectral maxims. Do the same also with two provided unknown samples of urine.

Task 4: Estimation of γ-glutamyl transferase (GGT) in serum

Reagents:

Commercial kit Gamma-Glutamyltransferase made by BioSystems S.A. Barcelona is used.

1. **Working solution:** L-γ-glutamyl-3-carboxy-4-nitroanilide 6.5 mmol/l in buffer consisting of glycyl-glycine 165 mmol/l and sodium hydroxide 104 mmol/l pH 7.9
2. **Serum** – unknown sample

Procedure:

The working solution and the cuvette for the measurement, must be pre-warmed for at least 5 minutes at 37 ˚C. Then proceed according to the table:

Measure directly to the cuvette:	
Working solution	1.0 ml
Serum	0.1 ml

Mix, incubate for about 30 sec. at 37 °C, and immediately measure the initial absorbance (A_0) at 405 nm against **solution of potassium dichromate**. Then keep reading the absorbance in (exactly) one-minute intervals for 3 minutes.

N.B.: Once the serum is added, the reaction is running and the measurement must be really started immediately after the 30 sec. incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{405}
0	A_0	
1 minute	A_1	$A_1 - A_0 \rightarrow \Delta A_1$ .....
2 minutes	A_2	$A_2 - A_1 \rightarrow \Delta A_2$ .....
3 minutes	A_3	$A_3 - A_2 \rightarrow \Delta A_3$ .....

$$\Delta A_{405}/\text{min.} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3}{3}$$

Next, catalytic activity of GGT is calculated using the molar absorption coefficient for the colored product 5-amino-2-nitrobenzoic acid:

$$\text{GGT } (\mu\text{kat/l}) = \Delta A_{405}/\text{min.} \times 18.52$$

Task 5: Examination of basic blood clotting parameters with coagulometer

Will be demonstrated by your instructor.

Task 6: POCT examination of troponin

Will be demonstrated by your instructor.