UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

LABORATORY PRACTICALS IN CLINICAL CHEMISTRY

TEXTBOOK AND LAB DIARY

Uniform master's study programme Pharmacy

Year 4

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Irena Prodan Žitnik, Lara Smrdel, Alenka Šmid, Dunja Urbančič

Ljubljana, 2025

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Assist. Dr. Tijana Markovic, MPharm

Assist. Dr. Irena Prodan Žitnik, MPharm, EuSPLM

SAFETY AND ETHICS IN CLINICAL LABORATORY

PERSONAL PROTECTION EQUIPMENT

When working in the laboratory, the use of a laboratory coat is mandatory. We always use protective gloves when working with biological samples. When working with dangerous substances, always use safety glasses.

LABORATORY RULES

• Jackets and bags should be stored in the cupboards/drawers intended for this purpose.

• Food and drinks are not allowed in the laboratory.

• Always use a fume cupboard when working with dangerous substances.

• Clean up the work surface properly after completing laboratory work.

When working in the laboratory, it is necessary to follow the rules, as this ensures our own safety and the safety of everyone who works in the laboratory. It is important that students follow the instructions of the teaching assistant. In order to ensure safety, the assistant can prevent a student who does not follow the rules from further performing laboratory practical work.

PICTOGRAMS

A hazard pictogram is an image on a label that includes a warning symbol in specific colors to provide information about the harm that a certain substance or mixture may cause to our health or the environment (Figure 1).

Figure 1. *Reagents marked with these pictograms can be dangerous if not handled properly. To ensure safe use, remember the meaning of the labels and read the instructions. Source: https://echa.europa.eu/sl/regulations/clp/clp-quiz; 6.7.2022*

HAZARD (H) AND PRECAUTIONARY (P) STATEMENTS

The risk (R) and safety (S) statements were replaced by hazard (H) statements and precautionary (P) statements in the course of harmonising classification, labelling and packaging of chemicals. Always check H and P statements to ensure the safety while working in the laboratory.

HANDLING AND ETHICS IN THE LABORATORY WITH BIOLOGICAL SAMPLES AND WITH ANALYSIS´ RESULTS

Laboratory medicine experts are constantly in indirect contact with patients - from receiving their biological samples and personal data to obtaining and reporting results. This raises many questions regarding an expert's ethics, attitude, and actions. The principles of ethical behavior, respect for the patient and biological material, and confidentiality of test results, etc., are discussed in The Deontology Codex in Laboratory Medicine.

In the laboratory practicals, you will work with biological samples from patients or use your own sample for testing. In this case, you will be asked to sign a consent form prior to sample collection and analysis. You are free to decide whether to participate in the analysis or not. In either case, your decision will not affect the results or grade of the laboratory practical.

PIPETTING

PIPETTES

Pipette is a laboratory tool, which is used for more precise dosing of solutions. Pipettes come in several designs for various purposes, the main types are: filling pipettes, measuring pipettes and burettes. Presentation of pipette parts can be seen in Figure 2. For pipetting, we use pipettes that differ from each other depending on the volume that we want to pipet. First, we need to choose the right pipette that corresponds to the volume that we want to pipette. Each pipette has a defined volume range and can only be used for pipetting within the defined measuring range.

Common measuring ranges of pipettes are:

- 0,5 µL – 10 µL
- 0,5 µL – 20 µL
- 10 µL- 100 µL
- 20 µL – 200 µL
- 100 µL – 1000 µL
- 1 ml -5 ml

The appropriate volume on the pipette is set by moving the volume adjustment button until the desired volume appears in the window. Be careful not to set the volume outside the measuring range of the pipette.

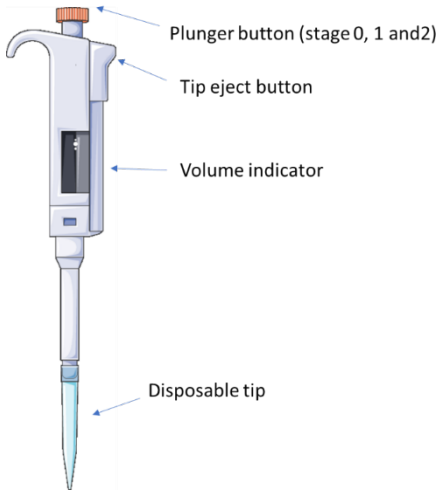

Figure 2. *Schematic representation of the pipette. The scheme was prepared using SMART - Servier Medical ART 3000.*

DISPOSABLE PIPETTE TIPS

Disposable tips differ from each other in terms of the volume that can be pipetted. We need to choose the right tip for the pipette as well as check that the tip fits well to the pipette. The tip is always replaced when another liquid is pipetted to prevent contamination of reagents, samples. After pipetting, the tip is thrown into the biological waste bin by pressing the tip removal button.

PIPETTING PROCEDURE

Before starting pipetting (Figure 3), select the appropriate pipette according to the volume to be pipetted and set the appropriate volume. According to the position of the button, the pipette has 3 stages (neutral 0, stage 1 and stage 2). First, test the button for all 3 stages on the pipette to get a feeling how the selected pipette works. The button is at first in neutral (0) stage. By pressing gently on the button, the button moves to the stage 1. When we press the button to the end, the button moves to the stage 2. Next, select the appropriate pipette tip and place it on the pipette. Make sure that the pipette tip fits well. Next, place the pipette in a vertical position above the liquid and slowly press the button to level 1. Then dip the pipette into the solution so that the tip of the pipette tip is a few mm below the surface of the liquid. The liquid volume is captured by slowly lowering the button to the neutral position (i.e. from level 1 to level 0) and waiting for 1 second. Before releasing the liquid, lean the pipette tip against the wall of the container at an angle of 20°-45°. When the pipette is at an angle, slowly press the button to level 2 and wait 1 second to transfer the entire volume of liquid. After pipetting, discard the pipette tip in the bin for biological waste by pressing the button to remove the pipette tip.

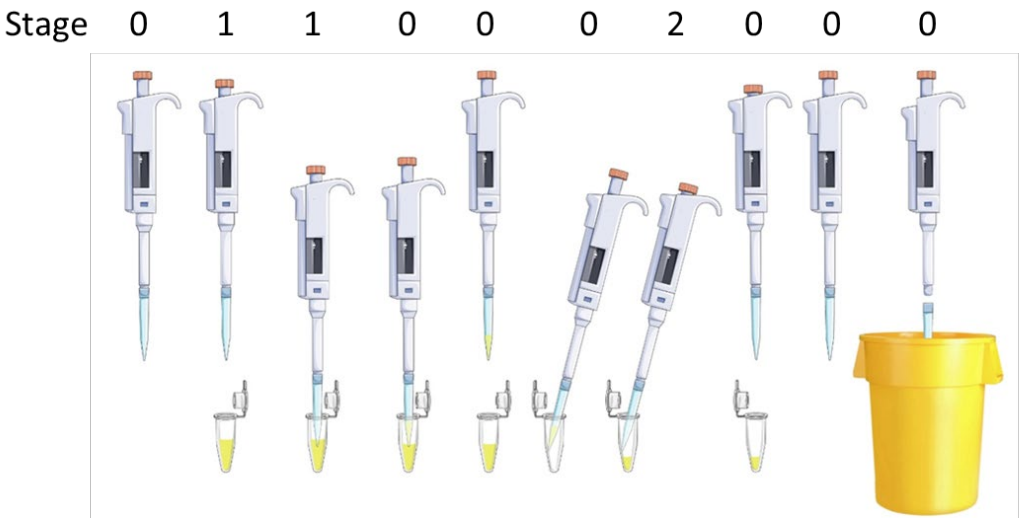

Figure 3. *Schematic representation of the pipetting procedure. The scheme was prepared using SMART - Servier Medical ART 3000.*

IMPORTANT THINGS TO PAY ATTENTION TO WHEN PIPPETING

When pipetting, always check the measuring range of the pipette and make sure that the pipette is never set outside the measuring range, otherwise recalibration of the pipette is necessary. Before pipetting, get familiar with the selected pipette by testing the stages on the pipette (0, 1, 2) by pressing the button. Before each pipetting, put a new tip on the pipette to avoid contamination of reagents and samples. Before dipping the pipette into the solution, always make sure that the button is pressed to level 1. It is important not to dip the pipette too deep into the solution, since it can lead to capturing too large volume due to the capillary effect. On the other hand, if the pipette tip is not immersed deep enough, bubbles can be trapped. Therefore, we dip the pipette tip to an appropriate depth into the solution, which ensures that at the end of pipetting tip is always immersed into the solution and that air would not be drawn into the pipette instead of the solution due to the lowering of the level of the solution during pipetting. It is also important that the pipette is in a vertical position when capturing the liquid, otherwise the wrong volume may be captured. When pipetting, press the button slowly and then wait 1 second so that no bubbles or liquid spills occur. When dispensing, we must keep the pipette against the wall at an angle of 20°-45° to ensure optimal liquid flow.

COMMON PIPETTING MISTAKES

- Incorrectly set volume – e.g. we want to pipette 100 µL of the solution, and we chose a pipette that has a measurement range of 2 µL -20 µL. We set the volume on the pipette to 10.0 (µL) - the pipetted volume will be 10 times too low.
- Incorrectly placed pipette tip - if the pipette tip is not firmly attached, it may fall from the pipette or the wrong volume may be pipetted.
- Intrusion of liquid into the pipette - if the pipette is not held downwards.
- Capturing the bubbles – this can occur if we drop the button from level 1 to the neutral position too quickly. The pipetted volume will be incorrect.
- Pipetting incorrect volume - if we press the button to level 2 instead of level 1 before capturing the solution. The pipetted volume of solution will be incorrect (too high).

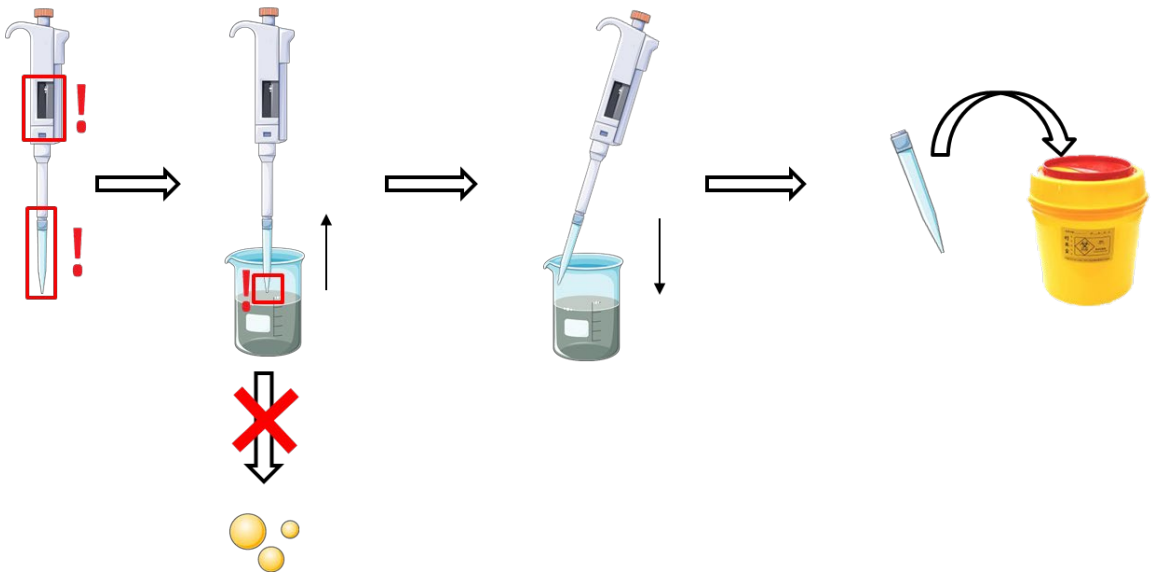

Figure 4. *Schematic representation of individual procedures that we must pay attention to when pipetting. The scheme was prepared using SMART - Servier Medical ART 3000.*

REVERSE PIPETTING

Reverse pipetting is used for viscous liquids as well as to prevent the formation of bubbles.

Reverse pipetting procedure:

1. Set the button on the pipette to stage 2, immerse the pipette tip into the solution and slowly move the button to the neutral position 0.
2. Put the pipette tip at an angle of 20°-45° against the wall of the container into which you will pipette the solution.
3. Lower the button to position 1 and use this to measure the volume of liquid. It is important to realize that with the reverse pipetting technique, some liquid will always remain in the pipette tip at this point.
4. At the end, press the button to level 2 against the wall of the waist container and throw away the rest of the liquid from the pipette tip.

DO YOU WANT TO LEARN MORE?

Watch the following video lectures that demonstrate the correct pipetting technique and point out common pipetting mistakes.

Pipetting technique

https://www.youtube.com/watch?v=QGX490kuKjg

https://www.youtube.com/watch?v=OOQWsCMQuRw

https://www.youtube.com/watch?v=nPjt1ZUNkFQ

Pipetts

https://www.youtube.com/watch?v=wlO4zJLR8R8

Common pipetting mistakes

https://www.youtube.com/watch?v=05oolv_y4Pk

Reverse pipetting

https://www.youtube.com/watch?v=1Y0U9jf5ZbI

TEST YOUR KNOWLEDGE

1. List the personal protective equipment in the laboratory.
2. Test your knowledge of pictograms in the following quiz: https://echa.europa.eu/regulations/clp/clp-quiz
3. Describe the pipetting procedure.
4. Which pipette (measuring range) would you choose for pipetting the following volumes: 0,5 µL; 10,5 µL; 20 µL; 135 µL; 200 µL; 1000,5 µL, 4,5ml?
5. Schematically show the pipetting procedure. Mark the important steps that we need to pay attention to when pipetting.
6. List 5 common pipetting mistakes.
7. Describe the process of reverse pipetting.
8. While watching the video lectures, write down 5 important findings for each clip.

BASIC LABORATORY HEMATOLOGY

Assist. Dr. Irena Prodan Žitnik, MPharm., EuSpLM

LEARNING OUTCOMES

The purpose of this practical is for students to learn the practical application of basic hematology procedures. After this lab practice, the student will know how to:

- prepare and stain a peripheral blood film for microscopic examination,
- identify different normal/healthy blood cells under a microscope and perform a manual differential leucocyte count,
- determine the erythrocyte sedimentation rate,
- perform one of the screening tests in hemostasis - prothrombin time measurement,
- evaluate and interpret the results.

Basic laboratory tests in hematology include complete blood count with evaluation of erythrocytes, leukocytes and platelets, screening tests for hemostasis and erythrocyte sedimentation rate.

COMPLETE BLOOD COUNT (CBC)

Complete blood count (CBC) is a commonly ordered laboratory test that provides useful information on the number and characteristics of different blood cell types. A standard CBC includes:

- Evaluation of red blood cells (RBC) – RBC count, hemoglobin concentration in blood, hematocrit and RBC indices
- Evaluation of white blood cells (WBC) – WBC count, WBC differential
- Evaluation of platelets – platelet count, platelet indices.

CBC is usually performed on automated hematology analyzers; however, a manual microscopic examination of stained blood film is necessary in a number of circumstances (to

verify flagged automated results, in case of deviation of certain parameters from normal values, in case of suspected hematologic disease, *etc.*)

The sample of choice for CBC and peripheral blood film is venous blood, anticoagulated with tri-potassium or di-potassium salt of ethylenediaminetetra-acetic acid (K_3EDTA or K_2EDTA). Thorough mixing of the blood immediately after collection is essential. EDTA in usual concentration does not influence the shape and volume of the blood cells, except immediately after blood collection. The tests are therefore performed at least 20-30 minutes after collecting the sample. Prolonged storage influences some test results, therefore, ideally, the samples should be tested within 6 hours after collection, but sufficiently reliable results can be obtained when the sample is stored for up to 24 hours at 4 °C.

For microscopic examination of blood film, the smear is usually stained using May-Grünwald–Giemsa (Pappenheim) stain. The blood film should be prepared not more than 4 hours after blood collection.

EVALUATION OF RED BLOOD CELLS

Red blood cell count

RBC count is usually measured on automated or semiautomated analyzers, but can also be performed by counting the cells microscopically in a counting chamber. It is is expressed as number of RBC per liter of blood (x10^{12}/L). RBC count is used to calculate some RBC indices like mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH).

Reference values[1]:	M	4.5 – 5.5 x 10^{12}/L
	F	3.8 – 4.8 x 10^{12}/L

Hemoglobin concentration (Hb)

Hemiglobincyanide method is the reference method for determination of hemoglobin concentration. Using potassium cyanide, hemoglobin is converted to a stable hemiglobincyanide (cyanmethemoglobin) and measured spectrophotometrically at 540 nm.

[1] *The reference values cited in this document were obtained from laboratories in the University Medical Centre Ljubljana, Slovenia.*

Alternatively, hemoglobin can be converted into hemiglobinsulphate, with maximum absorbance at 534 nm by addition of sodium lauryl sulphate.

Reference values:	M	130 - 170 g/L
	F	120 - 150 g/L

Packed red cell volume (PCV) or hematocrit value (HCT)

Packed cell volume or hematocrit is a measurement of the proportion of centrifuged blood that is occupied by red blood cells. A small amount of plasma trapped between red cells after centrifugation can impact the results, but the error caused by this is usually less than 0,01 PCV units. The PCV is expressed as a decimal fraction. In conjunction with estimations of Hb and RBC count, it can be used in the calculation of red cell indices.

Reference values:	M	0.400 – 0.50
	F	0.360 – 0.460

Red blood cell indices

RBC indices are parameters that describe the population of RBC in the patient and provide information on physical characteristics of RBC, such as average size (volume) and average hemoglobin content per RBC. These values are important for differentiation of anemias. RBC indices include:

a) Mean corpuscular volume (MCV)

MCV is the average size (volume) of RBCs in the sample. It can be measured directly by hematology analyzers or calculated as follows:

$$\text{MCV} = \frac{PCV \times 1000}{RBC\ count\ (\times\ 10^{12}\ /L)}$$

MCV is expressed in femtoliters (fL). One femtoliter is 10^{-15} L.

Reference values:	83.0 -101.0 fL

b) Mean corpuscular hemoglobin (MCH)

MCH measures the average amount of hemoglobin present in each RBC. It is expressed in picograms (pg). One picogram is 10^{-12} grams. The parameter is calculated as follows:

$$\text{MCH} = \frac{Hb\ (g/L)}{RBC\ count\ (\times\ 10^{12}\ /L)}$$

Reference values:	27.0 – 32.0 pg

c) Mean corpuscular hemoglobin concentration (MCHC)

MCHC refers to the average concentration of hemoglobin in the RBCs contained within the sample. It is expressed in g/L and calculated as follows:

$$\text{MCHC} = \frac{Hb\ (g/L)}{PCV}$$

Reference values:	315 - 345 g/L

Red cell distribution width (RDW)

The red cell distribution width is a measure of variation in cell volume within the RBC population. It is calculated using standard deviation of erythrocyte volumes (SD) and MCV, as follows:

$$\text{RDW} = \frac{SD}{MCV} \times 100$$

Reference values:	11.6-14.0 %

Reticulocyte count

Reticulocyte count is an indirect marker of RBC production in the bone marrow. It can be measured directly by hematology analyzers or calculated from the percentage of the total RBC, counted under a microscope.

Reference values:	20.0 – 100.0 10^9/L 0.5 – 2.5 %

EVALUATION OF WHITE BLOOD CELLS

WBC or leucocytes are cells of the immune system that can be categorized into mononuclear cells (monocytes and lymphocytes) and polymorphonuclear cells (neutrophil, basophil and eosinophil granulocytes).

Evaluation of WBC includes the WBC count and WBC differential count. WBC count measures the total number of WBC in blood and is expressed as number of WBC per liter (x 10^9/L). WBC differential count gives the percentage or the absolute count of each white blood cell type in the sample. The percentage is determined by counting the cells in peripheral blood smear under the microscope and the absolute value is calculated by multiplying the determined percentage with the total WBC count.

Reference values:		
WBC count	4-10 x 10^9/L	
Differential white cell count		
Neutrophils	1.50 – 7.40 x 10^9/L	40.0 – 80.0 %
Lymphocytes	1.10 – 3.50 x 10^9/L	20.0 – 40.0 %
Monocytes	0.21 – 0.92 x 10^9/L	2.0 – 10.0 %
Eosinophils	0.02 – 0.67 x 10^9/L	1.0 – 6.0 %
Basophils	0.00 – 0.13 x 10^9/L	0.0 – 2.0 %

Evaluation of the WBC can be performed using automated hematology analyzers. However, in certain cases the analyzer cannot accurately differentiate the cells and a manual microscopic examination of stained blood film is recommended. The criteria for manual examinations are as follows:

- Suspected hematologic disease
- WBC count above 30 x10^9/L or below 3 x10^9/L
- Platelet count below 100 x10^9/L or above 1000 x10^9/L
- Neutrophil count below 1 x10^9/L or above 20 x10^9/L
- Lymphocyte count above 5 x10^9/L
- Monocyte count above 1.5 x10^9/L
- Eosinophil count above 1.5 x10^9/L
- Basophil count above 0.3 x10^9/L or above 3%
- Flagged automated results for morphologic abnormalities, immature cells, platelet clumps or giant platelets, presence of erythroblasts

EVALUATION OF PLATELETS

Evaluation of platelets includes platelet count and mean platelet volume (MPV). Platelet count can be measured with automated analyzer or in counting chamber under the microscope. It is expressed in number of platelets per liter of blood (x10^9/L). MPV can be measured directly by the analyzer or calculated as follows:

$$\text{MPV} = \frac{PCT}{platelet\ count\ (\times\ 10^9\ /L)}$$

Reference values for MPV	7.8 – 11.0 fL
Platelet count	150 – 410 x 10^9/L

MICROSCOPIC EXAMINATION OF BLOOD FILMS – BLOOD CELL MORPHOLOGY

Red blood cell morphology

Normal RBCs are round and vary relatively little in shape, color or size. They measure 7-8 µm in diameter and their cytoplasm is pink and lighter in the center of the cell, compared to cell border. The central pallor occupies approximately one third of the cell.

