2 INVESTIGATION OF HARD TISSUE METABOLISM

Topics to be reviewed:
Bone and teeth tissue composition. Bone remodelling. Mineralization and demineralization process. Calcium and phosphorus metabolism. ALP and its isoenzymes. Bone markers.

Pre-lab questions

1. Describe the forms of calcium in blood plasma.
2. What is the purpose of thermal serum inactivation in ALP assay?
3. Describe significance of determination of bone ALP isoenzyme
4. Describe ALP isoenzymes physiologically occurring in plasma in adults and children/adolescents.
5. Describe forms of phosphate in blood plasma.

Introduction

Bones and teeth are hard tissues. Bones consist of mineral components, predominantly biological apatite (hydroxyapatite with contaminants), and of an organic part comprising collagen I and other non-collagen proteins (*e.g.* osteocalcin). Teeth are formed by three different hard tissues (enamel, dentin, and cement). While all contain the organic component, they differ in the amount of mineral component (96 % in enamel). Bone tissue is metabolically active, it can undergo a process of bone remodelling, in which the mature bone tissue is resorbed (predominate activity of osteoclasts) and new bone tissue is formed (predominate activity of osteoblasts). Mineralization and demineralization process has basic significance for teeth tissue. The essential role in this process is performed by saliva that serve as a reservoir of ions (calcium ions, phosphate, fluoride) for remineralizaton process.

Basic investigation of the hard tissue metabolism involves serum calcium and phosphate determination, as their metabolisms are tightly associated. Biochemical markers of bone formation include bone ALP isoenzyme (bALP), osteocalcin and procollagen I N-terminal propeptide (PICP); bone resorption markers include collagen I C-terminal telopeptide (ICTP), bone acid phosphatise (bACP) or urinary free deoxypyridinoline.

2.1 Determination of serum calcium

Majority of calcium (99 %) is contained in hard tissues – bones and teeth, the remaining 1 % circulates in blood. There are three forms of Ca^{2+} in blood plasma. Approximately 50 % of total calcium exists as ionized (freely diffusible), 45 % are protein-bound, and the remainder is coordinately bound with organic ligands such as citrates, malates, oxalates etc. Only free ionized Ca^{2+} ions exhibit biological activity and regulatory effects.

Total serum calcium is determined by spectrophotometry or atomic absorption spectrometry. Ionized calcium (biologically active form of calcium) is determined by potentiometry using an ion-selective electrode.

Spectrophotometric determination principle: Calcium ions react with specific reagent (known as Arsenazo III) to form purple-colored complex. The absorbance of the complex is then proportional to serum calcium concentration.

Materials: Kit Calcium Liquid 250 (Erba Lachema*) containing: *Reagent-calcium* (containing MES buffer 100 mmol/L; Arsenazo III 2,7-bis(2-arsonophenylazo)-1,8-dihydroxynaphtaline-3,6-disulfonic acid 200 μmol/L), *calibrator-calcium* (2.5 mmol/L), blood serum specimens. Micropipettors 10 μL and 1000 μL, water bath at 37 °C, spectrophotometer Spekol 1300 and software WinAspect, or spectrophotometer Helios Delta and software VisionLite Fixed. *Alternatively, a kit of BioVendor or Roche Diagnostics can be used; composition of reagents may then be different.

Procedure

☞ Nonhaemolytic blood serum without any other treatment is used for determination. Use clean labelled test tubes and pipette the reagents as described in the following table:

Reagents (μL)	**Blank**	**Test**	**Standard**
Reagent-calcium	1 000	1 000	1 000
Demi-water	20	-	-
Serum	-	20	-
Calibrator-calcium	-	-	20
Shake gently and incubate for 5 minutes at room temperature (or 1 minute at 37 °C)			
Measure absorbance* of the sample (A_x) and the standard (A_{STD}) at 650 nm against the blank within 60 minutes.			

* Measure absorbance according to enclosed instructions to the spectrophotometer and use relevant software.

Calculation of serum calcium concentration (mmol/L): $c_x = \frac{A_x}{A_{STD}} \times c_{STD}$

where c_{STD} represents concentration of calcium in calibrator (2.5 mmol/L).

Evaluation

Reference interval for total calcium concentration in serum of adults: 2.00 – 2.75 mmol/L.

Hypercalcemia may outcome from hyperparathyroidism and increased mobilization of calcium from bones. Increased levels of calcium are found at malignant diseases, such as metastasizing tumours or multiple myeloma. Overdosing on vitamin D may result in increased calcium concentration as well. Values over 3.5 – 4.0 mmol/L are considered as life threatening.

Hypocalcemia is typically caused by a lack of vitamin D. It might be also associated with hypoparathyroidism. Serious hypocalcemia leads to tetany and seizures.

Importance of total calcium determination lies in the correct diagnosis of hyper- or hypocalcemia. From the clinical point of view, determination of ionized calcium yields a more precise and thus valuable piece of information especially in severe cases.

2.2 Determination of catalytic concentration of serum ALP and its bone isoenzyme

Alkaline phosphatase (ALP) is a hydrolase responsible for cleaving phosphate from various phosphate esters, provided that Mg^{2+} ions and alkaline pH are present. It can be found in various tissues and organs, and has wide substrate specificity. There are four genes for ALP. The tissue non-specific gene produces ALP which is located in liver, bones, and kidneys. The other three genes are responsible for expression of intestinal, placental, and embryonic ALP isoenzymes. Catalytic activity of ALP in serum is commonly determined when considering bone or hepatobiliary diseases.

