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

Instructions for the practical lesson on biochemistry

Topic: Calcium, phosphorus, sodium, metabolism of bone tissue

Task 1: Estimation of total calcium in serum and urine

Reagents:

Commercial kit Bio-La-Test CALCIUM Liquid 250, made by Erba-Lachema s.r.o.

1. **Working solution:** MES buffer pH 6.5 100 mmol/l, arsenazo III 200 µmol/l
2. **Standard solution of calcium 2.5 mmol/l**
3. **Serum** – unknown sample (infectious material)
4. **Urine** – unknown sample (infectious material) **diluted 4×**

Procedure:

N.B.: Ultrapure deionized water must be used for this experiment both for the blank and for rinsing the cuvettes in between measurements.

Measure reagents to **plastic (!) single-use test tubes** according to the following table:

Measure in ml:	Serum sample (Tube No. 1)	Urine sample (Tube No. 2)	Standard (Tube No. 3)	Blank (Tube No. 4)
Working solution	1.0	1.0	1.0	1.0
Serum	0.01	-	-	-
Urine	-	0.01	-	-
Standard solution	-	-	0.01	-
Ultrapure water	-	-	-	0.01
Mix and incubate 1 minute at 37 °C. Measure the absorbances of serum, urine and standard against the blank at 650 nm in new 1 cm plastic cuvette. The coloration is stable for one hour.				

Calculations:

Concentration of total calcium in the serum (S-Ca):

$$\text{S-Calcium (mmol/l)} = \frac{A_{serum}}{A_{standard}} \times c_{standard}$$

Concentration of calcium in the urine (U-Calcium):

$$\text{U- Calcium (mmol/l)} = \frac{A_{urine}}{A_{standard}} \times c_{standard} \times \text{Dilution of urine}$$

Daily output of calcium into urine (dU-Calcium):

dU-Calcium (mmol/24 hrs) = U-Calcium (mmol/l) × Volume of urine (liters/24 hrs)

Task 2: Estimation of inorganic phosphate in serum and urine

Reagents:

Commercial kit Bio-La-Test PHOSPHORUS UV Liquid 250, made by Erba-Lachema s.r.o.

1. **Working solution:** Sulfuric acid 210 mmol/l, ammonium molybdate 650 mmol/l
1. **Standard solution of phosphate 1.61 mmol/l**
2. **Serum** – unknown sample (infectious material)
3. **Urine** – unknown sample (infectious material) **diluted 25×**

Procedure:

N.B.: Ultrapure deionized water must be used for this experiment both for the blank and for rinsing the cuvettes in between measurements.

Measure reagents to **plastic (!) single-use test tubes** according to the following table:

Measure in ml:	Serum sample (Tube No. 1)	Urine sample (Tube No. 2)	Standard (Tube No. 3)	Blank (Tube No. 4)
Working solution	1.0	1.0	1.0	1.0
Serum	0.01	-	-	-
Urine (diluted)	-	0.01	-	-
Standard solution	-	-	0.01	-
Ultrapure water	-	-	-	0.01
Mix and incubate 5 minutes at 37 °C. Measure the absorbances of serum, urine and standard against the blank at 340 nm in new 1 cm plastic cuvette. The coloration is stable for one hour.				

Calculations:

Concentration of inorganic phosphate in serum (fS-P inorg.):

$$\text{fS-Inorganic phosphate (mmol/l)} = \frac{A_{serum}}{A_{standard}} \times c_{standard}$$

Concentration of inorganic phosphate in urine (U-P inorg.):

$$\text{U- Inorganic phosphate (mmol/l)} = \frac{A_{urine}}{A_{standard}} \times c_{standard} \times \text{Dilution of urine}$$

Daily output of inorganic phosphate into urine (dU-P):

dU-Inorg. phosphate (mmol/24 hrs) = U-Inorg. phosphate (mmol/l) × Volume of urine (liters/24 hrs)

Task 3: Estimation of catalytic concentration of alkaline phosphatase and its isoforms

Reagents:

Commercial kit Bio-La-Test Alkalická fosfatáza 120 manufactured by Erba-Lachema s.r.o.. *is used.*

1. **Buffer:** N-methyl-D-glucamine pH 10.1, 0.35 mol/l, NaCl 70.0 mmol/l, $MgCl_2$ 0.5 mmol/l
2. **Substrate:** 4-nitrophenyl phosphate disodium salt 15 mmol/l in the buffer described above
   (Concentrations in the final reaction mixture)

3. **Inhibitor:** Sodium hydroxide 1 mol/l, Chelaton 3 30 mmol/l
4. **Serum** – unknown sample (infectious material)

Procedure:

Two serum aliquots are prepared for this experiment: 'S' (native) and 'T'(subjected to heat inactivation at 56 ˚C for 10 minutes).

Proceed according to the table:

Measure in ml:	Native serum sample (S1)	Control for native sample (S2)	Heat-inactivated sample (T1)	Control for heat-inactivated (T2)
Buffer	1.0	1.0	1.0	1.0
Native serum (S)	0.02	-	-	-
Heat-inactivated serum (T)	-	-	0.02	-
Mix and incubate 5 minutes at 37 ˚C				
Substrate	0.2	0.2	0.2	0.2
Mix and incubate exactly 10 minutes at 37 ˚C				
Inhibitor	0.5	0.5	0.5	0.5
Native serum (S)	-	0.02	-	-
Heat-inactivated serum (T)	-	-	-	0.02
Mix and within 30 minutes measure the absorbances of S1, S2, T1, and T2 against deionized water at 420 nm in 1 cm cuvette.				