Stained blood film should be evaluated for changes in size, color and shape of RBC and for abnormal structures within the cells. Any visible aggregation of the RBCs should be reported as well as the presence of erythroblasts (immature nucleated RBCs). RBCs larger than 9 µm in diameter are considered **macrocytes** and RBCs smaller than 6 µm in diameter are **microcytes**. **Anisocytosis** is a condition in which the RBCs are not even in size and **poikilocytosis** is characterized by deviation from normal RBC shape.

Platelet morphology

Normal platelets are 1–3 µm in diameter. They are irregular in outline with blue-grey cytoplasm and fine purple-red granules that may be scattered or centralized. A small number of larger platelets, up to 5 µm in diameter, may be seen in normal films.

When performing manual blood film evaluation, the observed morphological changes in platelets (size, granules, aggregation) should be described.

White blood cell morphology

In May-Grünwald–Giemsa (Pappenheim) stained blood films, the WBC nuclei are colored purple, cytoplasm pink or blue, neutrophil granules are purple-grey, eosinophil granules orange and basophil granules dark purple. For WBC differential, the following morphologic characteristics of the WBCs should be examined:

- size and shape of the cell
- segmentation of the nucleus and chromatin structure
- color of the cytoplasm
- color of the granules
- granulation and inclusions in the cytoplasm

WBC or leukocytes can be further categorized into mononuclear cells (monocytes and lymphocytes) and polymorphonuclear cells (neutrophil, basophil and eosinophil granulocytes). Normal neutrophils are uniform in size, with an apparent diameter of about 12-15 µm in a blood film. They have a segmented nucleus and, when stained, pink cytoplasm with fine granulation. The neutrophils have 2-5 nuclear segments connected by chromatin strands. Up to 8% of circulating neutrophils have unsegmented nucleus (band like shape, with diameter of the nucleus at the narrowest point not less than 1/3 that of its widest part) and are called band neutrophils. Band cells are less mature neutrophils, but are still counted as mature cells.

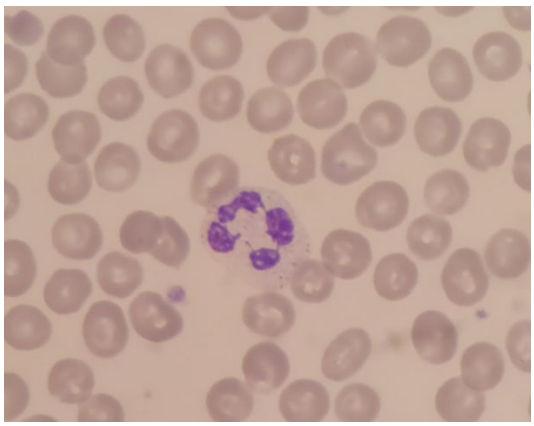

Figure 1. *Segmented neutrophil granulocyte*

Eosinophil granulocytes are a little larger than neutrophils (12-17 µm in diameter) and round or slightly oval in shape. They usually have two nuclear lobes with condensed chromatin and the cytoplasm is packed with distinctive orange (eosinophilic) granules.

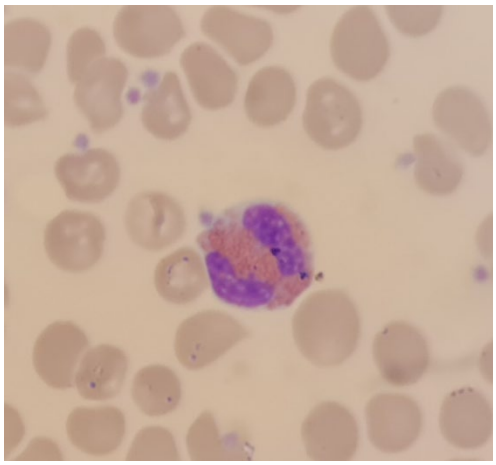

Figure2. *Eosinophil granulocyte*

Basophils are the rarest (<1%) of the circulating leucocytes. Their nuclear segments tend to fold up on each other, resulting in a compact irregular dense nucleus resembling a closed lotus flower. The distinctive, large, variably sized purple granules of the cytoplasm often obscure the nucleus.

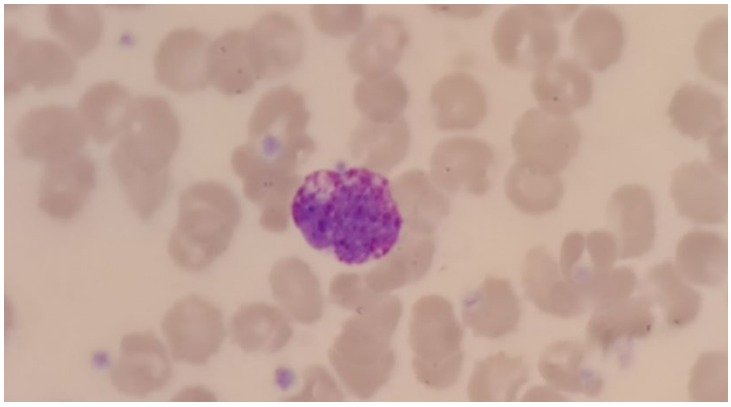

Figure3. *Basophil granulocyte*

The majority of circulating lymphocytes are small cells (7 do 12 µm) with a thin rim of cytoplasm, occasionally containing some azurophilic granules. Nuclei are remarkably uniform in size (about 7-9 µm in diameter). This provides a useful guide for estimating red cell size (normally about 7–8 µm) from the blood film. About 10% of circulating lymphocytes are larger (9 do 15 µm in diameter), with more abundant pale blue cytoplasm containing azurophilic granules. The nuclei of lymphocytes have homogeneous chromatin with some clumping at the periphery of the nucleus.

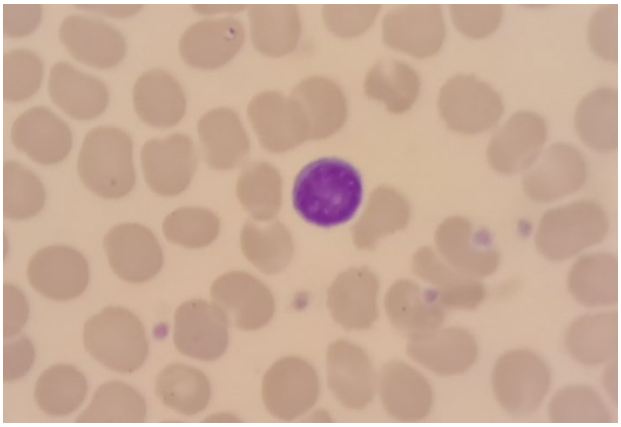

Figure 4. *Lymphocyte*

Monocytes are the largest of the circulating leucocytes and vary in size from smaller 12–20 µm in diameter to large monocytes 40 µm in diameter. They have bluish grey cytoplasm that contains variable number of fine purplish red granules and often also vacuoles. The nucleus is large and curved, often in the shape of a horseshoe, but it may be folded or curled. The chromatin is finer and more evenly distributed than that in neutrophil nuclei.

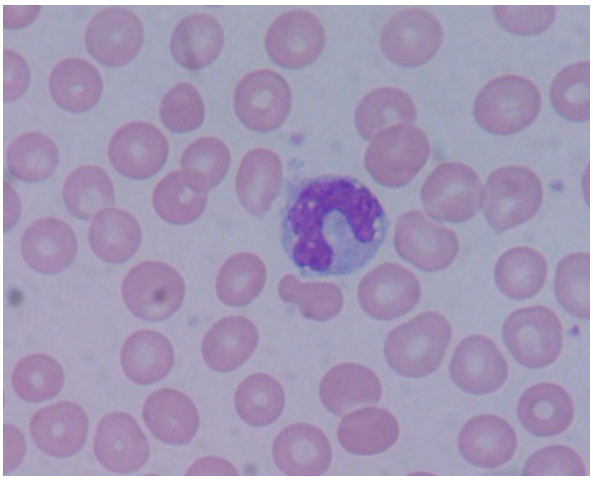

Figure 5. *Monocyte.*

BASIC METHODS IN HEMATOLOGY

Automated blood cell count

The traditional method for counting cells is electrical impedance, also known as the Coulter Principle. Diluted whole blood passes between two electrodes through an aperture so narrow that only one cell can pass through at a time. The impedance changes as a cell passes through.

The change in impedance is proportional to cell volume, resulting in a cell count and measure of volume. Impedance analysis returns CBCs and three-part WBC differentials (granulocytes, lymphocytes, and monocytes) but cannot distinguish between the similarly sized granular leukocytes: eosinophils, basophils, and neutrophils.

Flow cytometry is more expensive than impedance analysis but returns detailed information about the morphology of blood cells. It is an excellent method for determining five-part WBC differentials. A single-cell stream passes through a laser beam and light is absorbed and scattered. The absorbance and scattered light at multiple angles are measured to determine the cell's granularity, diameter, and inner complexity, which helps to differentiate between different WBCs.

Preparation of blood films

Blood films should be made on clean glass slides, from fresh blood or EDTA anticoagulated blood. To prepare blood film, place a small drop of blood in the center line of a slide about 1 cm from one end (Figure 6). Then, without delay, place a spreader (cover glass or glass slide) in front of the drop at an angle of about 30-45 degrees to the slide and move it back until it touches the drop. The drop should spread quickly along the line of contact. With a steady movement of the hand, spread the drop of blood along the slide. The thickness of the film depends on the angle between the glass slide and the spreader, the pressure of spreader against the glass slide and velocity of the pull.

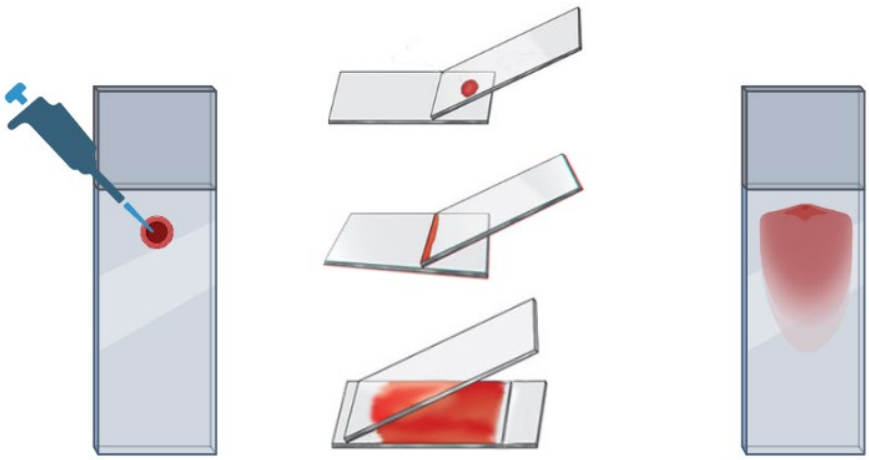

Figure 6. *Blood film preparation (Created with BioRender.com)*

A blood film has three parts: head, body and tail. It has to be thin, even and with straight side edges. The tail edge should be feathered and should not reach the end edge of the glass slide. After preparation, the blood films should be left to air dry (up to 30 minutes).

Blood film staining according to Pappenheim (May-Grünwald-Giemsa staining)

Blood films are most commonly stained according to Pappenheim, using May-Grünwald and Giemsa stains.

Reagents:

- May- Grünwald: eosin and methylen blue in methanol
- Giemsa: azur, methylene blue and eosin methanol
- Sorensen phosphate buffer (0.066M, pH=6.8-7)

Place freshly prepared and air-dried blood smears in a manual staining rack and place the rack in May-Grünwald solution for 5 minutes. Transfer the rack to diluted May-Grünwald solution (1:2 dillution with phosphate buffer) and after 1 minute to the diluted Giemsa solution (1:10 dillution with phosphate buffer) for 20 minutes. Wash with buffer and allow air to dry before examination.

Stained blood film examination

Blood films should be examined systematically, starting with macroscopic observation of the stained film and then microscopic examination progressing from low to high magnification. First, the film should be examined macroscopically to assess whether the spreading technique was satisfactory and to judge its staining characteristics. Next inspect the film under a low magnification (100x) to (a) get an idea of the quality of the preparation; (b) assess whether red cell agglutination, excessive rouleaux formation or platelet aggregation is present; (c) assess the number, distribution and staining of the leucocytes; and (d) find an area where the red cells are evenly distributed and are not distorted. Having selected a suitable area, a 1000x magnification with oil-immersion objective should be used for WBC differential and examination of WBC, RBC and platelet morphology. The suitable area is in the feathered tail edge of the blood smear, where RBCs are close together, but do not overlap.

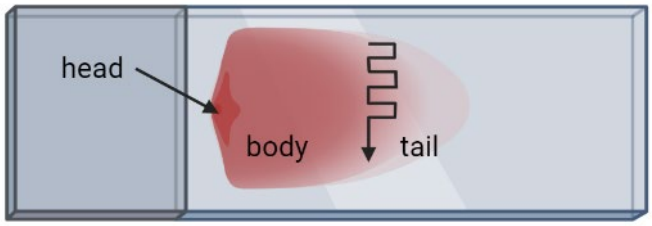

Figure 7. *Zig-zag manner of blood film examination (Created with BioRender.com)*

Blood cells are unevenly distributed across the blood film, the examination should thus be performed in a zig-zag manner, starting from one side of the suitable area of the smear and moving through the slide as shown in Figure 7. Less mature cells and monocytes can be found in higher numbers on the edges of the spread. At least 100 WBCs should be examined for WBC differential and all WBCs with nucleus and cytoplasm with clear margins should be included. Special attention should be given to the morphological changes of cytoplasm and nucleus.

TEST YOUR KNOWLEDGE

1. What is complete blood count? Which parameters are included in CBC?
2. Explain the preparation of blood film for microscopic examination.
3. What are the morphological characteristics of band neutrophils, segmented neutrophils, eosinophils, basophils, lymphocytes and monocytes in stained smear, prepared from the blood of a healthy individual?
4. Blood for complete blood count should normally be anticoagulated with:
    a) heparin
    b) citrate
    c) calcium
    d) chloride
    e) EDTA

ERYTHROCYTE SEDIMENTATION RATE (ESR)

Erythrocyte sedimentation rate (ESR) is a test that indirectly measures the degree of inflammation present in the body. The test measures the rate of sedimentation of erythrocytes in a sample of blood placed into a thin, vertical tube. Results are reported as the millimeters of clear fluid (plasma) present at the top portion of the tube, above RBCs, after one hour. The rate of sedimentation depends on mass, volume and shape of the RBCs, the constitution of plasma, temperature, time and angle of sedimentation and blood/anticoagulant ratio. Presence of elevated amounts of certain proteins in plasma, such as fibrinogen and immunoglobulins elevate ESR.

ESR depends on numerous factors, so its diagnostic usefulness is limited. It is a non-specific test that can be elevated in a number of different conditions and provides only general information about the presence or absence of an inflammatory condition. Normal ESR does not exclude the presence of a disease. An elevated ESR is often observed in cases of infection, malignancy, and autoimmune disease and anemia. In pregnancy, during menstruation and in older age, ESR can be physiologically elevated.

Reference values	M	under 50 years	0-15 mm/h
		≥ 50 years	0-20 mm/h
	F	under 50 years	0-20 mm/h
		≥ 50 years	0-28 mm/h

METHOD FOR MEASURMENT OF ERYTHROCYTE SEDIMENTATION RATE

ESR is determined in venous blood, anticoagulated with citrate in anticoagulant/blood ratio =1:5 (1 part anticoagulant, 4 parts blood). The test should be performed at room temperature between 18 and 24^{O}C, ideally at 20 ^{O}C. Mix the blood sample thoroughly, pull-up the stopper and then put the rigid transparent plastic tube into the blood. The blood must reach the zero mark on the tube. Place the tube vertically and leave undisturbed for exactly 60 min, free from vibrations. Then read the height of the clear plasma above the upper edge of the erythrocyte column. The result is expressed in mm per hour.

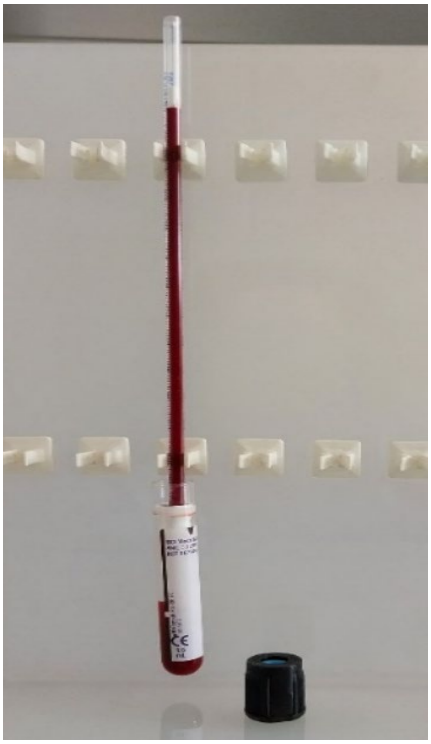

Figure 8. *Measurement of erythrocyte sedimentation rate*

Possible sources of false ESR results can be obtain duet to:

- Incorrect concentration of anticoagulant
- Hemolysis
- tube not dry and/or clean
- tube not filled to the zero mark or air bubbles in the tube
- Position/angle of the tubes during the test is not completely vertical
- Too long/too short time of measurement
- Temperature below 18 or above 24 ^{O}C
- Time from collection of the sample and ESR test exceeded 1 hour

PROTHROMBIN TIME (PT)

Prothrombin time (PT) measures the clotting time of recalcified plasma in the presence of an optimal concentration of tissue extract (thromboplastin). It indicates the overall efficiency of the extrinsic clotting system. The test results depend on activities of factors V, VII and X and on the fibrinogen concentration of the plasma.

The sample for prothrombin time and other test of hemostasis is venous blood anticoagulated with sodium citrate (0,105-0,109 M sodium citrate, light blue tube top). Other anticoagulants, including oxalate, heparin and EDTA are unacceptable. Factors V and VIII are unstable in oxalate anticoagulated samples and heparin and EDTA directly inhibit the coagulation process and interfere with determinations. Venous samples should be collected without a pressure cuff or with a light pressure tourniquet. Venous occlusion causes hemoconcentration, increase of fibrinolytic activity, platelet release reaction and activation of some clotting factors.

Blood should be transported to the laboratory within 4 hours after collection, at room temperature. Most routine coagulation investigations are performed on platelet-poor plasma (PPP), which is prepared by centrifugation at 2000 g for 15 min at 4 °C

PT can be expressed in seconds, as activity percentage of normal pooled plasma, or as international normalized ration (INR).

Thromboplastins used for PT determination vary greatly in their responsiveness to reduced activity of clotting factors, and consequently, the results of the PT tests are less comparable between laboratories if PT is expressed in seconds or % of activity. The use of the International Sensitivity Index (ISI) which represents the sensitivity of any given thromboplastin and the International Normalized Ratio (INR) to report the results, has minimized these difficulties and greatly improved uniformity of results between laboratories. This is

ISI values for thromboplastins are reported by the manufacturers in package inserts and INR can be calculated as follows:

$$INR = \left(\frac{patient\ PT\ (s)}{mean\ normal\ PT\ (s)}\right)^{ISI}$$

The recommended thromboplastin sensitivity for prothrombin time measurements are 0.9-1.7 ISI.

METHOD FOR DETERMINATION OF PROTROMBIN TIME

The one-stage PT of Quick is the most commonly used test. Platelet poor plasma is mixed with complete thromboplastin containing tissue factor (TF) and phospholipid at 37°C and an excess

of calcium chloride (25 mM) is added to initiate coagulation. The time taken from the addition of calcium to the formation of the fibrin clot is measured in seconds.

To express PT as activity percentage of normal pooled plasma, calibration curve with reference plasma should be prepared. PT is measured in triplicates in the following dilutions of reference plasma: undiluted, 50%, 25% and 12,5% and the values are plotted on a reciprocal curve sheet.

Reference values	
PT	0.7-1.2 (70-120%)
INR	0.9-1.3 2-3 (for patients on anticoagulant therapy for deep vein thrombosis prevention, atrial fibrillation, after AMI) 2,5-3,5 (for prevention of embolisms in patients with mechanical heart valve)

TEST YOUR KNOWLEDGE

1. What does the erythrocyte sedimentation rate measure?
2. Explain clinical significance of the ESR test.
3. How do we measure ESR?
4. What does PT test measure and what is its significance?
5. Which anticoagulants can be used in samples for PT measurement?
6. How is INR calculated?

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LAB REPORT: BASIC LABORATORY HEMATOLOGY

COMPLETE BLOOD COUNT

Automated CBC

Perform an automated CBC for three controls and your sample. Results of the controls should be in range, provided by the manufacturer. Write down and comment on the results obtained from the hematology analyzer (for all blood cell types).

Results and comments:

Blood film preparation and staining

Prepare blood films and stain one of them (the best one) with May–Grünwald–Giemsa stain. Check the quality of the blood film and write down your observation.

Results and comments:

Manual WBC differential count

Examine at least 100 WBCs in the blood film and compare your results with the results provided by your supervisor. Comment on the deviations between the results. Calculate the absolute values for all WBC types.

Results and comments:

Erythrocyte Sedimentation Rate

Mix the blood sample thoroughly, pull-up the tube cap and then insert the rigid transparent plastic tube into the blood. The blood must reach the zero mark on the tube. Place the tube vertically and leave undisturbed for exactly 60 min. Then read to the nearest 1 mm the height of the clear plasma above the upper limit of the column of sedimenting cells. The result is expressed in mm per hour. Compare the results with reference values and comment on them.

Results and comments:

Prothrombin time

Perform the test for two controls and your sample. Check if control results are within the range, provided by the manufacturer. Use the calibration curve to express the results in activity percentage of normal plasma. Calculate INR.

Results and comments:

Signature: Date:

FLOW CYTOMETRY IN HEMATOLOGY

Assist. Dr. Dunja Urbančič, MPharm

Assoc. Prof. Dr. Martina Gobec, MPharm

LEARNING OUTCOMES

After successfully completing this laboratory practical, student will be able to:

- describe the principles of flow cytometry,
- describe the principles of immunofluorescence,
- list the implication of flow cytometry in haematological and oncological examinations,
- describe how to use flow cytometry in the diagnosis of leukaemia and lymphoma,
- list most characteristic CD markers of blood cells,
- interpret the dot-plot of whole blood,
- distinguish cell populations based on immunofluorescent analysis.