The catalytic activity of total ALP in serum is a sum of liver and bone isoenzyme. As the bone isoenzyme can be easily inactivated by temperature (~56 °C), the difference in activity of serum ALP before and after inactivation determines the catalytic activity of bone isoenzyme.

Principle of determination: In alkaline pH, ALP cleaves phosphate from 4-nitrophenyl phosphate resulting in 4-nitrophenol and free phosphate. The catalytic activity is then proportional to the amount of 4-nitrophenol which is, after stopping the reaction by addition of an inhibitor, measured by spectrophotometry at 420 nm.

Material: Kit ALP 120 (Erba Lachema) containing: *Buffer* (*N*-methylglucamine pH 10.2; 427 mmol/L), *calibrator* (2.4 mmol/L 4-nitrophenol), *substrate* (4-nitrophenylphosphate disodium salt 91.5 mmol/L), *inhibitor* (1 mol/L NaOH, 3.3 mmol/L $EDTA.Na_2$), serum specimens. Pipettors 20, 200, 500 and 1 000 μL, water bath at 37 °C, spectrophotometer Spekol 1300 and software WinAspect, or spectrophotometer Helios Delta and software VisionLite Fixed.

Procedure

☞ Serum samples preparation: 1 mL of serum is halved to two aliquots. One 0.5 mL-aliquot becomes inactivated by incubating in a water bath at 56 °C for 15 minutes. Both samples have been already prepared and are labelled as follows.

Serum 1 = before inactivation

Serum 2 = after inactivation

☞ Proceed as described in the following table.

Reagencie (µl)	**Sample 1**	**Sample 2**	**Standard**
Buffer	1 000	1 000	1 000
Serum 1	20	-	-
Serum 2	-	20	-
Calibrator	-	-	20
Shake gently and preincubate for 5 minutes in water bath at 37 °C.			
Substrate	200	200	-
Demi-water	-	-	200
Shake gently and incubate for exactly 10 minutes in water bath at 37 °C.			
Inhibitor	500	500	500
Shake well and measure absorbances* of both samples and standard at 420 nm against demi-water within 30 minutes.			

* Measure absorbance according to enclosed instructions to spectrophotometer, use relevant software.

Calculation of catalytic concentration of ALP:

The concetration of 4-nitrophenol in calibrator (2400 µmol/L) at 10 min incubation (600 s) corresponds to ALP catalytic concentration (2400/600) 4 µkat/l. The ratio of absorbances of test and standard corresponds to the ratio of catalytic concentrations.

$$\text{ALP (µkat/L)} = \frac{A_x}{A_{\text{STD}}} \times 4$$

☞ Calculate the catalytic concentration of total ALP and its bone isoenzyme.

Catalytic concentration of bone ALP isoenzyme can be calculated as the difference of catalytic concentration of ALP in the serum before inactivation (Serum 1) and the serum after inactivation (Serum 2).

Evaluation

Reference values of catalytic concentration of serum ALP range from 0.7 – 2.2 μkat/L in adults; levels for children are below 8 μkat/L (indicating increased osteoblastic activity as bone growth occurs).

Increased ALP values are indicative for bone diseases (rickets, bone tumours, bone metastasis, Paget's disease) and for liver or bile duct diseases. Decreased levels of ALP can be observed in children with bone growth impairment.

ALP bone isoenzyme reference values are 0.1 – 1.6 μkat/L in adults. The bone isoenzyme has been considered to be a better marker of bone formation than the total serum ALP.

🕮 Inorganic phosphate and significance its determination in serum

Majority (80 %) of phosphate is contained in bones; about 1 % is in extracellular fluid. There are two phosphate fractions in plasma: **inorganic phosphate** consists of a mixture of HPO_4^{2-} and $H_2PO_4^-$, while **organic phosphate** is mostly found in phospholipids.

Reference interval for inorganic phosphate in serum of healthy adults: 0.84 – 1.45 mmol/L. Serum phosphate levels in children are physiologically increased because active bone growth occurs.

Low phosphate levels, known as hypophosphatemia, are indicative of hypovitaminosis D or hyperparathyroidism. High blood phosphate levels, hyperphosphatemia, could be indicative of severe kidney disease, bone disease, hypoparathyroidism or could be caused by an excessive uptake of phosphate-rich food supplements.

🕮 ALP isoenzymes

Different physical and chemical properties of ALP isoenzymes are used when distinguishing ALP isoenzymes. The most commonly employed method is electrophoresis; however, immunochemistry or thermal inactivation might be used as well (see task 2.2).

The bone and liver isoenzymes are usually the only isoenzymes found (cca 50/50 ratio) in the serum of healthy subjects. Intestinal isoenzymes are either absent or below 10 %. Placental isoenzymes are observed in gravidity; their elevated levels outside of pregnancy may indicate various malignant diseases (*e.g.* ovarian, pancreatic or gastric cancer, sarcoma). Typically, the bone isoenzyme is dominant in serum of children and adolescents at physiological conditions.

Whereas hepatobiliary diseases (*e.g.* cholestasis, viral hepatitis) cause elevated levels of liver isoenzymes, elevated values of the bone isoenzyme are indicative for rickets, bone tumours, bone metastasis, Paget's disease, *etc.*