Date ..... Name ..... Group .....

Instructions for the practical lesson on biochemistry

Topic: **Bile pigments, porphyrins**

Task 1: Diazotation and azo coupling

Reagents:

1. Sulfanilic acid (Diazo I reagent) 5 g/100 ml 0.6 mol/l HCl 
2. Sodium nitrite (Diazo II reagent) 5 g/100 ml deionized water 
3. Sodium carbonate 10 g/100 ml deionized water
4. β-naphthol 2 g/100 ml ethanol 
5. Tyrosine 0.2 g/100 ml 0.1 mol/l HCl 

Procedure:

1) Take about 1 ml of sulfanilic acid (Diazo I) and add about 5 drops of sodium nitrite (Diazo II). Then add solution of β-naphthol drop wise. A red-orange color of the azo dye Orange II should develop.
2) To about 1 ml of sulfanilic acid (Diazo I) add about 5 drops of sodium nitrite (Diazo II) and mix. Add about 0.5 ml of sodium carbonate drop wise in order to make the solution weakly acidic/neutral. To this mixture add some drops of tyrosine solution to produce another red azo dye. If the color does not appear, add carefully more sodium carbonate.

Task 2: 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 3: 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 4: Fluorescence 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.

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.