FLOW CYTOMETRY

Flow cytometry is a technique that provides information about the physical and biochemical properties of a single cell. When a cell passes through a laser beam, it sends a light signal to the detector. The signal depends on the structural properties of the cell. Only cells in a suspension can be used for flow cytometry. After a cell suspension is slowly injected into a sheath fluid, the cells are sorted to form a queue that allows them to pass individually through a laser beam (Figure 1). The process of such cell sorting is called hydrodynamic focusing.

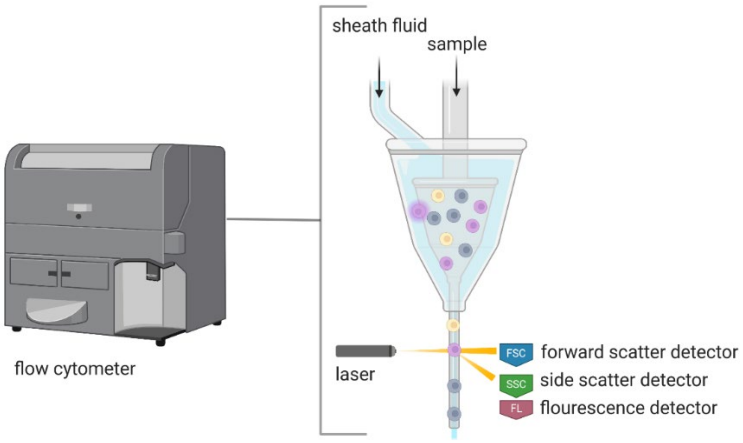

Figure 1. *Drops of injected sample migrate from injector towards narrow and strong current of the sheath fluid. The precise control of the velocity of both currents enables the alignment of cells, which then individually pass the source of light. Created with BioRender.com*

FORWARD AND SIDE SCATTERING

The light from the laser beam is scattered in all directions as it passes through the cell-containing sample (Figure 1). The scattered light has the same wavelength as the excitation laser. Two photodetectors in the flow cytometer measure the light scattered from individual cells in the sample. The first detector, called the forward scattered light detector (FALS), is aligned with the plane of the laser beam and is located on the opposite side of the light source (Figure 1). It detects the light scattered at small angles, i.e., the light scattered from the surface of the plasma membrane of the passing cell (Figure 2). The measured parameter is called forward scattering (FSC) and is proportional to the size of the cell - the larger the cell, the larger the FSC signal on the FALS detector. On the other hand, light from the membranes of organelles in the cell (e.g., lysosomes, endoplasmic reticulum, phagosomes) is scattered at larger angles (Figure 2). This light is detected by the detector, which is called right-angle light scattering detector (RALS) and is oriented perpendicular to the plane of the laser path. The parameter measured by RALS is called side scattering (SSC) and is proportional to the complexity (especially granulation) of a given cell - the larger the number of organelles and inner membrane structures, the larger the SSC signal on the RALS detector.

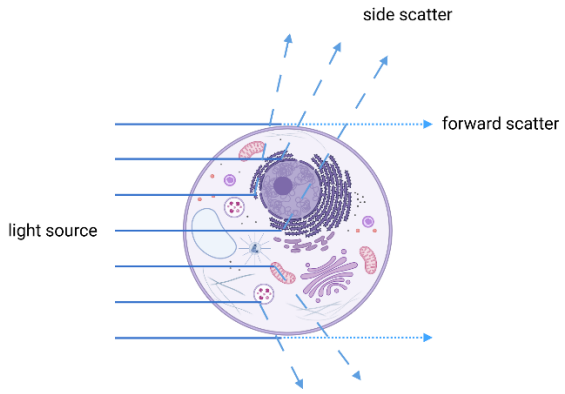

Figure 2. *The scattering of the light interacting with the cell. Created with BioRender.com*

Based on the scattering parameters mentioned above, we can distinguish between individual cell populations in blood. As an example, let's see how flow cytometer sorts leukocyte fraction of whole blood. The signals from FALS and RALS detector can be presented in a dot-plot diagram. The x-axis represents the front scatter parameter (FSC) while the y-axis shows the side scatter (SSC). Each dot in the graph, an "event", is a cell detected by the detectors. According to FSC and SSC signals of cells in the leukocyte fraction of the whole blood (Figure 3), we can distinguish three populations of cells:

- small and non-granulated lymphocytes,
- large monocytes, which express greater granulation with respect to lymphocytes,
- structurally most complex and middle-sized granulocytes.

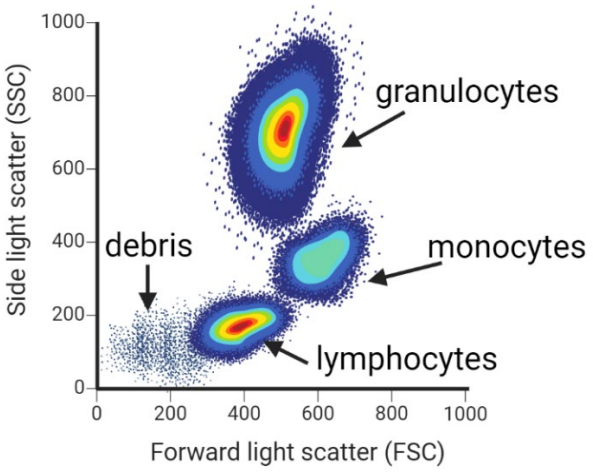

Figure 3. *Dot-plot diagram of leukocytes analysed on a flow cytometer using forward and side scattering. Created with BioRender.com*

IMMUNOFLUORESCENCE FOR SPECIFIC LABELLING OF CELLS

Beside morphological features of the cell, flow cytometer is an excellent tool to observe functional characteristics of cells as well. Alongside FALS and RALS detectors, there are at least three fluorescence detectors, measuring the light emitted from fluorescently labelled cells in the flow cytometer (Figure 1). Cells can be labelled either with fluorescent probes or by using immunofluorescence technics. Fluorescent dyes enable observation of functional features by emitting the light when in contact with certain molecule or organelle in the cell. For example, propidium iodide, a small fluorochrome, stoichiometrically interacts with DNA and can be therefore used for DNA quantification.

In clinical practice, immunofluorescence is commonly used to detect the presence of antigens on the surface or inside the cell. This can be done by specific fluorescently labelled antibodies targeting the antigen of interest. When the fluorescently labelled cell is exposed to the excitation light, it emits light at a higher wavelength compared to the excitation wavelength (Figure 4). When the photons of the emitted light reach the corresponding detector, their energy is converted into an electrical signal that is displayed as a graphical feature on the monitor after computational processing (Figure 1). A combination of multiple fluorescent dyes and/or fluorescently labelled antibodies can be used in the same sample. However, a careful experimental design should be considered to avoid false positives due to overlap of emission spectra from simultaneously excited fluorochromes (Figure 4). Taking this consideration into account, the number of parameters that can be measured by flow cytometry is limited only by the availability of fluorescent biomarkers and the configuration of the instrument (number and wavelengths of lasers and detectors).

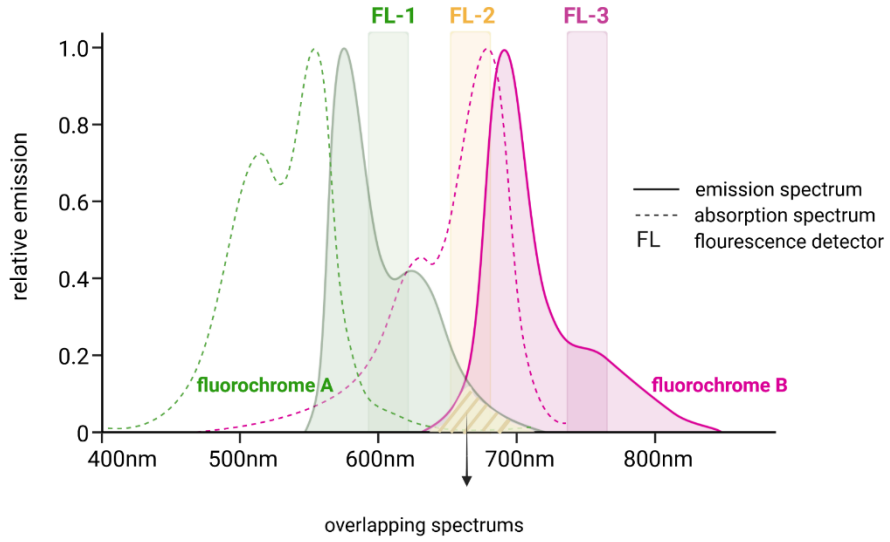

Figure 4. *Overlap of emission spectra from two different fluorescence probes. The fluorescence detector FL -2 can detect the emission of both fluorochromes (A and B). Detection of both dyes with the same detector appears to enhance the fluorescence intensity of fluorochrome B, leading to false positives for cell features labelled with this fluorochrome.*

Flow cytometry is a rapid, objective, and quantitative method that is indispensable in clinical diagnostics in many fields of medicine (Table 1). The main advantages are the use of a small sample volume and the simultaneous measurement of several parameters in the same sample.

Table 1. *Clinical applications of flow cytometry*

Field	Clinical application	Parameters
immunology	histocompatibility	IgG, IgM
	Transplant rejections	CD3
	Studies of immunodeficiency	CD4, CD8
	Diagnosis and follow-up of autoimmune disorders	IgG, IgM, complement
oncology	DNA content	DNA
	Marker proliferation	Ki-67, PCNA
	Phenotyping of leukaemia and lymphoma	leukocyte antigens
hematology	Reticulocyte determination	RNA
	Thrombocyte functionality	CD61, CD63, PAC-1
	Foetal haemolytic diseases	Rhesus D antigen

FLOW CYTOMETRY IN HEMATOLOGY – EXAMPLES

Blood cells exhibit distinct functions and can be differentiated through various properties, primarily surface molecules, typically proteins. These proteins serve as targets for fluorescently labeled antibodies, which selectively adhere to them. Subsequent detection of these molecules is achieved using flow cytometry. Proteins exclusively located on the surfaces of specific cell types are denoted as **clusters of differentiation** (CDs). Analyzing the surface expression of particular CD molecules proves invaluable in characterizing cell phenotypes. A technique employed to identify cells based on specific markers or antigens present on their surface, using specific antibodies is referred to as **immunophenotyping**. Figure 5 denotes specific antigens (CD markers) that are characteristic for specific blood cells.

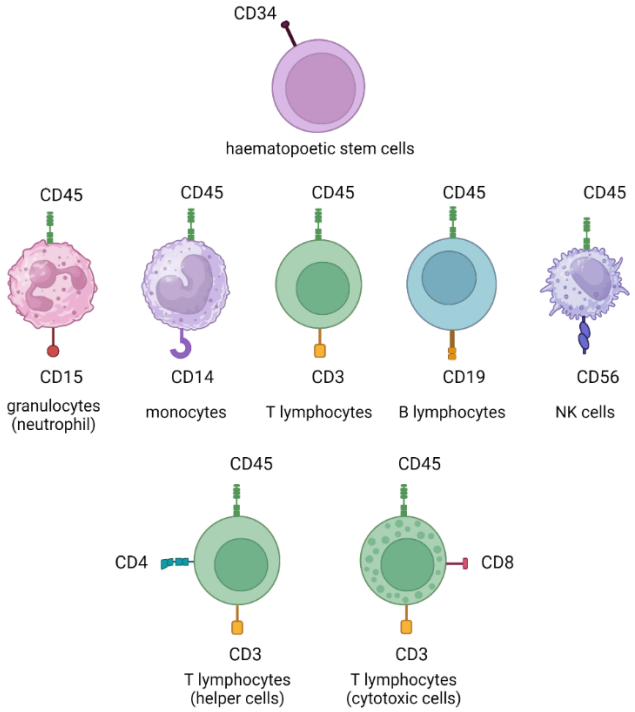

Figure 5. *Antigens specific for selected leukocytes. Created with BioRender.com*

Immunophenotyping of T lymphocytes

Analysis of T lymphocytes. Lymphocytes were labelled by antibodies against CD4 (fluorochrome: allophycocyanin – APC) and against CD8 (fluorochrome: phycoerythrin – PE) and analysed by flow cytometry.

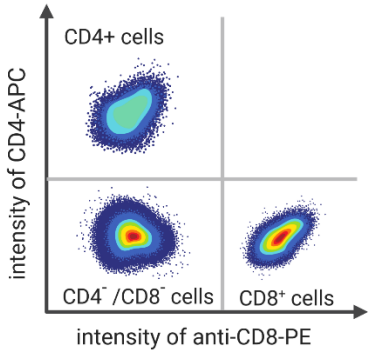

Figure 6. *Dot-plot diagram of lymphocytes labelled by CD8-PE and CD4-APC antibodies. Created with BioRender.com*

We can distinguish three different populations on the dot-plot diagram with x and y axes being the intensities of both fluorescent dyes (Figure 6):

- cells, that only emit the photons of APC – CD4+/CD8- cells (T-helper cells)
- cells, that only emit the photons of PE – CD8+/CD4- cells (T-cytotoxic cells)
- cells, that emit neither of the photons – CD4-/CD8- cells (natural killer cells)

In the diagnosis and monitoring of immunodeficiencies or autoimmune diseases, the percentage of lymphocytes and the ratio of T-helper and T-cytotoxic cells are frequently determined (Table 2). In HIV-infected patients, for example, a decrease in T-helper cells is a reliable indicator of disease exacerbation and progression.

Table 2. *Representative clinical conditions with significantly altered lymphocyte populations.*

Immunodeficiency	Change in the population
CVI	lymphocytes B ↓, T helper cells ↓
SCID	lymphocytes B ↓, T helper cells ↓, T cytotoxic cells ↑
syndrome DiGeorge	lymphocytes T ↓
Syndrome Nezelof	lymphocytes T↓
AIDS	T helper cells ↓, T cytotoxic cells ↑

CVI – *common variable immunodeficiency*; SCID – *severe combined immunodeficiency*

Immunophenotyping of leukaemia and lymphoma

Together with clinical data and the results of cytologic, histologic, cytogenetic, and molecular genetic testing, the diagnosis of leukaemia and certain lymphomas is based on immunophenotypic characteristics of abnormal, malignant cells. Flow cytometry allows us to determine the presence of multiple cell markers that allow identification of a malignant population. The markers can identify:

a) cell linage (myelocytic, monocytic, B-lymphocytic, T-lymphocytic),
b) the differentiation stage of the malignant cells,
c) the clonality of the cells as evidence of malignant growth, and
d) the phenotype(s) of the malignantly transformed cells.

Biological samples used for immunophenotyping of leukaemia and lymphoma are usually blood, puncture fluids (bone marrow, lymph nodes, cerebrospinal fluid, etc.), or biopsy samples (lymph nodes or other tissues) that are appropriately prepared prior to analysis. The erythrocytes must be removed from the whole blood. Tissue from the biopsy must be mechanically and enzymatically processed before leukocytes are isolated and analysed. After leukocyte isolation, fluorescently labelled antibodies are used to stain the cells. Each cell expresses specific biomarkers, and their expression varies depending on the cell type (linage) and stage of development. For leukaemia and lymphoma, a stepwise diagnostic analysis is usually performed. The first step is to determine the cell lineage - myeloid, B-lymphocytic or T-lymphocytic - followed by determination of the subtype and differentiation stage of the cells.

Acute leukaemia

According to the immunophenotype and cytomorphology of leukemic cells, assigned by molecules found on the surface of cells (i.e. clusters of differentiation – CD), the acute leukaemias are classified as:

a. Lymphoblastic leukaemias (ALL):
    - B-lymphoblastic (B-ALL) (CD19+)
    - T-lymphocytic (T-ALL) (CD2+, CD3+)

b. Myeloblastic leukaemias (AML) – This is very heterogenous group of leukaemia, within which several subgroups classified according to differentiation stage exist (FAB classification):
   - myeloblastic – M0, M1, and M2 (CD34+, CD13+, CD33+)
   - pro-myelocytic – M3 (negative to HLA-DR and CD34+)
   - myelomonocytic – M4 (myeloid and monocytic biomarkers)
   - monoblastic – M5 (CD14+, CD64+)
   - erythroblastic – M6 (glycophorin A – CD235+)
   - megakaryoblastic – M7 (CD41+, CD61+)

c. Leukaemias of uncleared linage: biomarkers of lymphoblastic and myeloblastic linages are present; the division between ALL and AML is not possible.

Table 3. *Expression of antigens characteristic for ALL.*

	B-lymphoblastic (B-ALL)			
	pro (B-I)	common (B-II)	pre (B-III)	mature (B-IV)
HLA-DR	positive			
CD22				
CD79a				
CD19				
TdT	positive			negative
CD10	negative	positive		negative
cIgM	negative		positive	negative
s/m IgM	negative			positive

	T-lymphocytic (T-ALL)			
	pro (T-I)	pre (T-II)	cortical (T-III)	mature (T-IV)
TdT	positive			negative
cCD3	positive			
CD7	positive			
CD2	negative	positive		
CD5	negative	positive		
CD4	negative		positive for CD4 **and** CD8	positive za CD4 **or** CD8
CD8	negative			
CD1a	negative		positive	negative
sCD3	negative			positive

Chronic leukaemia and malignant lymphoma

In chronic leukaemia and lymphoma, the malignant cells have characteristics of mature lymphocytes. The most common malignancies are B-lymphocyte derived. Below are some of the most common biomarkers used in diagnosis (Figure 5). In addition to biomarkers, determining the clonality of cells and the ratio of lymphocytes expressing light K or λ chains are very important in the diagnosis of chronic leukaemia and malignant lymphoma. This ratio, which is normally 1.4:1, is greatly altered in chronic malignant B cells.

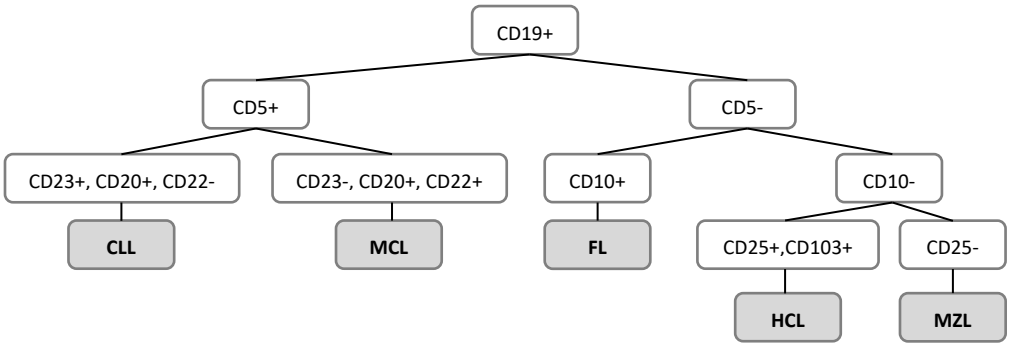

Figure 5. *Diagnostic diagram of selected chronic leukaemia and lymphoma. CLL (chronic lymphocytic leukaemia), MCL (mantle cell lymphoma), FL (follicular lymphoma), HCL (hairy cell leukaemia), MZL (marginal zone lymphoma).*

TEST YOUR KNOWLEDGE

1. The cells were labelled with two fluorochromes, both excited by 488 nm laser. The emission spectra of fluorochromes are overlapping. Why this can be an issue at the analysis and how can we avoid the crosstalk of two fluorochromes?
2. What are the disadvantages of flow cytometry?
3. What are clusters of differentiation (CD molecules)?
4. Reflect on the haematopoiesis.

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LAB REPORT: FLOW CYTOMETRY

In this lab practical, we will use direct immunofluorescence to identify T lymphocyte populations. Selected antigens on the surface of cells will be determined by corresponding monoclonal fluorescently labelled antibodies. Antibody binding, and thus the presence of antigens, is detected using a flow cytometer.

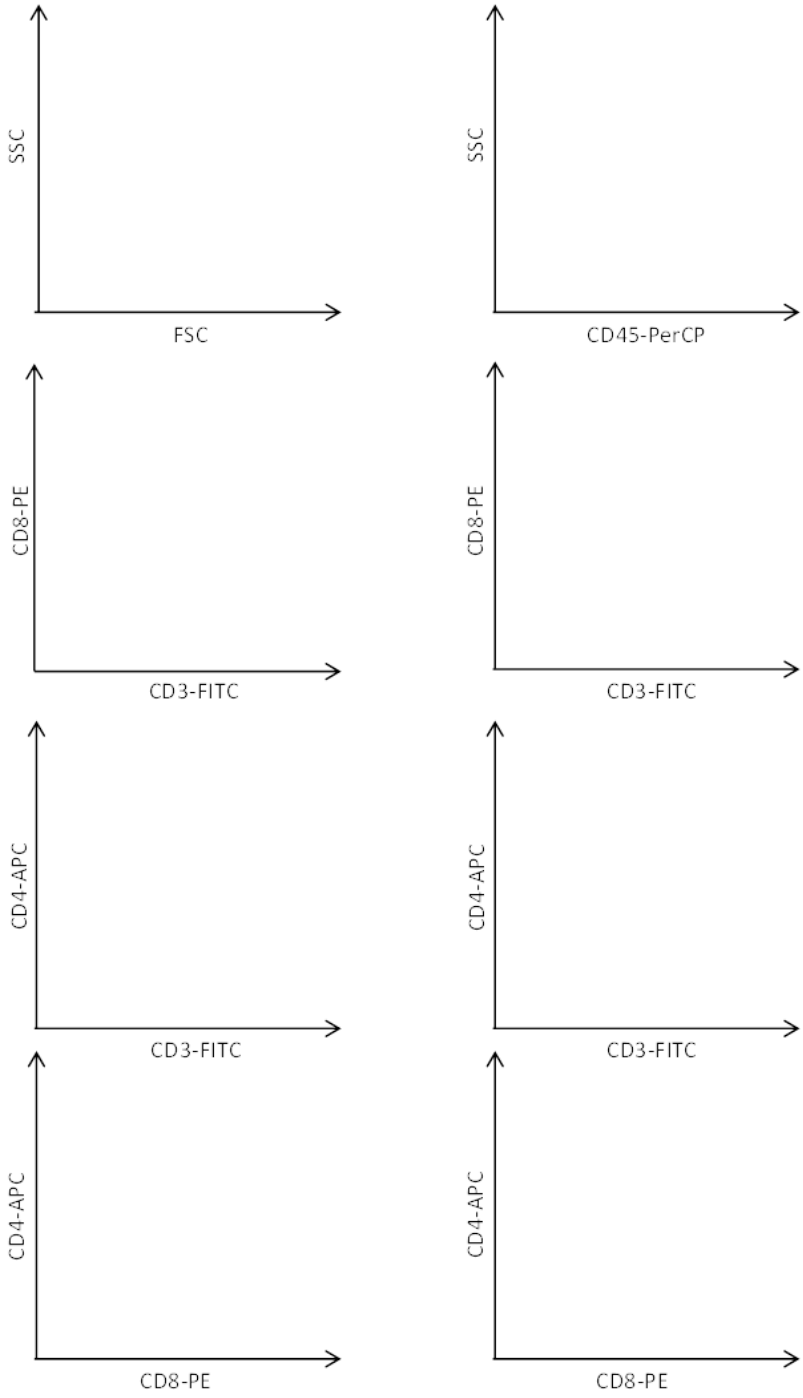
Protocol:

1. Label the tube for flow cytometric analysis (12x75 mm).
2. Transfer 10 µL of MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent to the bottom of the tube.
3. Transfer 50 µL of whole blood to the bottom of the tube. Do not allow the blood to stay on the walls of the tube. *The blood was drawn with K_2EDTA.*
4. Cover the tube and vortex.
5. Incubate for 15 min protected from light.
6. Dilute FACSLysing solution 10x with bi-distilled water.
7. Add 450 µL of diluted lysis solution (FACSLysing solution).
8. Cover the tube and vortex.
9. Incubate for 15 min protected from light.
10. Vortex the sample just before the analysis on flow cytometer to avoid aggregation of cells.
11. Observe different two-parameter dot-plot diagrams and histograms and determine the percentage of selected lymphocytic populations.