Calculations:

Subtraction of control absorbances:

$\Delta A_S = A_{S1} - A_{S2}$ $\Delta A_T = A_{T1} - A_{T2}$

Total catalytic concentration of ALP:

Calculation is based on the molar absorption coefficient of 4-nitrophenol.

Total ALP (μkat/l) $= \Delta A_S \times 10.263$

Catalytic concentration of the liver isoenzyme:

Calculation assumes that 100 % of the bone and one third of the liver isoenzyme have been inactivated by heat.

Liver isoenzyme ALP (μkat/l) = $1.5 \times \Delta A_T \times 10.263$

Catalytic concentration of the bone isoenzyme:

Bone isoenzyme ALP (μkat/l) = Total ALP (μkat/l) − Liver isoenzyme ALP (μkat/l)

Task 4 Solubility of various calcium salts

Reagents:

1. **Calcium chloride**, $CaCl_2$, solid substance
2. **Calcium carbonate**, $CaCO_3$, solid substance
3. **Calcium phosphate**, $Ca_2(PO_4)_3$, solid substance
4. **Ammonium oxalate**, $(NH_4)_2(COO)_2$, saturated solution 
5. **Hydrochloric acid**, HCl, diluted 
6. **Sodium hydrogen carbonate**, $NaHCO_3$, 10 g/l
7. **Na_2EDTA**, saturated solution
8. **Lactose**, 10 g/l

A. Solubility of calcium salts in water and HCl

Procedure:

a. Take four short glass test tubes and pour a pinch of solid calcium chloride (tube No. 1), calcium carbonate (tube No. 2) or calcium phosphate (tubes No. 3 and 4).

b. Add about 1.5 ml of deionized water to each tube.

c. Mix and observe whether the different calcium salts dissolve in water.

d. To the tubes No. 2, 3, and 4 try to add diluted hydrochloric acid drop wise until the precipitate just dissolves; record the number of drops to the table in your report.

e. Next, to the tube No. 3 containing dissolved $Ca_3(PO_4)_2$ add sodium hydrogen carbonate solution drop wise and observe formation of a precipitate.

f. To the tube No. 4 add about 8 drops of saturated Na_2EDTA solution followed by the sodium hydrogen carbonate solution (the same number of drops as to the tube No. 3). Na_2EDTA prevents precipitation.

g. Summarize all your observations to the table in your report.

B. Influence of some food components on solubility of calcium salts

Procedure:

Prepare two mixtures according to the table and observe whether a precipitate of calcium salt results.

	Tube No. 1	Tube No. 2
$CaCl_2$	1 measure	1 measure
Deionized water	1 ml	1 ml
Ammonium oxalate	cca 5 drops	-
Lactose	-	cca 5 drops

Task 5 Quantitative estimation of Na^+ in urine

Reagents:

1. **Dilution buffer**: 1.98 g of acetic acid 100 % and 4.09 g of ethanolamine in 1,000 ml, pH alkaline.
2. **Standard NaCl solutions** 0.1 mol/l, 0.01 mol/l, and 0.001 mol/l in the dilution buffer.
3. **Urine sample** of unknown Na^+ concentration, **diluted 10×** with the dilution buffer.

Procedure:

a. Dilute the urine sample 10× with the buffer: mix 5 ml of sample with 45 ml of buffer.

b. Follow the provided instructions for use of pH-meter as a mV-meter and measure the electrode potentials in the calibration solutions of NaCl 1 – 3 as well as in the diluted urine sample. Do not rinse the electrode with distilled water in between measurements; wipe it gently (avoid rubbing!) instead. Record all measured values of E (mV) into table in your report.

c. Create a calibration graph from the measured values of the electrode potentials and the corresponding pNa values of the standard solutions. Plot the pNa values on the x axis and the potential in mV on the y axis. Next, use the calibration graph to read the pNa for the analyzed urine sample.

d. Convert the pNa value of diluted urine sample into Na^+ concentration:

$$\mathbf{pNa = -\log[Na^+]} \qquad \mathbf{[Na^+]_{diluted\ urine} = 10^{-pNa}}$$

e. Correct for dilution of urine:

$$\mathbf{U\text{-}Na^+ = 10 \times [Na^+]_{diluted\ urine}}$$

Further calculations:

Daily output of Na^+ into urine:

dU- Na^+ = U-Na^+ (mol/l) × Vol. urine (liters/24 hrs)

Fractional excretion (FE) of Na^+:

$$FE_{Na} = \frac{U_{Na} \times P_{Cr}}{U_{Cr} \times P_{Na}}$$

U_{Cr} Creatinine in urine (mmol/l)

P_{Cr} Creatinine in serum (mmol/l)

U_{Na} Sodium in urine (mmol/l)

P_{Na} Sodium in serum (mmol/l)

Tubular resorption (TR) of Na^+:

$TR_{Na} = 1 - FE_{Na}$