Results and comments

Comment on two-parameter histograms and percentages of selected lymphocytic populations.

Lymphocyte	**reference values (%)**	**sample (%)**
B-lymphocytes	5-26	
NK cells	5-22	
T-lymphocytes	56-86	
T-helper cells	33-58	
T-cytotoxic cells	13-39	



Signature: Date:

DETERMINATION OF C-REACTIVE PROTEIN IN SERUM

Assoc. Prof. Dr. Martina Gobec, MPharm

Assist. Dr. Tijana Markovic, MPharm

LEARNING OUTCOMES

After successfully completing this laboratory practical, student will be able to:

- describe the importance of CRP measurement for therapeutic intervention,
- explain the basic principle of turbidimetric measurements,
- summarize the Heidelberger-Kendall curve,
- justify the concentration range in which CRP can be measured,
- apply the obtained knowledge to perform CRP analysis in the serum using anti-CRP antibodies.

C-REACTIVE PROTEIN

CRP is an acute phase protein whose concentration is elevated upon inflammation. It is pentameric circular plasma protein and an important element of innate immunity. Upon inflammation-induced stimulus, such as bacterial or viral infection, surgical procedure, burns, hypoxia or systemic inflammation in autoimmune disorders, innate immune response in macrophages, adipocytes as well as other cells triggers the production of IL-6 (Figure 1). This cytokine is released into systemic blood circulation and stimulates the synthesis of CRP in liver. When CRP reaches the inflamed location, it binds to phosphocholine, polysaccharides and nucleic acids, and opsonizes viral particles or bacteria, activating complement system and recruiting immune cells, especially macrophages, to eliminate intruders by phagocytosis. It is an early marker of acute inflammation. Its concentration begins to increase already 4-8 hours after the inflammation trigger and reaches peak levels on day 1 to day 3. Elevated CRP level

can still be detected several days after the triggering inflammation; however, once inflammation subsides, CRP level falls quickly.

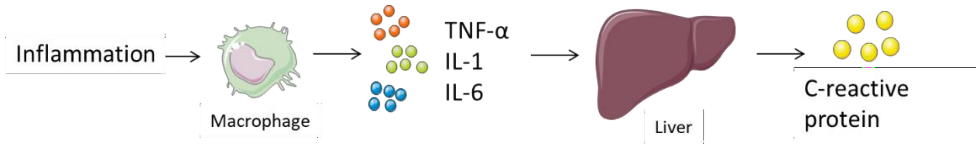


Figure 1. The signalling pathway in production of C-reactive protein (CRP). The scheme was prepared using SMART - Servier Medical ART 3000.

In healthy individuals, CRP is present in concentration lower than 5 mg/L. In Table 1, different pathologies and corresponding expected CRP levels are presented. During viral infection, CRP concentration in plasma is usually not or only mildly elevated. Nevertheless, certain viral infections, can induce CRP levels towards 50 mg/L. The CRP levels can increase 1000-fold or even 10,000-fold with bacterial infection. The physician will suspect bacterial infection in patient when CRP concentration is higher than 40 mg/L, while CRP concentration higher than 100 mg/L is considered highly predictive of inflammation due to bacterial infection. In severe invasive infection or sepsis CRP values can spike up to 500 mg/L.

Taking this information in to account, measuring CPR can prove useful in detecting bacterial infection and in monitoring the efficacy of the treatment with antibiotics. Determination of CRP can also help distinguish between latent and active phase of rheumatic diseases and efficiency of (immunosuppressive) treatment in these patients. Measuring its concentration is helpful also when evaluating effectiveness of post-operative procedures. Additionally, high-sensitivity CRP test (hs- CRP) can determine the risk of developing cardiovascular disease. CRP values in cardiovasculardisorders are low and usually within normal range. However, only small changes in CRP level can indicate a risk for developing e.g. coronary heart disease. That is why high sensitivity method for CRP detection is used when assessing risk for cardiovascular diseases. Despite its significance in diagnosis of bacterial infection, elevated concentration of CRP is, notably, disease-unspecific and has to be interpreted together with other clinical parameters.

Other laboratory parameters indicating acute inflammation:

- White blood cell count
- Erythrocyte sedimentation
- Other acute phase proteins (e.g. procalcitonin)

Table 1. *Expected CRP values for different pathologies triggering (acute) inflammation.*

Pathology		**CRP concentration in serum (mg/L)**
Bacterial infectionsg	Localized (tonsillitis, middle-ear inflammation)	20-100
	Severe (pneumonia, meningitis, pyelonephritis) Sepsis	>100
Viral infections	Viral pneumonia, meningoencephalitis, influenza	10- 20
	Cold	10
Inflammatory and autoimmune disorders	Rheumatoid arthritis	10-20
	Systemic lupus erythematosus	10-30
	Acute pancreatitis	10-30
Necrosis	Myocardial infraction	10-30
	Cancer	10-40

Reference values: *Serum CRP*	< 5 mg/L

METHODS FOR DETERMINATION OF CRP

CRP is determined immunochemically. CRP reacts with anti-CRP antibodies forming immune complexes. CRP concentration can be measured as light scattered from these complexes. Two most commonly used methods exploiting light scattering for detection of immune complexes in general are nephelometry and turbidimetry.

IMMUNOCHEMISTRY OF IMMUNE COMPLEXES

Interaction between antigen (Ag) and antibody (Ab) and the consequent formation of immune complexes is the basis for several physiological responses of immune system. Besides, their formation is well-exploited in molecular biology, e.g. the usage of antibodies as reagents to detect specific antigen in the sample. High specificity and avidity of antibodies are two major advantages for which immune complexes gained importance in analysis of biological and clinical samples. The interaction between Ab and Ag is possible on account of several different noncovalent bonds: hydrogen bonds, van der Waals bonds, electrostatic bonds and hydrophobic interactions. The extent of this interaction is dependant on avidity and affinity of the Ab for antigen of interest.

NEPHELOMETRY AND TURBIDIMETRY

The immune complexes are small multimolecular particles that can be detected by measuring light scattered from their surface. The light scattering occurs upon exposure of particles to the laser beam and is directed in all angles in respect to the laser beam. The light scattering from immune complexes is independent of the wavelength of the laser, however the direction depends on the ratio between size of the immue complex and wavelength. During scattering the laser light loses its intensity in the direction of the laser and gains intensity in the other directions. The extent of scattering can be measured using detector positioned either perpendicularly or parallelly to the laser beam (Figure 2). In the first case, the light scattered from the particle is measured directly and the method is called nephelometry (Figure 2, b). In the second case, we detect the residual light – the light that was not scattered by the analysed sample and remained in the direction of the beam (Figure 2, a). This method is called turbidimetry. For turbidimetry any spectrophotometer can be used and the procedure is similar to measuring the absorbance of the sample.

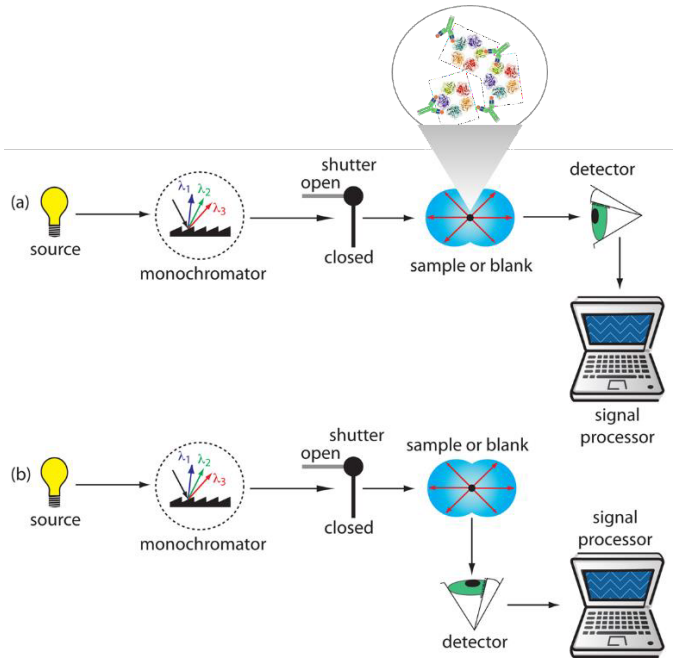


Figure 2. *The light path and the detection of scattering by turbidimetry (a) and nephelometry (b). Created with BioRender.*

HEIDELBERGER-KENDALL CURVE

Imagine different biological samples with an antigen whose concentration we want to measure. The same amount of antibody against this antigen is added to each sample. In samples with a very low antigen concentration, only a few immune complexes are formed, and the intensity of the light scattered in this sample is low (Figure 3). As the antigen concentration increases, the number and size of the immune complexes increases, resulting in more scattering. The scattering signal increases linearly with the concentration of the antigen as long as the concentration of the antibodies exceeds the concentration of the antigen in the sample. This area is called the "zone of antibody excess". In the sample where the concentration of the antigen is similar to the concentration of the antibodies, the "zone of equivalence" is reached. This means that all binding sites on antibodies are connected with all antigens in the maximum possible network of immune complexes. Therefore, the scatter signal in this sample is as high as possible. If you analyse the sample with an antigen concentration that is higher than the antibody concentration, the immune complexes will start to dissolve because there are more binding sites for antibodies in the sample. The number and, more importantly, the size of the immune complexes decreases, resulting in a

decrease in the amount of light scattered in the sample. The area where the scattered signal decreases with antigen concentration is called "the zone of antigen excess".

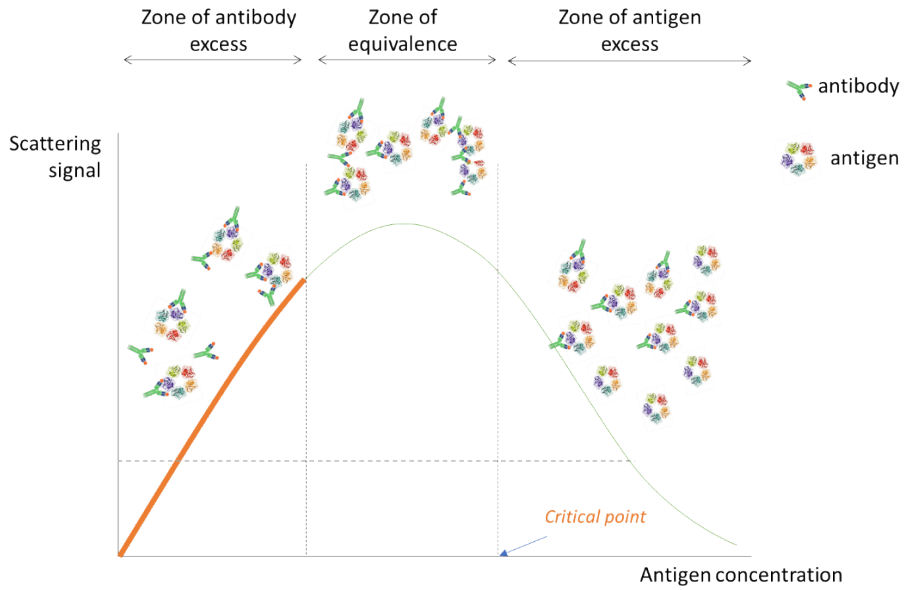


Figure 3. Heidelberger-Kendall curve. The scheme was prepared using SMART - Servier Medical ART 3000.

The biological phenomena described can be represented in a Hiedelberger-Kendall curve (Figure 3). We can observe that the sample with a relatively low antigen concentration can produce a similar scattering signal as the sample with an extremely high antigen concentration when both samples are exposed to the same amount of antibody (Figure 3, horizontal dashed line). If we use the immunochemical method to determine CRP concentration, the sample from a patient with severe sepsis would have a very low scatter signal although the CRP concentration is extremely high. Lower CRP concentration, calculated from this sample, will indicate a diagnosis other than bacterial infection. To avoid ambiguity, such samples should be diluted to reach the zone of antibody excess where the relationship between scatter signal and CRP concentration is linear.

TEST YOUR KNOWLEDGE

1. Draw a plot of scattering signal with respect to the CRP concentration, if the amount of anti-CRP antibodies is constant. Indicate the region in which CRP level is determined.

2. Which diagnosis is highly possible at CRP concentration in serum >100 mg/L?

3. Name three parameters beside CRP that are elevated in inflammation.

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2. Black S, Kushner I, Samols D. C-reactive protein. The journal of biological chemistry. 279, 47; 48487-48490. 2004.

3. Servier Medical Art. https://smart.servier.com/ Accessed: 28.2.2019

LAB REPORT: C-REACTIVE PROTEIN

During this practical work, CRP will be determined immunochemically: Ag + Ab →Ag-Ab. CRP in serum reacts as an antigen with antibodies against CRP. The concentration of immune complexes formed in the sample is proportional to the concentration of CRP in the sample. Immune complexes will be measured at 340 nm using turbidimetry. The concentration of CRP will be determined from the linear curve obtained from standars of known CRP concentration.

Protocol

1. Dilute standard No 5 twice with Assay Buffer (R1) to obtain concentration of 40 mg/l.
2. Pipette according to the scheme below in 1.5 ml tube (in µL):

	Standards	**Sample**	**Control**
Assay Buffer (R1)	200	200	200
Diluted standard	10		
Sample (serum)		10	
Control			10

3. Mix and transfer 100 µL of each reaction mixture into a cuvette.
4. Measure the absorbance (scattering) of blank solutions at 340 nm.
5. Add 20 µL of Antibody reagent (R2) into the residual solution in the first tube. Mix well and incubate for 5 min at room temperature.
6. Measure the absorbance (scattering) of final solutions at 340 nm after exactly 5 min. *Mix well before the measurement.*

$$c_s = c_{st} \cdot \frac{(A_s - A_{s\,blank})}{(A_{st} - A_{st\,blank})}$$

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Measurements

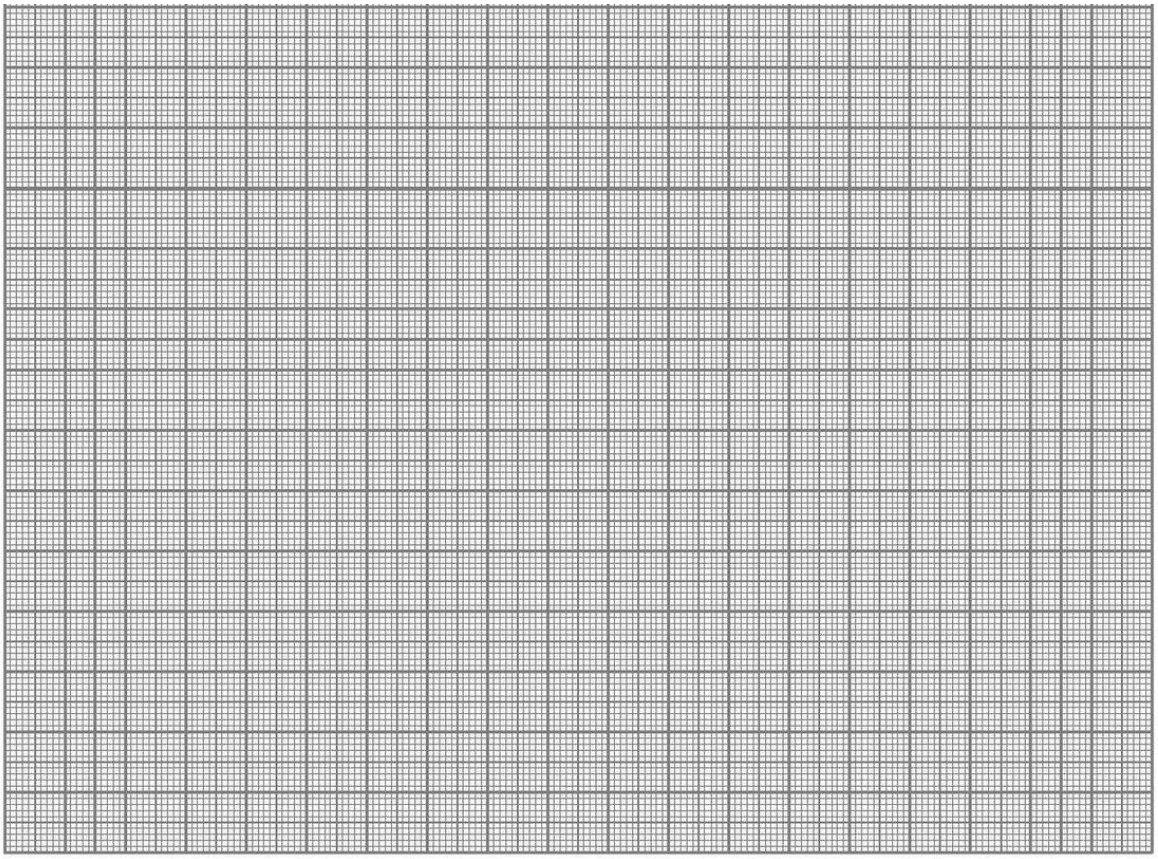
Blank	**A (340nm)**	**Sample**	**A (340nm)**
Control		Control	
Standard 6 (160 mg/l)		Standard 6 (160 mg/l)	
Standard 5 (80 mg/l)		Standard 5 (80 mg/l)	
Standard 5' (40 mg/)l		Standard 5' (40 mg/)l	
Standard 4 (20 mg/l)		Standard 4 (20 mg/l)	
Standard 3 (10 mg/l)		Standard 3 (10 mg/l)	
Standard 2 (2.5 mg/l)		Standard 2 (2.5 mg/l)	
Standard 1 (0 mg/l)		Standard 1 (0 mg/l)	
Serum sample		Serum sample	

Values for standards and controls

Standard:

Control:

Graphs and calculations



Analytical evaluation (samples, controls, graph):

Clinical interpretation:

Signature: Date:

BASIC URINALYSIS

Asist. Dr. Dunja Urbančič, MPharm

LEARNING OUTCOMES

After completing this laboratory practical, the student will be able to:

- list in detail the components of the basic urin analysis,
- describe the organoleptic properties of urine and stat most common pathological causes for their deviation from normal,
- explain in detail each pad on the dipstic, in terms of analytes, chemical/enzymatic reaction, physiological readings and pathological conditions for positive reaction of particular analyte,
- recognize pathological urine sediment and tell the underlying potential couse(s),
- apply the knowledge obtain in this practical to perform urine dipstic test, organoleptic examination and microscopic examination of a urine sample in the laboratory,
- synthesize results from all three parts of basic urinanalysis and interpret the results from clinical samples.

BASIC ANALYSIS OF THE URINE SAMPLE

Basic urinalysis (Figure 1) contains a description of a physical and physicochemical properties of urine, a chemical analysis, and a microscopic examination of the urine sediment.

Urine collection, storage, stabilization, and transport are the most important elements of the preanalytical phase. For screening tests random sample is normaly used. However, random urine specimens are associated with many false negative and some false positive results. The first (6-8h in the bladder) or second (2-4h in the bladder) morning urine is the sample of choice.

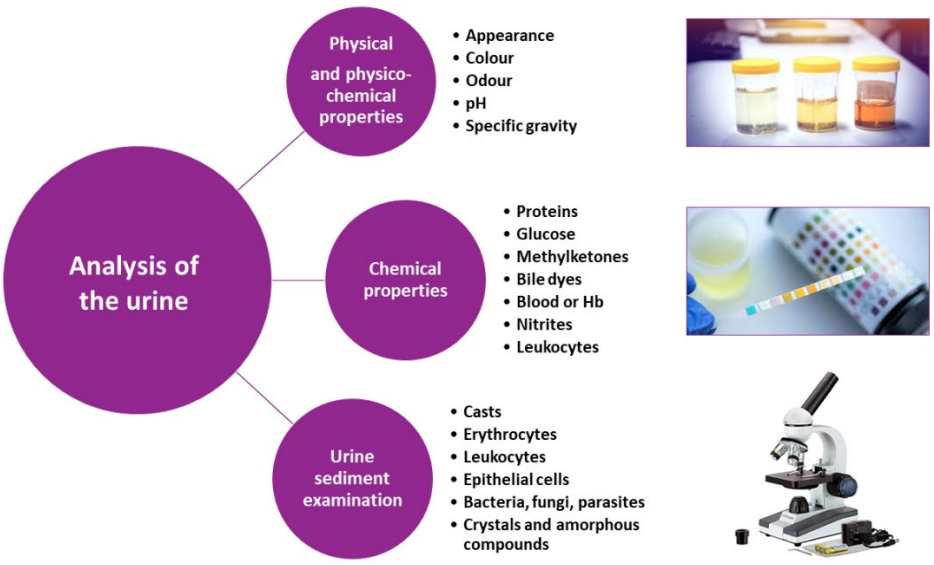


Figure 1. *Elements of basic urin analysis.*

Proper sample collection is very important for reliable urinalysis. The first urine stream is always contaminated with the normal bacterial flora of the urethra (in both genders) or the vaginal flora in women, therefore, a sample of a midstream urine must be collected.

Only freshly collected urine can be examined, which means that the urine sample should be tested within two hours upon the receipt. If the specimen cannot be analyzed in 2-6 hours, it can be refrigerated for not more than 4 hours before examination for the analysis on reagent strip and for not more than 8 hours for microscopic examination of the urine. Note, that urates and phosphates may precipitate in refrigerated samples, however, they dissolve at room temperature.

Improper collection of urine sample may result in changes in physical properties (darker colour, turbidity, foul odour), chemical properties (lower or higher pH), and microscopic changes (lysis of erythrocytes, leukocytes, and casts, presence of bacteria, precipitation of uric acid).

ORGANOLEPTIC EVALUATION OF THE URINE

The physical characteristics of the urine sample, such as the appearance (**turbidity** and **colour**), and **odour**, are evaluated organoleptically. The fresh urine sample from a healthy patient is usually clear, yellow, and has a characteristic odour reminiscent of the smell of a beef broth.

Table 1. *Most common urine odours and their causes.*

Odour	Potential couses
»beef soup«	normal urine
ammonia	old or incorrectly stored urine
ammonia or feet	urinary infection
sweet or fruity	production of ketones due to: • diabetes • starvation, dieting, malnutrition • excessive physical activitiesv • omiting, diarrhea
musty or mousy	phenylketonuria
maple syrup	maple syrup disease (leucine, isoleucine, valine)
rotten / old fish	trimethylaminuria
rancid/pungent	tyrosinemia
characteristic odours	food ingredients, medicines: asparagus, garlic, onion medicines containing phenol (menthol)

Urine turbidity

Urine of a healthy individual is clear (transparent) and has no visible particles. Any constituents present are soluble. If not clear, urine may be described as:

- slightly cloudy: some particles are present, the typed text is not cloudy when viewed through the sample;
- cloudy: particles are visible, when viewed through the sample typed text is visible but cloudy;
- very cloudy (opaque): when viewed through the sample typed text is not visible;
- flocculent: containing numerous shreds or fluffy particles.

Turbidity of the urine depends on the concentration and type of urinary component. Common causes of abnormal turbidity include:

- non-pathological components: urates, phosphates, mucus, mucin, epithelial cells, sperm, prostatic fluid, fecal contamination, radiographic dye, lotions, creams, talc.
- pathological components: unusual crystals, leukocytes, erythrocytes, bacteria, fungi, renal epithelial cells, fats (lipids, chylomicrons), kidney stones.

Table 2. *Selected urine colours and potential causes.*

Appearance	Cause	Remarks
colourless, light yellow	dilulted urine	polyuria, non-fasting specimen
yellow	normal urine	
	flavines	vitamin B intake
	concentrated urine	dehidration, heat, yellow foam
yellow-orange	urochrome/urobilin	
	sulfasalazine, phenytoin, nitrofurantoin	
yellow-green  yellow-brown	Bilirubin, biliverdin	yellow foam, beer-like
red 	haemoglobin, Ery	positive strip result, menstruation
	myoglobin	positive strip result, muscle damage
	porphyrins	porphyria, may be colourless
	beetroot, carotenes	food
red-pink	urate	may be associated with crystalluria
brown or black 	haemoglobin	positive strip result
	myoglobin	muscle damage
	methaemoglobin	acidic pH
	melanin	melanoma

Urine odour

The urine of a healthy person, containing mostly water and very small amount of other constituents, has a mild odour similar to that of beef broth. There are many different endogenous metabolites, food components, and medications that can cause changes in the odour of urine. Some urine odors are characteristic for certain metabolic diseases (Table 1).

Urine colour

Normal urine colour ranges from pale yellow to deep amber. This colour is caused by a compound called urochrome. The intensity of colour depends on how dilute or concentrated the urine is. Other pigments and compounds in certain foods and drugs can also change the colour of urine. Beets, berries and fava beans are among the foods most likely to affect colour. Many over-the-counter and prescription drugs impart vivid hues to urine, such as red, yellow or even greenish-blue. An unusual urine colour can also be a sign of disease. For example, deep red to brown urine is a tell-tale sign of porphyria, a rare, inherited disorder of red blood cells. Some examples of unusual urine colour are listed in Table 2.

PHYSICOCHEMICAL PROPERTIES AND CHEMICAL ANALYTES IN URINE

Physicochemical properties and chemical analytes in urine can be rapidly measured using urine test strips or »dipsticks«. The reagent strips for basic urinalysis comprise pads for 10 analytes such as proteins, blood, leukocytes, nitrites, glucose, ketones (acetoacetic acid), bilirubin and urobilinogen as well as physical properties such as pH and specific gravity.

Methodology behind reagent strips

Reagent strips is a basic diagnostic tool in which the reagents are incorporated into carriers in a dry state. The reagent strips consist of up to 10 different chemical pads, that are made of cube-shaped cellulose matrices, which contain dry chemical reagents. When immersed into the urine sample, the reagents dissolve in the water from the urine and a chemical reaction occurs between the analyte and the reagents, resulting in the formation of a chromogen. The resulting change in colour or its intensity can be read by the analist, comparing colour scale on the container of the reagent strip, or using a reflectometer (Figure 2).

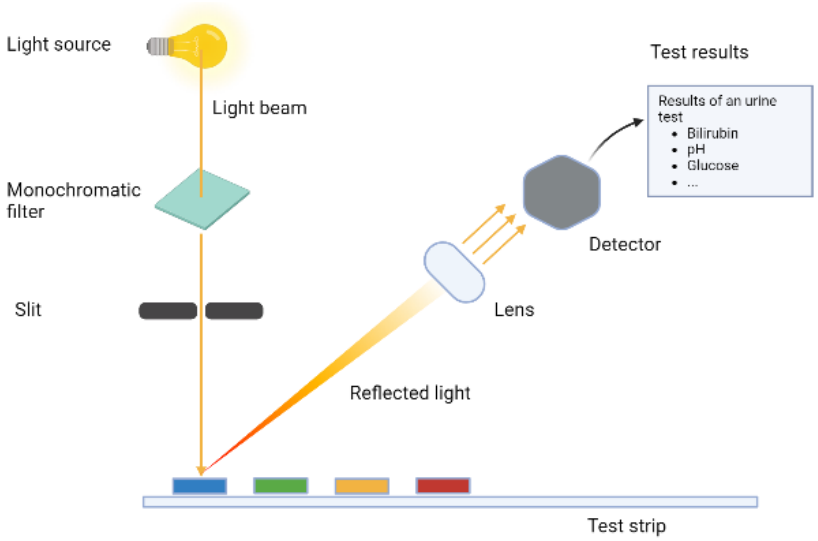


Figure 2. *Schematic representation of reflectometer. Created with BioRender.com*

Instrumental readings are obtained by absorption-reflection spectroscopy, which measures the light absorbed and scattered by the reagent strip. Just as in classical photometry, the intensity of the reflected light (I_R) is compared to the intensity of the light emmited to the reagent strip (I_0). When the light beam reaches the reagent strip, the light is absorbed by the resulting product (chromogen), and/or scattered by particles on the reagent field. The intensity of reflected light measured by the reflectometer is a function of absorption and scattering (so I_R is always less than I_0). The reflectance (R) is the ratio of I_R to I_0, and the decimal logarithm of R is called reflection density (DR). Due to differences in the perception of the human eye and the optical system of the measuring instrument, there is not always perfect agreement between visually and instrumentally determined results. However, the use of reflectometers has greatly improved the accuracy and reliability of the results.

pH

Reference values	4.5-8.0

The reagent strip contains a dual indicator system (methyl red and bromothymol blue) that changes colour depending on the pH of the urine. The pH also depends on the person's diet. An acidic pH in urine is present in people who eat mainly proteins, while it is alkaline in vegetarians and people who eat few carbohydrates. An acidic urine may also indicate metabolic or respiratory acidosis, while an alkaline pH in urine may indicate alkalosis. The pH

also determines what amorphous salts and crystals will be present in the urine. At acidic pH we may observe urates, while at alkaline pH we observe phosphates.

Interferences: The growth of certain bacteria in the urine specimen (due to infection or contamination) can result in a very alkaline urine (pH > 8), mainly due to the conversion of urea to ammonia. The intake of medication can also influence the pH of the urine.

Specific gravity

Reference values	1.001 – 1.035

The polyelectrolytes on the specific gravity reagent pad act as ion exchangers, releasing hydrogen cations in exchange for urine cations. The hydrogen ions released are determined by the pH indicator bromothymol blue applied to the same pad on the test strip. The amount of hydrogen ions released is proportional to the concentration of cations in the urine and is only an estimate of the osmolality of the urine. The result of the specific gravity provides information about the hydration status of the subject and the ability of his kidneys to concentrate the urine. Values depend on the amount of fluid ingested, ambient temperature, and physical activity. Lower values can be expected if you have been drinking a lot of fluid, in diseases where the ability to concentrate urine is impaired, and in diseases such as diabetes insipidus. Higher values may be due to excessive sweating (physical activity, febrile conditions), dehydration, and various diseases. In isostenuria, in which the kidneys lose their ability to concentrate urine, the specific gravity of the final urine is equal to the weight of the primary plasma ultrafiltrate (1.010), regardless of fluid intake.

Interferences: The test determines only the ionized components of urine, so increases in non-ionized components, such as glucose (which also contributes to osmolality), cannot be detected. Proteinuria can lead to falsely elevated results.

Proteins

Reference values	up to 0.1 g/L

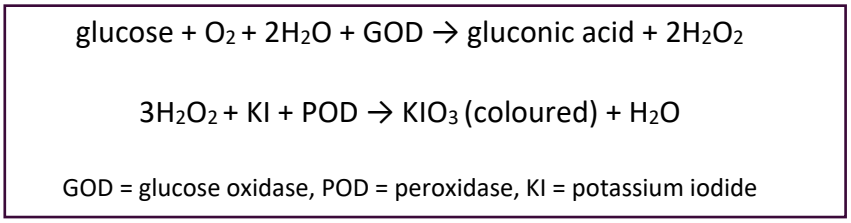
The main component of the protein reagent pad is a buffer solution that maintains a constant pH of 3. Tetrabromophenol blue indicator is used in the same pad. The amino groups of proteins are proton acceptors. In an acidic environment, amino groups in proteins accept proton from tetrabromophenol and become protonated to the form $-NH3^{+}$. Deprotonation of

tetrabromophenol results in a change of colour to blue-green. There are many possible causes for the presence of proteins in urine, which are divided into prerenal (increased haemoglobin concentration, myoglobin, Ig light chains), renal (glomerulonephritis or secondary glomerular damage due to diabetes, infectious diseases, drugs ...) and postrenal (inflammation, injury or malignancy of the urinary tract). Proteins may also be transiently elevated after strenuous physical activity, orthostatic proteinuria, urinary tract infections, dehydration, and fever-related illness.

Interferences: The test is not specific for any particular type of protein, but is most sensitive to albumin (as it is most abundant in the blood and contains many amino groups). The lower limit of detection for albumin is 15-30 mg/dL. The test is less sensitive to mucoproteins and globulins, which can usually be detected at a concentration of 60 mg/dL or more. A negative result does not exclude the presence of proteins.

Glucose

Reference values	up to 0.8 mmol/L



Normal concentratons of glucose in urine range between 0 and 0.8 mmol/L, however, kidneys can excrete up to 1.7 mmol/L (i.e. 30 mg/dL) of glucose in physiological conditions. These amounts are below the detection limit of the reagent strip. The sensitivity for glucose on the reagent strip is between 4.2 and 7.0 mmol/L (75–125 mg/dL) and this state is called glucosuria. Glucosuria occurs when the threshold for tubular glucose reabsorption is exceeded; that occurs when blood glucose reaches approximately 10 mmol/L. Causes may be prerenal (hyperglycemia due to diabetes, hormonal disorders such as Cushing's disease, pancreatic disorders ...) or renal (tubular damage due to Fanconi syndrome, heavy metal poisoning ...). Diabetes is the most common cause of glucosuria.

Interferences: The test is glucose specific, but oxidants and peroxides or antioxidants (on laboratory materials, glassware) can cause false positive or false negative results, respectively.

Ketones

Reference values	up to 2 mg/dL (0.2 mmol/L) of acetoacetic acid

Acetoacetic acid reacts with sodium nitroprusside at alkaline pH to form a purple coloured complex. Normally, ketones are not detected in urine. A ketone concentration of 10 mg/dL or more occurs in ketoacidosis, starvation, or other abnormalities of carbohydrate or lipid metabolism. Trace amounts of ketones may be present in physiological stress situations such as starvation, pregnancy, or exercise.

Interferences: The reaction proceeds only with acetic acid in urine and not with acetone or β-hydroxybutyric acid.

Bilirubin

Reference values	negative (0.34 µM)

In the pad on the reaction strip bilirubin reacts with diazotized dichloroaniline in a strongly acidic medium to form a coloured azo dye.

bilirubin glucuronide +	Ar - N + ≡N (acidic pH)	→	azobilirubin (aromatic compound)
	(diazonium salt)		(brown azo dye)

Bilirubin is not detected in the urine of healthy individuals. Bilirubin in urine may be a sign of liver disease such as hepatitis or cirrhosis, or may indicate obstruction of the bile ducts.

Interferences: The sensitivity of the test is too low to detect early stages of liver disease, in which very small amounts of bilirubin (1.71 µM) may be excreted in the urine. Due to oxidation upon the exposure to UV light, the specimen must be protected from light.

Urobilinogen

Reference values	up to 16 µM

The reaction pad for urobilinogen contains reagents for a modified Ehrlich reaction. P-Diethylaminobenzaldehyde reacts with urobilinogen in a strongly acidic medium to form a

pink colour. A value of 34 µM represents a transition from a normal to a pathologic state requiring further investigation to determine hemolytic or liver disease. Joint evaluation of bilirubin and urobilinogen is helpful in the differential diagnosis of jaundice and other diseases of the hepatobiliary tract.

Interferences: False positive results can result from other compounds that react with the Ehrlich reagent, such as p-aminosalicylic acid and sulfonamides. Atypical colour reactions may occur in the presence of high concentrations of p-aminobenzoic acid. The test detects urobilinogen in urine at very low concentrations (3.4 µM).

Blood (haemoglobin)

Reference values	< 0.1 mg/L Hb (3 Erci/µL)

The basis of haemoglobin detection on the test strip is the peroxidase activity of haemoglobin, which catalyses the oxidation of chromogen to a green coloured product. The appearance of green dots in the test area indicates the presence of intact erythrocytes, while a uniform green colour indicates the presence of free haemoglobin. Haemoglobin is not detected in normal urine. The so-called "occult" blood (haemoglobin or intact erythrocytes) appears in urine due to internal bleeding and/or diseases of the urinary tract and kidneys. The presence of traces of blood already indicates a pathological condition, and further investigations are required to clarify the causes. The most common causes of hematuria are diseases of the kidney and urinary tract (glomerulonephritis, cystitis, stones, tumours), hemoglobinuria is caused by intravascular hemolysis (transfusion reactions, hemolytic anaemia, infections), and myoglobinuria is caused by damage to the skeletal or cardiac muscle (surgery, ischemia...), toxins (heroin, snake venom). All these conditions can be caused by excessive physical exertion.

Interferences: Myoglobin and oxidants (hypochlorite) can cause false positives. Microbial peroxidases, which can occur in urinary tract infections, can also cause a false positive result. False negative results are caused by increased amounts of ascorbic acid in the urine. Menstrual bleeding should be excluded as a positive result.

Nitrites

Reference values	negative

The reagent pad for nitrite determination contains an aromatic amine (p-arsanilic acid), which forms the diazonium salt with nitrites in acidic medium. The diazonium salt then reacts with the aromatic compound to form a pink azo dye.

$$Ar - NH_2 + NO_2^- \text{ (acidic pH)} \rightarrow Ar - N + \equiv N$$

(p-arsanylic acid) (diazonium salt)

$$Ar - N^+ \equiv N \quad + \quad Ar' \rightarrow Ar - N = N - Ar' \text{ (azo dye)}$$

Gramme-negative bacteria convert nitrates, which come from the patient's diet, into nitrites. During infection, the concentration of nitrites increases over time due to the retention of bacteria in the bladder. Detection of nitrites on the reagent strip depends on when the urine specimen is collected. The manufacturer recommends that the urine remain in the bladder for at least 4 hours before the sample is collected for analysis.

Interferences: A negative result does not mean that no bacterial infection is present, as this may be due to insufficient incubation time in the urine, insufficient nitrate uptake, or the presence of non-nitrate reducing bacteria.

Leukocytes

Reference values	negative

The reagent pad for leukocyte detection in urine utilizes the esterase activity of granulocytes, which catalyzes the hydrolysis of the ester and releases the aromatic compound. The latter binds to the diazonium salt in the pad and forms a purple azo dye.

$$\text{Ester} + \text{leukocyte esterase} \rightarrow \text{aromatic compound } (Ar') + \underbrace{Ar - N^+ \equiv N}_{\text{diazonium salt}} \rightarrow \underbrace{Ar - N = N - Ar'}_{\text{azo dye (purple)}}$$

Elevated leukocyte levels (≥10 leukocytes/µl) are indicative of pyuria (leukocyturia) and occur in almost all renal or urinary diseases. Leukocyturia can also occur in the absence of bacteria (i.e., sterile pyuria).

Interferences: Some antibiotics can cause false-negative results (tetracyclines, cephalosporins). False-positive results may be due to contamination with vaginal discharge (which may be observed as a greater number of squamous epithelia on microscopic examination of the urine sediment).

MICROSCOPIC EXAMINATION OF URINE SEDIMENT

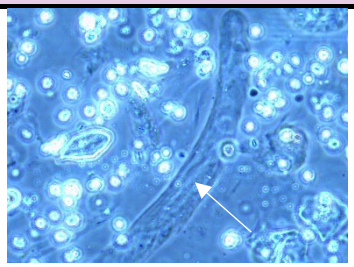
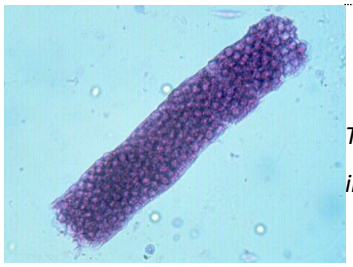
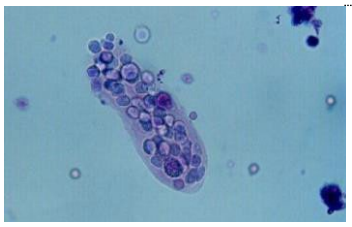
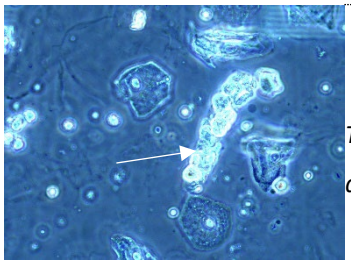
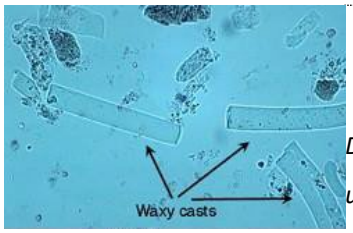
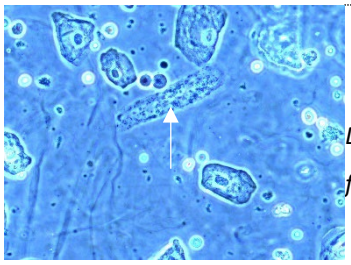
Microscopic examination of urine sediment is an essential part of basic urinalysis, as it can confirm and clarify the results obtained with the reagent strip (e.g. leukocytes, blood). However, it can also provide data on the structures in the urine that cannot be deduced from the chemical analysis of the urine. The structures that can be seen in the urine sediment under the microscope are casts, cells and crystals. Prior to examination, the urine is centrifuged to concentrate the solid components. A drop of the concentrated sample is then placed under a coverslip and examined, usually at 100x and 400x magnification. The microscopic constituents of urine are reported as an amount of a component present in the field of view. Urine is traditionally examined using light microscopy, but some laboratories use phase contrast microscopes, which can better visualize elements such as urinary crystals and mucus.

Casts

Reference values	negative (the exception being hyaline casts)

Casts are peel-offs of the distal tubules and collecting ducts in the kidney. They have rounded ends and may vary in length and thickness. They are composed mainly of proteins secreted from the renal tubules under physiological conditions (Tamm-Horsfall mucoproteins). Non-pathological hyaline casts (formed in healthy tubules) are narrow, whereas we observe thicker casts when the tubules are damaged. The formation of casts is influenced by factors that contribute to protein denaturation and precipitation: low urine flow, increased salt concentration, fever, and low pH. If other components (cells, proteins, fat droplets, bacteria, etc.) are present in the lumen of the tubule during precipitation of tubule proteins, they will become entrapped in the protein matrix of the cast and form complex casts. Table 3 summerizes the most common forms of casts.

Table 3. *Type of casts and their (patho)physiological relevance.*

Type of cast	Microscopic view	Potential causes
hyaline		• in most kidney diseases • after physical exertion, during heat conditions, dehydration…
erythrocyte		• in hematuria *The presence of even a single erythrocyte cast is an indicator of glomerular bleeding*.
leukocyte		• especially in acute infection of the upper urinary tract • also in non-bacterial kidney infections (e.g. glomerulonephritis)
epithelial		• in nephrotic syndrome, acute necrosis of tubules *They contain tubular epithelial cells and are difficult to distinguish from leukocyte casts.*
waxy		• in renal failure, rapidly progressing glomerular diseases *Derived from cellular or hyaline casts that are transformed under the influence of substances present in the urine.*
granular		• in kidney diseases *Dark granule casts are common in patients with renal failure.*

Erythrocytes

Reference values	up to 3 per field of view (400 x magnification)

Under the microscope, erythrocytes can be seen as biconcave cells without a nucleus. With an average diameter of 6 µm, they are smaller than leukocytes and epithelial cells, but slightly larger than yeasts. Their appearance is influenced by the osmolality of the urine: in hypertonic urine, erythrocytes are contracted and wrinkled; in hypotonic urine, however, they swell and lose haemoglobin. In the he latter conditions, they can be seen as empty cells surrounded by a membrane, but they are difficult to see under a light microscope. Alkaline and hypotonic urine (specific gravity ≤ 1.009) promotes lysis and degradation of erythrocytes (this may occur in the urinary tract itself), so only haemoglobin can be determined in such samples. Erythrocytes appear in urine as a result of damage to the vascular barrier in the kidney and urinary tract due to disease or mechanical injury and when capillaries are more permeable during the inflammatory process.

Interferences: Other components of urinary sediment may resemble erythrocytes: yeasts, some forms of calcium oxalate crystals, fat droplets, or air bubbles. If erythrocytes are visible under the microscope but not identified with a reagent strip, interference with ascorbic acid is possible.

Leukocytes

Reference values	up to 5 per field of view (400 x magnification)

Polymorphonuclear neutrophils are the most commonly observed leukocytes in urine sediment. They are round cells with an average diameter of 10-14 µm, a segmented nucleus and granular contents. They often appear in clumps in urinary tract infections.

Interferences: Eosinophils, lymphocytes, monocytes, and macrophages may also be present in urine sediment. In a routine examination of unstained urine sediment, it is difficult to distinguish between the different types of leukocytes. Some tubular epithelial cells also look very similar to leukocytes.

Epithelial cells

Reference values	normally present in small numbers

Different types of epithelial cells can be observed in the urine sediment: Squamous cells, transitional cells (uroepithelial cells), and epithelial cells of the renal tubules (cells of the collecting duct, cells of the distal spiral tubule, cells of the proximal spiral tubule). Typically, epithelial cells are larger than leukocytes and have a distinct nucleus. They arise from normal epithelial sloughing and regeneration or appear in the urine due to epithelial damage resulting from inflammation and renal disease.

Interferences: The most common epithelial cells in urine sediment are squamous cells, which may also occur with irregular sampling.

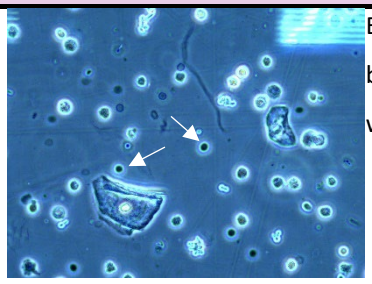
Microorganisms (bacteria, fungi, yeasts, parasites)

Reference values	negative

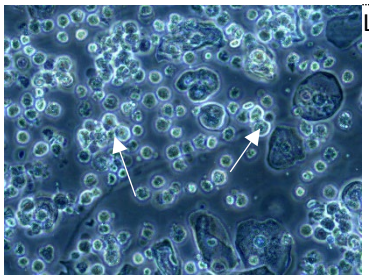
Bacteria are recognized as rods (rod bacteria) or small round cells that may flicker and form long strands of filamentous cells. Bacteria are present in urine during urinary tract infections when leukocytes are also elevated. Yeasts are recognized as oval, round, or elongated budding cells and may also form long filamentous mycelia. The most common yeast in urine is Candida albicans, which can also grow in the urinary tract, especially in patients with diabetes, after prolonged antibiotic or immunosuppressive therapy.

Interferences: When many squamous cells and bacteria are present, contamination during collection and wrong handling of the urine specimen are suspected . Round yeasts resemble erythrocytes but are slightly smaller and often contain a nucleus. Candida, the protozoan Trichomonas vaginalis, and the parasite Enterobius vermicularis most commonly occur due to contamination with genital secretions.

Table 4. *Types of cells and their relevance in urine sediment examination. Phase contrast microscopy. Fresh and unstained urine sediment. Original magnification 400x.*

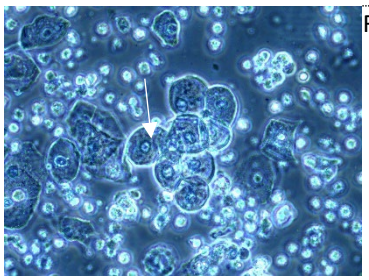
Type of cells	Microscopic view	Description
Erythrocytes		Erythrocytes can take many forms: round biconcave cells without nucleus or shrunken, wrinkled cells with outgrowths.

Leukocytes



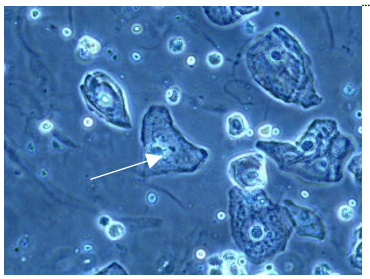
Leukocytes are round cells with nucleus.

Epithelial cells

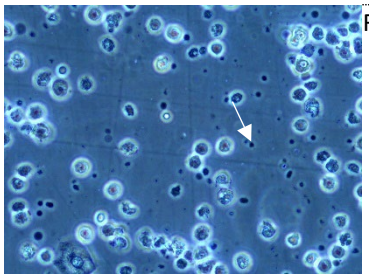


Round cells of the uroepithelium.

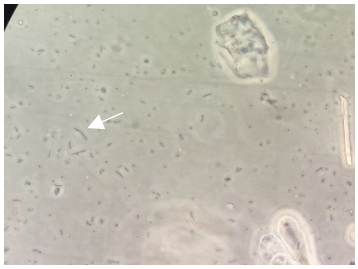
Squamous epithelial cells



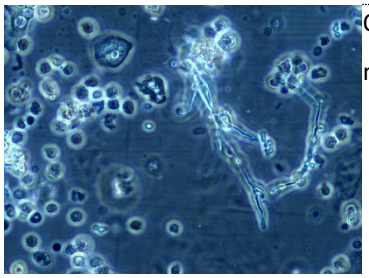
Bacteria



Round small cells or cells in the shape of rods.



Fungi (yeasts and hyphae)



Oval or round cells that "bud" or form filamentous mycelium.

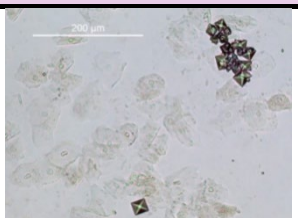
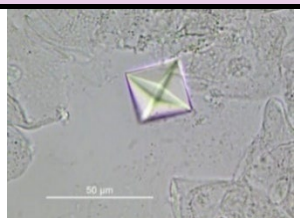
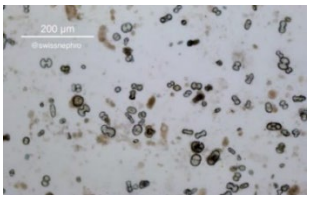
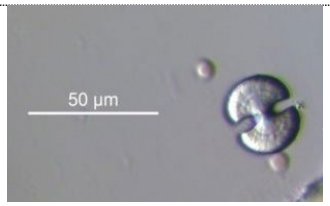
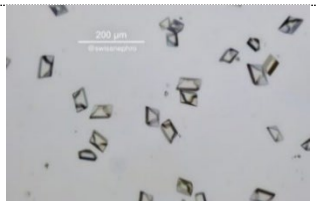
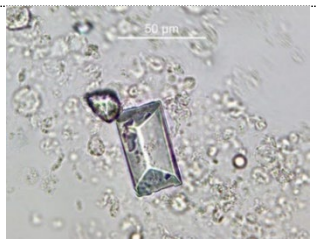
Crystals and amorphous salts

Reference values	only non-pathological

The salts present in urine may be deposited in amorphous or crystalline form (especially if the urine is allowed to stand). They are usually not pathological (carbonates, oxalates, phosphates, urates, ...) and have no clinical significance. Some crystals are found mainly in acidic urine (e.g. uric acid crystals), while others predominate in basic urine (e.g. phosphates). Certain substances that are pathologically present in urine (amino acids, bilirubin, cholesterol) may also crystallize and appear in urine sediment.

Some of the most commonly observed cells and crystals in urine sediment are listed in tables 4 and 5, respectively.

Table 5. *Representative crystals that can be found in urine sediment.*

Type of crystal	Microscopic view
Calcium oxalate dihydrate	 
Calcium oxalate monohydrate	 
Uric acid	
Struvit	

OTHER TEST FOR ANALYSIS OF THE URINE

Other tests are sometimes recommended to provide more accurate information about the composition of the urine sample.If bacteria are present in the urine, the analyst can perform a urine culture test to determine the exact bacteria causing the infection. Similarly, the presence of bilirubin can be further investigated using Harrison's method. In this lab we will take a closer look at a more sensitive method for protein determination in urine - the sulfosalicylic acid test. Also, we will see how to distinguish between hemoglobinuria and hematuria.

TOTAL PROTEIN IN URINE BY SULFOSALICYLIC ACID TEST

The main disadvantage of determining proteins with the dipstick is their higher sensitivity to albumin but not to low concentrations of γ-globulins and Bence-Jones proteins. Lower protein concentrations can be determined by a sulfosalicylic acid (SSA) test. The presence of acid causes the proteins to denature, which in turn reduces their solubility and therefore precipitates the proteins. The precipitation occurs at room temperature, the resulting turbidity ("cloudiness") is roughly proportional to protein concentration (Figure 3). The result can be evaluated visually or measured turbidimetrically.

This method is considered to be one of the most sensitive methods for detecting proteins in urine, as it leads to the precipitation of all proteins (both albumin and globulins). The positive result should be interpreted in conjunction with the specific gravity result. A large volume of dilute urine (polyuria) can result in a negative result for reaction with proteins (proteins are too dilute to be detected) despite the presence of proteinuria. In addition, slightly elevated proteins in diluted urine indicate greater pathology than slightly elevated proteins in concentrated urine.

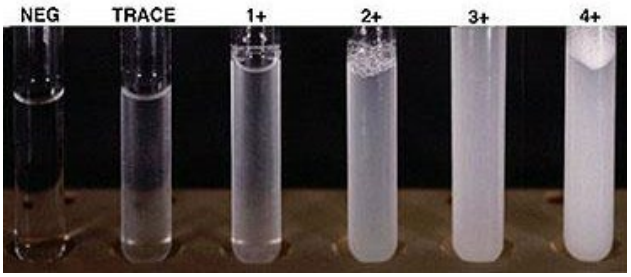


Figure 3. *The change in urine turbidity after addition of sulphosalicylic acid. The amount of protein in urine can be graded as trace – false turbidity, +1 – definite turbidity, +2 – heavy turbidity but no flocculation, +3 – heavy turbidity with light flocculation, +4 – heavy turbidity with heavy flocculation.*

DIFFERENTIATING HEMATURIA FROM HAEMOGLOBINURIA

Hematuria can be distinguished from haemoglobinuria organoleptically, microscopically and chemically (see above). Organoleptically, we examine the colour and cloudiness before and after centrifugation of the urine (Table 6). Microscopic examination of the sediment verifies the presence and number of erythrocytes.

Table 6. *Schematic protocol on how to distinguish between hematuria and haemoglobinuria.*

		HEMATURIA		HAEMOGLOBINURIA	
		alkaline pH	acidic pH	alkaline pH	acidic pH
organoleptic examination	before centrifugation	red, cloudy urine	brown, cloudy urine	red, clear urine	brown, clear urine
	after centrifugation	no longer red, clear urine	no longer brown, clear urine	red, clear urine	brown, clear urine

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2. Kouri T, Hofmann W, Falbo R, Oyaert M, Pestel-Caron M, Schubert S, Berg Gertsen J, and Merens A, on behalf of the Task and Finish Group Urinalysis of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). The EFLM European Urinalysis Guideline, update 2023. https://www.hdmblm.hr/images/vijesti/-2023/31-

01/EFLM_European_Urinalysis_Guidelines_Draft.pdf
3. Package insert for Siemens, Multistix 10 SG dipstic: https://wexnermedical.osu.edu/-/media/files/wexnermedical/healthcare-professionals/clinical-labs/forms-policies-procedures/point-of-care/clinitek/multistix-package-insert.pdf?%20la=en&hash=66CBDB2DBCA88640757CECD8F58AD8D70E666DB8, September 1, 2024
4. Urine fellow network. https://www.renalfellow.org/, September 23, 2024

TEST YOUR KNOWLEDGE

1. Reflect which components are:
    - Normally present in the urine
    - Pathologically present in the urine
    - Randomly present in the urine
2. What proteins (in terms of size) are expected in the urine for kidney disease, which primarily affects glomeruli compared to tubular damage?
3. What physical, chemical and microscopic changes occur in the urine sample if it is not analyzed in a timely manner?
4. Which of the reagents in the pad for urine blood detection on a dipstic does ascorbic acid react with and why does it interfere with the analysis?

LAB REPORT: URINALYSIS

Protocol for determination of the physical, physicochemical and chemical properties of the urine sample:

1. Prepare a stopwatch and set it to 2 minutes.
2. Stir the urine sample and transfer 10 ml of urine to a test tube.
3. Evaluate the appearance, colour and odour organoleptically.
4. Remove a reagent strip (dipstick) from the container and check that the test fields are intact and dry.
5. Dip the reagent strip into the urine sample in a tube and immediately pull it along the test-free side around the edge of the test tube.
6. Start the stopwach.
7. Read the value of each analyte after the exact number of seconds specified on the container. Compare the colour on each pad on the reagent strip with the colour box instructions corresponding to the analyte and determine the value semi-quantitatively.
8. Repeat the dipstick analysis on a Clinitek reflectometer using a new strip. The reflectometer will scan the pads at corresponding time and print out the results for the analytes tested. Note: The value of 1 EU/dl used by the machine for measuring urobilinogen is 16 µmol/L.

Protocol for microscopic examination of urine sediment:

1. Transfer 10 mL of well-mixed urine into a conical tube (Figure 4A).
2. Centrifuge for 5-10 minutes at 1500-2000 rpm.
3. Discard the supernatant and mix the sediment (cca 0.5 mL) well.
4. Transfer 20 µL of the sediment onto the quick-read urinalysis test slide (Figure 4B).
5. First, examine the sediment at 100x magnification under the light microscope.
6. Next, examine 5-10 fields of view at 400x magnification.
7. Observe structures and changes when applying phase contrast.
8. Write down your observations.

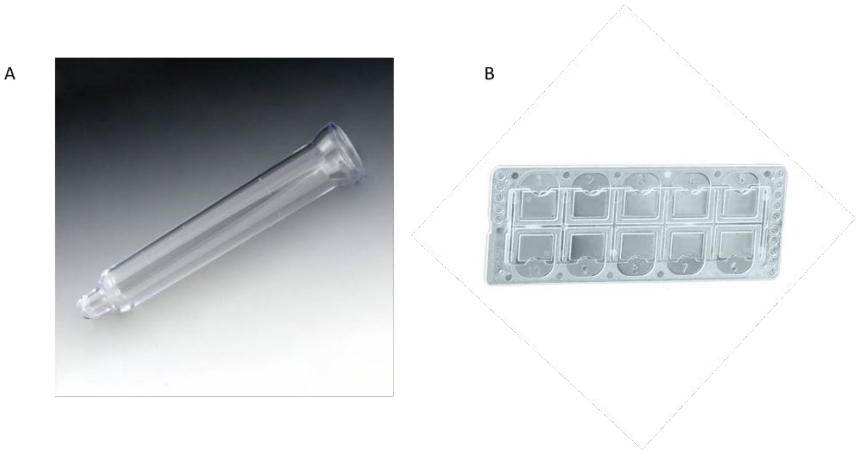

Figure 4. *Conical tube (A) and quick-read urinalysis test slide (B).*

Protocol for total proteins in urine with sulfosalicylic acid test

Reagent: Solution of sulfosalicylic acid in destilled water (200 g/L).

1. Transfer approximately 2 mL of centrifuged urine into two identical tubes.
2. Add 5-6 drops of the reagent into one of the tubes.
3. Mix and compare with urine in the control tube.
4. Determine the turbidity level.

Results and comments

Organoleptic examination of the urine:

Results from the dipstick:

component	visual inspection	reflectometer

Total proteins with sulfosalicylic acid test

Examination of the urine sediment

SEDIMENT COMPONENT	DESCRIPTION	RESULTS OF THE ANALYSIS
CASTS	The casts are counted and differentiated into hyaline, granular, erythrocyte, leukocyte, epithelial, waxy and mixed. The result is denoted as the number of individual types of casts per field of view under 100x magnification.	
ERYTHROCYTES AND LEUKOCYTES	The result is denoted as the number of Erci or. Lkci per the field of view under 400x magnification. If there are more than 50 Erci or Lkci in the field of view, this is denoted by "many". If there are more than 100 in the field of view, then we label it "very numerous". When Erci and Lkci are in clusters, we denote that as the number of clusters per field of view.	
BACTERIA, FUNGI, PARASITES	The result is denoted as the number of bacteri/fungi/parasites per the field of view under 400x magnification. We denote the presence of bacteria and fungi in terms of few, numerous, very numerous. The appearance of the bacteria is only described in the larger ones; e.g. rod-shaped, filamentous ... We denote all types of fungi only as fungi. For parasites, only name the parasite.	
EPITHELIAL CELLS	Cells are viewed under 400x magnification. We describe them only if there are many in the sediment and denote it as: numerous, very numerous.	
CRYSTALS	Crystals are viewed under 400x magnification. Common crystals are denoted as rare, moderately present or numerous. Pathological crystals are counted at low magnification and identified if possible.	

Clinical interpretation:

Signature: Date:

DETERMINATION OF SERUM CREATININE USING KINETIC JAFFE REACTION

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Assist. Dr. Jasna Omersel, MPharm, Spec. med. biokem., EuSpLM

LEARNING OUTCOMES

- describe the principles of the Jaffe reaction and its modifications for creatinine determination in serum samples,
- explain the theoretical background of the Jaffe reaction, its clinical significance, and the relationship between creatinine concentration and glomerular filtration rate (GFR),
- perform creatinine concentration measurement using the kinetic Jaffe reaction and apply the results to estimate GFR,
- evaluate the advantages and limitations of the kinetic Jaffe reaction for creatinine determination and compare the results with automated analyzing systems,
- apply the obtained knowledge to interpret creatinine measurement results, discuss their clinical implications, and troubleshoot common issues.

GLOMERULAR FILTRATION RATE AND CREATININE

Nowadays, glomerular filtration rate (GFR) represents the best marker for the estimation of kidney function. The determination of GFR is used for detecting kidney disease as well as for monitoring the course of an already diagnosed disease, the efficiency of treatment, and for predicting the need for transplantation. The estimation of GFR is also important for detecting the potential toxic effects of drugs undergoing renal excretion. As chronic kidney diseases significantly increase the economic burden on the health care system,

many studies were focused on the identification of an appropriate analyte and towards the development of analytical methods. One of such analytes commonly used is **creatinine**, an **endogenous marker for GFR**. The concentration of creatinine is usually determined in serum and its clearance is calculated by additionally measuring its concentration in daily urine.

CLINICAL SIGNIFICANCE OF CREATININE AND GFR

Creatinine

Creatinine is a by-product of creatin degradation and is excreted from our body with urine. Creatinine is formed spontaneously from creatine and creatine phosphate in muscles and is excreted into the plasma at a constant rate. 1–2% of muscle creatine is transformed to creatinine every day. The quantity of creatinine produced is proportional to muscle mass, which is further dependent on gender, age and to a smaller extent on a diet (proteins). Plasma creatinine is excreted from blood into the urine mostly by glomerular filtration and additionally to a lesser extent (7–10 %) with the secretion in the proximal tubule. Daily creatinine excretion is stable.

Glomerular filtration rate

The best indicator for the assessment of glomerular rate and determination of kidney function, for evaluation of the dosage regimen of drugs, and for monitoring of the progression of kidney disease is the ability of glomeruli to filter plasma in a certain amount of time. The volume of plasma filtered by the glomerulus per unit of time is called glomerular filtration rate. If we assume that a substance is excreted exclusively by glomerulus and neither secreted nor absorbed in tubules and if we measure the concentration of this substance in plasma and 24-hour urine, we can calculate the GFR with the use of data of the substance clearance:

$$GFR = \frac{c(u) \cdot V(u)}{c(pl) \cdot t}$$

GFR: glomerular filtration rate (mL/min)

c (u): concentration of the substance in urine (µmol/L),

c (pl): concentration of the substance in plasma (µmol/L) V (u): volume of urine (mL)

t: time of urine collected

One of such substances excreted from plasma only by filtration in glomeruli is an exogenous polysaccharide inulin. Measuring the inulin in plasma and daily urine would thus give the exact value for GFR. However, due to intravenous application and analytical difficulties, inulin is only used for research purposes.

As creatinine is constantly and endogenously produced and **excreted almost exclusively by glomerular filtration**, urinary creatinine excretion has been used as a measure of GFR. We can estimate GFR by calculating creatinine clearance:

$$ClCr = \frac{c_{Cr}(u) \cdot V(u)}{c_{Cr}(pl) \cdot t}$$

ClCr: Creatinine clearance (mL/min),

cCr (u): creatinine concentration in urine (µmol/L), cCr (pl): creatinine concentration in plasma (µmol/L) V (u): volume of urine (mL)
t: time of urine collected

Creatinine clearance is the **volume of plasma** from which creatinine is removed per unit time. CrCl is usually reported in units of **mL/min** and can be corrected for body surface area:

$$ClCr = \frac{c_{Cr}(u) \cdot V(u) \cdot 1.73}{c_{Cr}(pl) \cdot t \cdot A}$$

ClCr: Creatinine clearance (mL/min/1.73m^2),

cCr (u): creatinine concentration in urine (µmol/L), cCr (pl): creatinine concentration in plasma (µmol/L) V (u): volume of urine (mL)

t: time of urine collected

A: patient's body surface (m^2)

On account of 7-10% of creatinine secreted by tubules, creatinine clearance overestimates the exact GFR. However, CrCl provides a reasonable approximation of GFR.

In normal kidney function the GFR exceeds 90 ml/min. GFR below reference values indicates acute or chronic kidney injury, decreased blood flow to the kidneys due to decreased blood volume or acute tubular damage. Increased creatinine clearance values

are clinically not relevant and most commonly result from errors in urine collection (incompletely emptied bladder before collection begins).

There are several possible sources of errors in determining creatinine clearance:

- insufficiently emptied bladder when collecting urine,
- inclusion of the first urine excreted in 24-hour urine,
- spontaneous excretion of urine during defecation,
- increased physical activity during urine collection,
- insufficient hydration of the patient (insufficient urine flow).

Estimation of GFR

To test for the errors stated above, to avoid the inconvenience of 24-hour urine collection and in cases where immediate results are required and there is no time to collect 24-hour urine (eg, renal function control during nephrotoxic drug therapy), nomograms and equations have been developed to estimate GFR. In addition to serum creatinine concentration, gender, age, body surface area and race are considered in these equations. The most commonly used are: the CKD-EPI equation (last version from 2021), the equation derived from the Modification of Diet and Renal Disease (MDRD) study and the Cockroft-Gault equation. Due to a good correlation of the calculated GFRs with measured values, the National Kidney Foundation and the National Kidney Disease Education Program recommends calculation of GFR based on **CKD-EPI equation (2021),** which includes paramters: sex, age and creatinine concentration. A complex formula is availabe for use on different on line calculators.

CKD – EPI equation calculator:

https://www.mdcalc.com/calc/3939/ckd-epi-equations-glomerular-filtration-rate-gfr

Cockroft-Gault equation:

$$eClCr = \frac{(140 - age) \cdot \mathrm{m}}{0.814 \cdot c_{\mathrm{Cr}}(s)} (\cdot\, 0.85\ for\ women)$$

eClCr: estimated creatinine clearance (ml/min/1.73m^2), cCr (s): creatinine concentration in serum (µmol/L)

age (years)

m: body mass (kg)

Limitation of estimated GFR:

- equations developed have been based on patients' measurements and not on healthy individuals
- the equations are not suitable for the assessment of GFR in **acute** kidney failure as serum creatinine concentration varies rapidly
- the equations are not suitable for estimating GFR in subjects with extreme deviations from the mean in height, weight, age
- the equations are not suitable for estimating GFR in children. In these cases, serum cystatin C is determined, as it correlates better with kidney function and GFR score than serum creatinine.

Table 1. *Stages of chronic kidney disease and corresponding GFR values*

Stage	Features	GFR (ml/min/1.73m^2)	Measures
1	kidney injury with normal or elevated GFR	> 90	- diagnostics, therapy of kidney disease - reduction of CVD risk factors - slowing the progression of CKD
2	kidney injury with a slight decrease in GFR	60-89	- dose adjustment of drugs according to GFR
3	moderate decrease in GFR	30-59	- assessment and treatment of CKD complications
4	severe reduction in GFR	15-29	- preparation for renal replacement therapy
5	renal failure	< 15	- renal replacement therapy

GFR-glomerular filtration rate; CKD- chronic kidney disease; CVD-cardio-vascular disease

Biological samples for creatinine determination

Creatinine is most commonly measured in serum, plasma or diluted urine (1: 100). Serum creatinine levels increase after high-protein meals, therefore, collecting blood under fasting conditions is recommended. The serum must be separated from erythrocytes and other cells in less than 14 hours after collection. Haemolyzed samples are also not suitable for Jaffe reaction analysis, and false lower concentrations are determined for icteric

samples. Samples can be stored at 4°C for more than 7 days and in the freezer for several months. False negative creatinine levels can be determined in contaminated samples, as bacterial metabolites slow down or inhibit the chemical reaction. When collecting 24-hour urine, keep the sample container on cold place or add a preservative to prevent bacterial breakdown of creatinine or conversion to creatine.

Reference values

Reference values for creatinine in serum depend on gender, age and method used (Table 2). Serum creatinine concentrations are lower in women than in men due to lower muscular mass. Babies and children have lower concentrations than adults. Creatinine clearance is lower in the elderly due to decreased renal function. GFR drops ~ 1% / year after the age of 40. Elderly patients with normal serum creatinine but reduced muscle mass may have GFR reduced for up to 30%. Serum creatinine concentrations can reach values above 1000 µmol/L in patients with severe kidney disease.

Table 2. *Creatinine reference values for Jaffe method.*

	Men	Women
Serum:	62-104 µmol/L	49-90 µmol/L
24-hour urine:	10-18 mmol/24 h	7.5-12.5 mmol/24 h
ClCr:	95-140 mL/min	90-130 mL/min

ANALYTICAL METHODS FOR CREATININE DETERMINATION

Jaffe reaction

The methods most frequently used to measure creatinine are based on the Jaffe reaction first described in 1886. In this reaction, creatinine reacts with **picric acid** in **alkaline solution** to form a red-orange chromogen (Figure 1). The reaction was later adopted for the measurement of blood creatinine by Folin and Wu in 1919. The complex formed has absorbance maximum at 485 nm. However, the absorbance of the coloured complex is usually measured at 510–520 nm, due to strong absorbance of picrate ion at wavelengths lower than 500 nm.

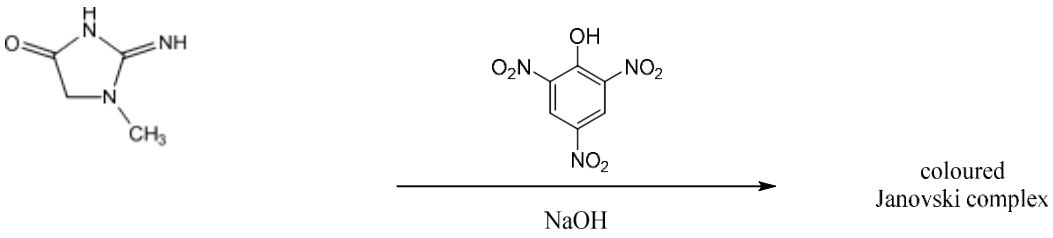

Figure 1: *Schematic representation of Jaffe reaction.*

The reaction is **nonspecific** and subject to **positive interference** by a large number of compounds (**pseudocreatinins**), including acetoacetate, acetone, ascorbate, glucose, guanidine, acetone, uric acid, cephalosporin and pyruvate. False positive results are also obtained if the sample was haemolyzed and when reaction temperature increases above 30°C. False negative results can be the result of bilirubin interference. In order to **improve the specificity, modified Jaffe methods** were developed.

Jaffe method with deproteinization and aluminium silicate

More accurate results are obtained by **removing proteins** from serum, following by the absorption of creatinine to aluminium silicate. Proteins are first precipitated by sodium tungstate and H_2SO_4 or trichloroacetic acid and removed in the following centrifugation step. The adsorbent is then introduced to the protein-free supernatant and creatinine is adsorbed within 1 min with >90% efficiency. Sodium aluminum silicate (Lloyd's reagent), aluminum magnesium silicate (Fuller's earth) and bentonite can be used as adsorbents. The interfering substances remain in solution. After additional centrifugation step the supernatant is discarded, and a solution of alkaline picrate added to the precipitate containing creatinine to form a coloured complex – Jaffe reaction. After centrifugation, the absorbance of the coloured complex in the supernatant is measured at 520 nm. Possible interferences: pyruvate (> 0.9 mmol/l) and 2-oxoglutarate (> 0.5 mmol / l), which can also be adsorbed to aluminium silicate. Because this method is time consuming and not readily automated, it is not routinely used.

Kinetic Jaffe method

Another approach used to increase the specificity of assay methods for creatinine is a kinetic Jaffe method. In this method, serum is mixed with alkaline picrate and the rate of change in absorbance within 25 to 60 s is measured. In this method, the contribution of several otherwise interfering substances (pseudocreatinines) is eliminated, as the fast reacting interfering substances interact with picrate in the first 25 s, while the slowly reacting interfering substances interact with picrate only after 60 s. By measuring the absorbance in the interval between 25-60 s, we subtract the absorbance of fast reacting substances, yielding contributing absorbance of creatinine only. The kinetic method requires a constant temperature and pH and can only be performed with a biochemical analyser. Nowadays, kinetic Jaffe method is still the most commonly used method for routine creatinine determination in biochemical laboratories. New, enzymatic methods, are on the horizon for the rutine determination of creatinine in sera.

TEST YOUR KNOWLEDGE

1. What are the risk factors for developing chronic kidney disease?
2. A man (45 years, 73 kg) has recently started chemotherapy treatment with potentiated nephrotoxic side effects. To monitor the therapy, a doctor ordered a determination of creatinine clearance. The serum creatinine concentration was 0.08 mg/ml, the patient excreted 50 ml of urine within 1 hour with a creatinine concentration of 0.45 mg/ml. Calculate creatinine clearance. What does creatinine clearance say about the patient's GFR? What does an estimated GFR say about kidney function? What tests might be worth performing to further elucidate the patient’s state?
3. List at least 5 advantages of the kinetic Jaffe method performed on the analyser over the classical Jaffe or modified Jaffe method.
4. While watching the video lectures (see additional materials), write down 5 important findings for each clip.

DO YOU WANT TO LEARN MORE?

Watch the following video lectures to learn more about chronic kidney disease.

Anatomy and physiology of kidney:

https://www.youtube.com/watch?v=805VoHIIQCs

https://www.youtube.com/watch?v=JCEwZCqvyNI

Assessing Kidney Function: Glomerular Filtration Rate (GFR):

https://www.youtube.com/watch?v=Uc150kdLNwg

https://www.youtube.com/watch?v=blHDsi6nm6E

Chronic kidney disease:

https://www.youtube.com/watch?v=fv53QZRk4hs

Clinical case:

https://www.youtube.com/watch?v=lKgfyCtikms

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LAB REPORT: DETERMINATION OF CREATININE IN SERUM USING KINETIC JAFFE METOD

Within this laboratory work you will measure serum creatinine concentration in two different ways, both based on kinetic Jaffe method. First you will perform analysis by using **(1) automated analysing system** Viva-Jr® (SIEMENS). After the determination of creatinine concentration with the automated system, you will also perform analysis **(2) manually** to compare the efficiency and precision of both methods. You can find the instructions for the manual creatinine determination on the separate paper provided.

Description of work with automated analysing system Viva-Jr® (SIEMENS)

Automated analysing system Viva-Jr® is used for *in vitro* diagnostic measurements of analytes in serum, plasma and urine samples. It can be used for general biochemical testing, testing of prohibited substances or toxicological studies and for the therapeutic drug monitoring (TDM) in biological samples. The analyser belongs to the group of discrete (each reaction and measurement happens in its own reaction vessel) and selective (analyser performs in the sample only the tests selected by the operator and not all available tests) analysers.

The analyser consists of an **analytical unit** (sample and reagent rotor and a rotor with cuvettes for spectrophotometric measurement) and a **control unit** (computer, display and printer). The inner part of the reagent rotor is covered and constantly cooled (8–12°C), allowing suitable storage of the reagents throughout the day. Reagent rotor is joined to the sample tube rotor, with 3 additional reagent sites (room temperature), and it is also possible to subsequently insert urgent samples without the interruption of the routine work. Rotor with cuvettes, in which reactions and spectrophotometric measurements of analytes happen, is heated to 37°C. The absorbance can be measured at the endpoint or kinetically, and monochromatically or bichromatically. We can choose from 8 different wavelengths in the UV-VIS spectrum. After the measurement reaction mixtures are transferred to the waste and the reaction cuvettes are rinsed, which enables their reuse. The analyser enables automatic calibration and its evaluation, automatic

evaluation of control samples within the batch, automatic measurement of the sample blank and dilution of the sample, and verification of the corresponding spectral properties of the cuvettes. The system can be on standby for 24 hours with minimal intervention by operator. Analyser can identify samples and reagents with scanning their bar codes or after manual entry of the code for each reagent.

The analyser is connected to a systemic computer supported by Microsoft Windows XP. On the LCD screen user windows are displayed, through which we perform search orders, calibrations and daily analysis of control samples, and maintenance of the analyser. All test results, results of test parameters, calibration results, and control results are stored on a computer disk or external memory.

Reagents:

Creatinine is determined according to the manufacturer's instructions using appropriate analysing kit. Appropriately trained operator is also required for the usage of the biochemical analyser. The reagents are ready for use:

- Reagent 1: **NaOH**, 0.2 mol/L
- Reagent 2: **picric acid**, 25 mmol/L
- Washing solution: distilled water and detergent
- Cooling solution
- Control solutions
- Standard solution

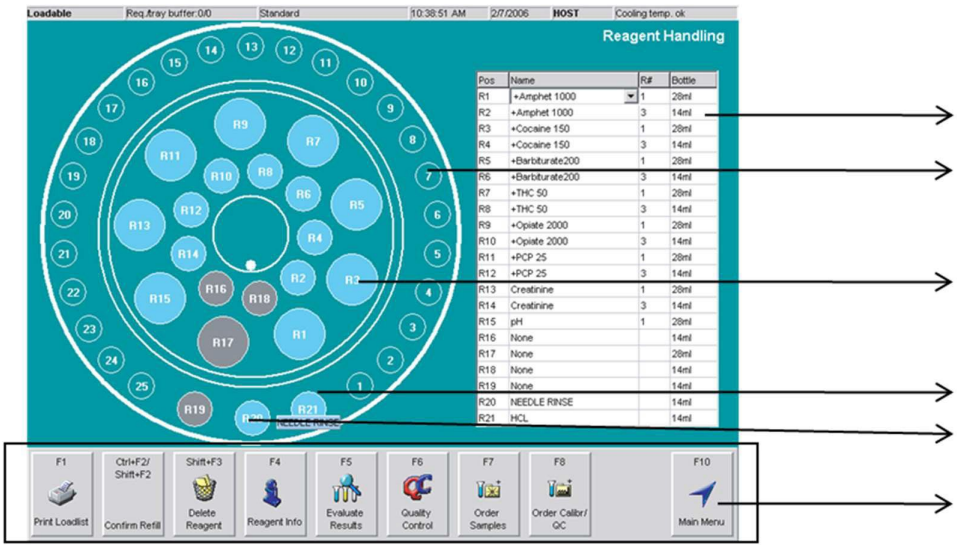
Before the analysis, turn the analyser on, check the operation of the system, check the level of the washing solution (distilled water + detergent), cooling solution and empty the containers with waste fluid.

Principle of the method:

The analyser uses the kinetic Jaffe method for the measurement of the serum creatinine in the samples. Creatinine reacts with an alkaline picrate solution to form an orange-coloured complex whose absorbance is measured at 505 nm and 571 nm (reference filter) at time points pre-set by the manufacturer.

Protocol for automated creatinine determination:

1. The assistant will introduce you to the biochemical analyser. Name the highlighted parts of the analyser displayed in the program dialog.



2. Pipette exactly 200 µL of the sample or the control into tubes provided for the analyser.
3. In the “ORDER SAMPLES” dialog box, enter the number or a label of the sample, tick off the desired test (creatinine, sample blank) and place the tube into the assigned location.
4. Separately, prepare a test tube with 200 µL of distilled water and put it into position 1.

5. Place the control, the standard and reagents in designated, well-defined locations in the reagent rotor.

Warning! Reagent 1 contains a base, NaOH, which is an eye and skin irritant. The use of protective gloves and clothing is required.

Protocol for manual creatinine determination:

1. Prepare working reagent. Warm it up to 37ºC.
2. Prepare four 2 mL tubes. Pipette into each tube (in µl)

	Blank	**Standard**	**Sample**	**Control**
Deminaralized water	100			
Standard solution		100		
Sample (serum)			100	
Control				100

3. Add 1 mL of working reagent to standard and meassure the absorbance at 37ºC kinetically – first meassurement at 30s (A_{st1}) and second meassurement at 60s (A_{st2}).
4. Add 1 mL of working reagent to sample and meassure the absorbance at 37ºC kinetically – first meassurement at 30s ($A_{Sample1}$) and second meassurement at 60s ($A_{Sample2}$).
5. Add 1 ml of working reagent to control and meassure the absorbance at 37ºC kinetically – first meassurement at 30s ($A_{Control1}$) and second meassurement at 60s ($A_{Control2}$).

Equations:

$$c_{sample} = (A_{sample2} - A_{sample1}) / (A_{st2}-A_{st1}) \times c_{st}$$

$$c_{control} = (A_{control2} - A_{control1}) / (A_{st2}-A_{st1}) \times c_{st}$$

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the method (Scheme of reaction time, graph of two-point calibration)

Measurements

Automated procedure

Control interval 1: Determined concentration of the control:

Control interval 2: Determined concentration of the control:

Sample: Determined concentration of the sample:

Manual procedure

	A1	**A2**
Control		
Standard		
Sample		

$C_{standard}$:

Control interval:

Calculations

Control:

Sample:

Analytical evaluation (controls, samples, comparison of the two methods):

Clinical interpretationot the result:

Signature: Date:

DETERMINATION OF SERUM IRON BY FERENE TEST

Assist. Dr. Irena Prodan Žitnik, MPharm, EuSpLM

Assoc. Prof. Dr. Barbara Ostanek, MPharm

LEARNING OUTCOMES

This laboratory practical will teach the students the significance or serum iron measurement and some other parameters for assessment of body iron status. After successfully completing this laboratory practical, the student will be able to:

- elaborate the clinical significance of serum iron measurements,
- perform analysis and measure serum iron,
- describe the possible interferences in this laboratory test,
- analytically and clinically interpret the results.

IRON AND ITS CLINICAL SIGNIFICANCE

Iron is classified as an essential trace element, meaning that it is present only in small amount in humans, but is indispensible for life due to involvement in many biological processes. It is an integral part of hemoglobin, myoglobin, cytochromes, catalases, peroxidases, and other enzymes and is involved in oxygen binding and transport, oxygen metabolism, cellular respiration as well as numerous other biological functions. It can however also be harmful, especially as it catalyzes the formation of highly reactive hydroxyl radicals which can damage cellular membranes, DNA and proteins.

IRON METABOLISM

Absorption of iron

The total amount of serum iron is regulated by absorption in the small intestine. Dietary iron can be in heme or non-heme form and is absorbed predominantly in the duodenum and proximal jejunum. Typically, only 10% of the dietary iron is absorbed. To be absorbed by intestinal cells, iron must be in the bivalent form (Fe^{2+}), so the non-heme trivalent dietary iron must first be reduced to bivalent state by duodenal cytochrome b reductase or other reducing agents present in the intestine e.g. ascorbic acid. Heme iron enters the enterocytes by means of vesicular transport. In the enterocytes, heme oxygenase cleaves the porphyrin ring and iron (Fe^{2+}) is released. After absorption to enterocytes, iron can be stored in cellular ferritin or transported to the basolateral side of enterocytes, where it is oxidized to Fe^{3+} and bound to ferroportin, the transmembrane transporter, regulated by hepcidin. After entering the blood, Fe^{2+} binds to apotransferrin for transport in the circulation.

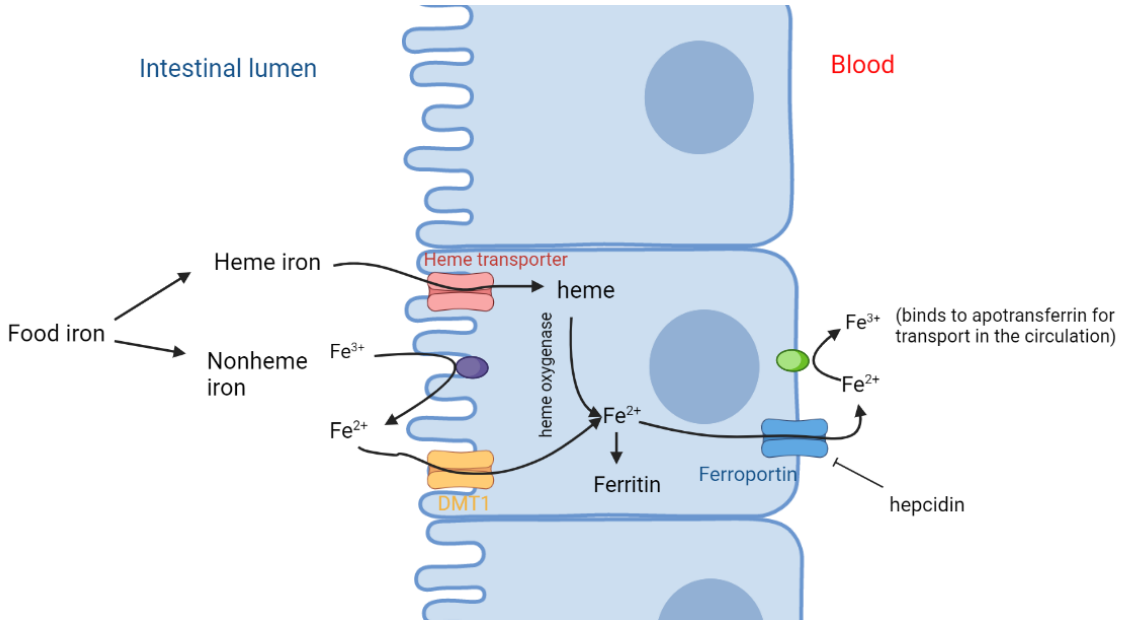


Figure 1. *Intestinal iron absorption (Created with BioRender.com)*

Distribution

Iron is present in the body in three main forms, as functional iron, storage iron and transport iron.

- Functional iron is mostly bound in hemoglobin (65 - 70 %), myoglobin (4 %) and several other enzymes, such as peroxidases, catalases and cytochromes (less than 1%).
- Storage iron: Iron is stored in the body as ferritin and hemosiderin, primarily in the bone marrow, spleen and liver. **Ferritin** consists of a protein shell (apoferritin) surrounding an iron core, where iron is stored as ferric oxyhydroxide phosphate. The majority of ferritin is located intracellularly, however small amounts of ferritin are also present in plasma in levels that reflect total body iron stores. **Hemosiderin** is aggregated and partially deproteinized ferritin. In contrast to ferritin, it is insoluble in water.
- Transport iron: Iron in the circulation is bound to Fe transport protein, transferrin. Each molecule of transferrin has two binding sites for Fe^{3+}. Binding to transferrin keeps Fe nonreactive in the circulation and enables delivery to cells via transferrin receptors.

DISORDERS OF IRON METABOLISM

Iron deficiency, iron overload and anemia of chronic disease are most prevalent disorders of iron metabolism. Some of the common causes leading to either iron deficiency or iron overload, are listed in Table 1.

Table 1. *Causes or iron deficiency or iron overload*

Iron deficiency	Iron overload
a) Physiological condition (elevated need of iron and/or elevated iron losses) - puberty - pregnancy and childbirth - frequent blood donations - period and hormonal changes in women **b) Disorders affecting iron absorption** - chronic diarrhoea - malabsorption syndrome **c) High blood loos in GIT and UGT** - chronic bleeding (peptic ulcer, gastritis, GIT tumours, hemorrhoids) - nephrosis	**d) Primary (inherited) hemochromatosis** Mutations in the *HFE gene*, gene for hepcidin (*HAMP*), TfR 2 or ferroportin (*SLC40A1*) **e) Chronic anemias,** due to - ineffective erythropoiesis (thalassemia, sideroblastic anemia) - hemolysis (hemolytic anemias) **f) High iron intake** - chronic transfusion - long-term Fe supplementation

PARAMETERS FOR BODY IRON STATUS ASSESSMENT

Serum iron

Reference values:	10.7 – 28.6 µmol/L

Measurement of serum iron concentration refers to Fe^{3+} bound to serum transferrin and not to iron circulating as free Hb in serum. Iron can be measured in serum or plasma collected using heparin as an anticoagulant. EDTA, oxalate or citrate cannot be used, due to binding of Fe ions, except when iron is measured using atomic absorption spectroscopy (AAS). Because of the diurnal variation in serum iron concentrations, sampling in the morning, when iron concentrations are the highest, is preferred. Various physiologic or pathologic conditions as well as age gender and medications influence serum iron concentration.

Total iron binding capacity (TIBC)

Reference values:	44.0 – 70.4 µmol/L

Total iron binding capacity (TIBC) refers to the amount of iron that would saturate all binding sites on transferrin. It relates to serum transferrin concentration. Normally, around 30% of iron binding sites are occupied. TIBC deviates from reference values in disorders of iron metabolism. It is usually increased in iron deficiency and decreased in iron overload (it follows the changes in transferrin production in these conditions). TIBC is also decreased in malnutrition, chronic inflammatory disorders or malignancies, in liver disease, if protein synthesis is impaired, and in kidney disease due to the loss through urine. Increased TIBC values can be found in patients taking oral contraceptives.

Transferrin saturation (sTf)

Reference values:	15 – 45 %

Transferrin saturation can be calculated from serum iron and TIBC using the following equation:

$$sTf\ (\%) = \frac{serum\ iron\ (\frac{\mu mol}{L})}{TIBC\ (\frac{\mu mol}{L})} \times 100$$

Ferritin

Reference values:	M	22 – 275 µg/L
	F	5 – 204 µg/L

Serum ferritin reflects total body iron stores. It is a sensitive marker of iron deficiency, as its values are decreased long before changes in serum iron or blood Hb concentration are observed. Measurements of serum ferritin is particularly valuable in distinguishing iron deficiency anemia from anemia of chronic disease. Increased serum ferritin is an indication iron overload, but can also be found in several disorders such as chronic infections, chronic inflammatory disorders, liver disease, heart disease, and malignancies.

Reticulocyte hemoglobin content (C-Hr) and proportion of hypochromic erythrocytes (% HYPO)

Reference values:	HYPO%	1-5%
	CHr	30-36 pg

Hematological markers are important complement to the biochemical markers of iron status. They range from basic markers like concentration of hemoglobin, number of erythrocytes, mean volume (MCV) and mean hemoglobin concentration (MCHC) of erythrocytes which are used to diagnose anemia and determine its morphologic character (i.e. iron deficiency anemia is characterised by microcytic and hypochromic erythrocytes). More sophisticated hematology analyzers enable also determination of additonal parameters like reticulocyte hemoglobin content (C-Hr) and proportion of hypochromic erythrocytes (% HYPO). These provide better insight into the functional availability of iron for erythropoesis and are indispensable especially in chronic kidney disease patients on hemodialysis receiving erythropoietin or other erythropoiesis-stimulating agents, anemia of chronic diseases and iron deficiency in infants and adolescents.

Reticulocytes are present in the blood for 18 to 36 hours before becoming mature erythrocytes. Measurement of C-Hr is thus considered the most sensitive indicator of current iron availability for erythropoiesis. The % HYPO is the proportion of hypochromic erythrocytes with a hemoglobin concentration of less than 280 g/L, expressed in % of the total erythrocytes. It is another important parameter for assessing iron deficient erythropoesis for longer period since the life-span of erythrocytes is around 120 days.

ANALYTIC METHOD FOR IRON DETERMINATION

In this practical, you will measure serum iron concentration using iron ferene test. In acidic medium, transferrin bound iron is released from transferrin and is then reduced to Fe^{2+} in the presence of ascorbic acid. Ferrous ion reacts with chromogen ferene to form a blue complex which you will measure spectrophotometrically at 593 nm (578-600 nm). The absorbance is directly proportional to the concentration of iron in serum.

Interferences:

- Hemoglobin interferes in the analysis, therefore hemolyzed samples should not be analyzed.
- Bilirubin up to 340 µmol/L does not interfere.
- Triglycerides up to 11.3 mmol/L do not interfere.
- Zinc, copper or cobalt up to 4 mg/L do not interfere.

Biological samples for iron determination

Serum or heparin plasma can be used for iron determination. Specimen should be collected before specimens requiring other anticoagulants which could interfere in iron determination. Sample is stable up to 7 days at 2-8°C, but must be separated from blood cells 2 hours after blood collection at the latest, to minimize hemolysis. The preferred time for sampling is in the morning, since the values decrease during the day.

TEST YOUR KNOWLEDGE

1. Explain the principle of iron ferene test for serum iron determination.
2. List the reagents used for serum iron determination in this practical and explain the purpose of each reagent.
3. Why is EDTA plasma not suitable for iron determination?

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4. Thomas L. Clinical Laboratory Diagnostics, electronic edition Release 3, 2022, dostopno na https://www.clinical-laboratory-diagnostics.com/

LAB REPORT: DETERMINATION OF IRON IN SERUM

Protocol for ferene reaction:

Pipette (in mL) in 10 mL test tubes:

	Blank	Sample	Control	Standard
Reagent 1	1	1	1	1
Distilled water	0.2	–	–	–
Sample	–	0.2	–	–
Control sample	–	–	0.2	–
Standard	–	–	–	0.2

Mix and incubate for 5 minutes at 37°C. Then read the extinction of the sample against reactive blank.

Reagent 2	0.25	0.25	0.25	0.25

Mix and incubate for 5 minutes at 37°C. Then read the extinction of the sample, control sample and standard against blank. The colour is stable for at least 15 minutes at room temperature.

Reagents:

Reagent 1: citric acid 180 mmol/L, ascorbic acid 100 mmol/L, copper-specific masking agent 100 mmol/L, surfactants

Reagent 2: citric acid 180 mmol/L, ferene 6 mmol/L, surfactancts and preservatives

Standard: iron 17.9 µmol/L

Performance characteristics

Linearity: 0.75 – 143.2 µmol/L

If the concentration is higher than 143.2 µmol/L, repeat the analysis on a diluted sample (1:2) and multiply the result by 2.

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the method (Scheme of reaction time, graph of two-point calibration)

Measurements

Control sample: Control sample blank:

Sample: Sample blank:

Standard: Reagent blank:

Given values for control and standard:

Control interval: Standard concentration:

Calculations

Results

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

DETERMINATION OF SERUM BILIRUBIN BY JENDRASSIK-GROF METHOD

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Assoc. Prof. Dr. Barbara Ostanek, MPharm

LEARNING OUTCOMES

Measurement of serum bilirubin is usually done as a part of the hepatic function panel. After successfully completing this lab practical, the student will be able to:

- describe the clinical significance of serum bilirubin measurements,
- perfom analysis and measure total and direct serum bilirubin,
- describe possible interferences in bilirubin analysis,
- analytically and clinically interpret the results.

BILIRUBIN AND ITS CLINICAL SIGNIFICANCE

METABOLISM OF BILIRUBIN

Bilirubin is the end product of heme metabolism. Its daily production is about 250 – 300 mg, of which approximately 85% is released during breakdown of senescent erythrocytes and 15% is derived from degradation of heme containing proteins such as the cytochrome P 450 isoenzymes, myoglobin, peroxidases etc. In reticuloendothelial system, heme is initially broken down by heme oxygenase into biliverdin, which is then reduced to bilirubin (BLR) by biliverdin reductase. BLR is poorly water-soluble, therefore, after release into circulation, it is transported to liver bound to albumin. Entry into the hepatocyte appears to be partly passive and partly mediated by organic anion transporter proteins like OATP1B1. In this process BLR

is released from albumin, which remains in the bloodstream. In hepatocytes, BLR is conjugated with glucuronic acid, forming monoconjugated or diconjugated bilirubin. The conjugation is catalyzed by UDP glucuronyltransferase 1A1 (UGT 1A1). The esterification disrupts the intramolecular hydrogen bonds, thereby opening the molecule and rendering the conjugated bilirubin more water-soluble, allowing excretion in the bile. Excretion to the bile happens against a concentration gradient and is mediated by anion transporter proteins mostly by MRP2. A fraction of conjugated BLR may be transported from hepatocytes back to blood, where it represents up to 20% of total BLR. In the intestine, conjugated BLR is broken down into urobilinogen by bacterial enzymes. Oxidation of urobilinogen gives rise to urobilin and stercobilin, which together with their degradation products give brown color to the stool. A portion of the urobilinogen is reabsorbed, transported through the portal vein circulation back to the liver and re-excreted via the bile (enterohepatic circulation). A small proportion of the urobilinogen in the blood is eliminated from the body by the urine. In case of long-lasting high concentrations of conjugated bilirubin in blood, some of it is covalently bound to albumin forming BLR δ. BLR metabolism is presented in Figure 1.

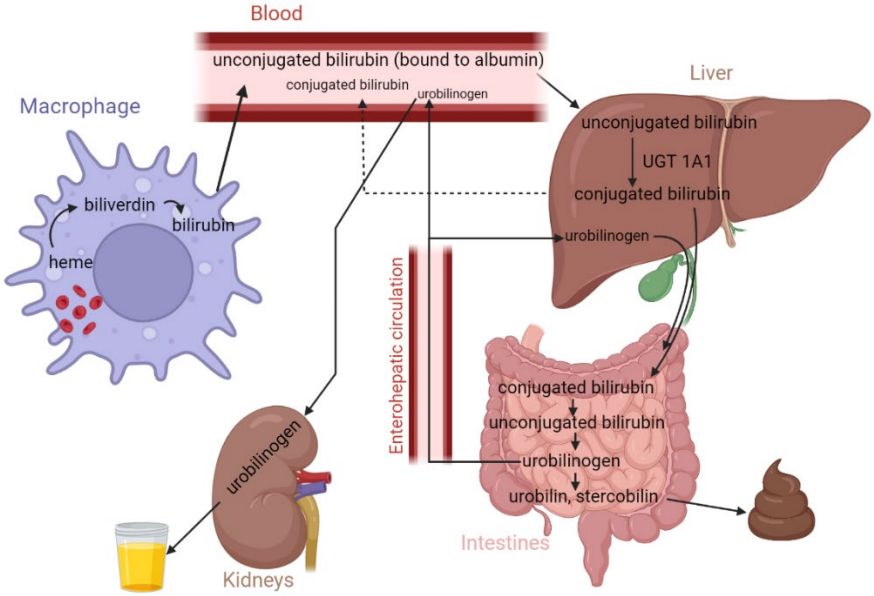

Figure 1. *Bilirubin metabolism. UGT 1A1 – UDP glucuronyltransferase 1A1 (Created with BioRender.com)*

Bilirubin is very unstable if it is not protected from light. When exposed to light, structural isomerisation, configurational isomerisation and photo-oxidation occur. The products of

these photochemical reactions are more hydrophilic and can be excreted in bile and urine without conjugation.

Bilirubin fractions:

- bilirubin α (non-covalently bound to albumin, unconjugated);
- bilirubin β (bilirubin monoglucuronide)
- bilirubin γ (bilirubin diglucuronide);
- bilirubin δ (covalently bound to albumin, conjugated);
- free bilirubin (IXα 4Z, 15Z); less than 0.1 %
- 3 photoisomers of bilirubin, formed when exposed to light: 4Z, 15E; 4E, 15Z; 4E, 15E

CLINICAL SIGNIFICANCE OF SERUM BILIRUBIN MEASUREMENT

Reference values:	
total bilirubin	up to 17 µmol/L
direct bilirubin	up to 5 µmol/L

Determination of bilirubin is important for:

- evaluation of liver function,
- differential diagnosis of hyperbilirubinemias / jaundice.

A condition in which there is too much bilirubin in the blood is called **hyperbilirubinemia**. Depending on the predominant form of BLR, hyperbilirubinemia can be classified as unconjugated and conjugated (Table 1).

Jaundice (icterus) is a yellowish discoloration of the skin, whites of the eyes, and other mucous membranes which can be observed at bilirubin levels above 35-50 µmol/L in adults. Jaundice may not be visible in the neonate's until the bilirubin concentration exceeds 80-120 µmol/L. Jaundice can be classified into three categories, depending on the cause of hyperbilirubinemia: pre-hepatic, hepatic and post-hepatic.

Hyperbilirubinemia with very high concentrations of unconjugated fraction may cause bilirubin to accumulate in the gray matter of the central nervous system, potentially causing irreversible neurological damage leading to a condition known as **kernicterus or bilirubin encephalopathy**.

Table 1. *Causes of unconjugated and conjugated hyperbilirubinemia.*

Unconjugated hyperbilirubinemia (unconjugated BLR >80 %)	Conjugated hyperbilirubinemia (conjugated BLR >50 %)
a) ↑ production • hemolysis (hereditary and acquired hemolytic anemias) • ineffective erythropoiesis (thalassemia, megaloblastic anemia) b) decreased uptake into hepatocytes • drugs • Gilbert syndrome c) ↓ conjugation in liver • Crigler-Najjar syndrome • Gilbert syndrome • neonatal jaundice • drugs (enzyme inhibitors) • hepatocellular dysfunction (hepatitis)	d) ↓ secretion into canaliculi • hepatocellular disease (hepatitis, cholestasis) • cirrhosis • drugs (oral contraceptives, estrogens) • Dubin-Johnson syndrome • Rotor syndrome e) bile duct obstruction • gall stones, • bile duct stenosis • tumors (bile duct, pancreas)

JENDRASSIK-GROF METHOD FOR DETERMINATION OF BILIRUBIN

In this lab practice, the concentration of total and direct bilirubin will be measured with Jendrassik-Grof method. The reaction mixture includes:

- DIAZO reagent: Sulfanilic acid reacts with sodium nitrite to produce diazotized sulfanilic acid (diazo reagent), which reacts with bilirubin and forms azobilirubin (Figure 2). All forms of bilirubin, except BLRα (non-covalently bound to albumin), react with diazo reagent.
- ACCELERATORS: Accelerators promote dissociation of unconjugated BLR from albumin without denaturation. Methanol, Na-acetate, Na-benzoate, caffeine, dyphylline are some of the possible accelerators.

- ADDITIONAL REAGENTS: Ascorbic acid is usually added to degrade excessive diazo reagent and prevent the interference of oxyhemoglobin. K-Na-tartarate and NaOH are used to make reaction mixture alkaline. Consequently, previously purple azobilirubin changes into green ionic form, which has higher extinction coefficient. This increases method sensitivity.

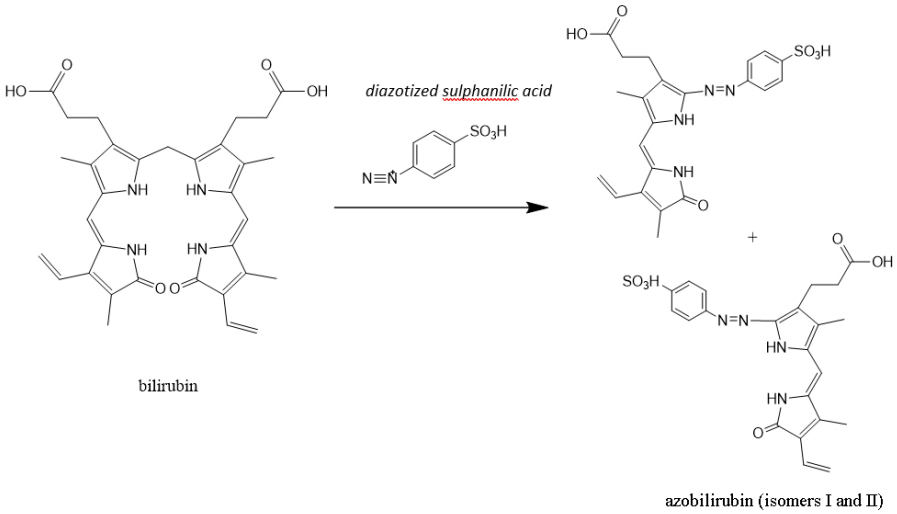

Figure 2. *Reaction of BLR with diazotized sulfanilic acid.*

When accelerator is not added to the reaction, all fractions except BLR α are measured (**direct bilirubin)**, which includes: BLR β, γ, δ, free BLR and photoisomers. When accelerator is added, **total BLR** (all fractions of BLR) is measured. The concentration of BLR α is calculated by subtracting the concentration of direct bilirubin from the concentration of total bilirubin (**indirect BLR**).

For determination of total BLR accelerator will be added to the sample before adding the diazo reagent. For the determination of direct BLR, the accelerator will be added after the ascorbic acid had already degraded the excess of diazo reagent.

Interferences: Falsely increased results can be expected in lipemic samples. Hemolysis may result in falsely decreased values. In the reaction of autooxidation of hemoglobin (Hb) H_2O_2 is produced, which degrades azobilirubin. This reaction is promoted by the presence of diazo reagent and nitrite, which are degraded with ascorbic acid. By adding ascorbic acid to the reaction mixture, the interference by oxiHb is decreased. Use of aminosalicylic acid and

levodopa can falsely increase the results. Use of oral contraceptives or aminophenazone and smoking can falsely decrease the measured serum bilirubin.

Biological samples

Bilirubin can be determined by the Jendrassik-Grof method in serum, plasma and urine. The usual anticoagulants (heparin, EDTA, oxalate, citrate) do not interfere with the measurement. In case of delayed analysis samples should be stored in dark (up to 3 days at 4° C or up to 3 months at -70° C). Direct light exposure can cause a decrease of total bilirubin levels for up to 50% per hour.

It is important to note, that elevated bilirubin levels in serum or plasma specimens can interfere with many laboratory tests, mostly via two mechanisms, spectral and chemical. Bilirubin has two absorption peaks, at 400 and 540 nm, and any laboratory test that measures near these wavelengths can be affected by the bilirubin interference. Additionally, bilirubin as an antioxidant can interfere with peroxidase-based tests and cause negative bias.

Determination of BLR by multilayer film-slide technology

One limitation of the Jendrassik-Grof method is that it cannot measure conjugated BLR separately, which is relevant for classification of hyperbilirubinemias. Namely, this fraction is included in the measurement of direct BLR, which represents only a surrogate for conjugated BLR. Methods based on the dry chemistry technology enable routine determination of conjugated, unconjugated and σ BLR fractions. The test slide consists of several layers (Figure 3) with specific functions. When sample is applied, reagents dissolve and unconjugated BLR is released from albumin in the spreading layer, while σ BLR is retained. The masking layer blocks potential interference from compounds trapped in the spreading layer like hemoglobin. In the registration layer unconjugated and conjugated BLR bind to a cationic polymer (mordant). These complexes have increased absorbance at specific wavelengths and sum of conjugated and unconjugated BLR is calculated from reflection densities at 400 nm and unconjugated BLR from reflection density at 460. The total BLR is also measured by a dedicated slide and uses a modification of the classical wet chemistry diazo reaction. This enables also the calculation of σ BLR which is increased in hepatic and post hepatic jaundice and remains elevated for several weeks due to its long half-life of 18 days.

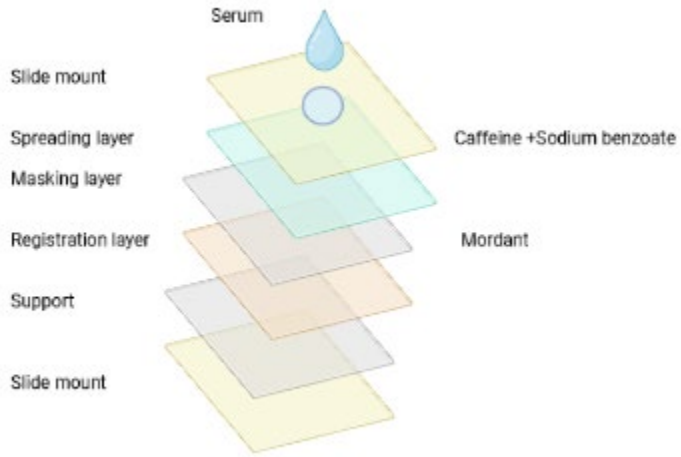

Figure 3. *Compositon of the VITROS®slide for conjugated and unconjugated bilirubin determination.*

TEST YOUR KNOWLEDGE

1. What kind of values (increased, decreased, normal) of conjugated and unconjugated BLR in serum and urobilinogen (UBG) and BLR in urine are expected in different types of hyperbilirubinemia? Complete the table!

hyperbilirubinemia	**conj. BLR (serum)**	**Unconj. BLR (serum)**	**UBG (urine)**	**BLR (urine)**
Pre-hepatic				
Hepatic				
Post-hepatic				

2. Why does the addition of Fehling reagent (K-Na-tartrat, NaOH) increase sensitivity of the method?
3. Why is accelerator added in the reaction mixture for measuring direct bilirubin and blank sample?
4. Write the formula for calculation of concentrations of total and direct bilirubin.

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LAB REPORT: DETERMINATION OF BILIRUBIN IN SERUM BY JENDRASSIK-GROF METHOD

Protocol of Jendrassik-Grof method:

1. Pipette (in μL) in 10 mL test tubes:

	Sample (tot. BLR)	**Sample (dir. BLR)**	**Sample-blank**	**Control (tot. BLR)**	**Control (dir. BLR)**	**Control-blank**
accelerator	400	-	400	400	-	400
sample (serum)	200	200	-	-	-	-
control sample	-	-	-	200	200	-
reagent 1	-	-	20	-	-	20
diazo reagent	100	100	100	100	100	100
sample (serum)	-	-	200	-	-	-
control sample	-	-	-	-	-	200

2. Vortex, incubate for 10 minutes at room temperature, then add:

reagent 1	20	20	-	20	20	-
accelerator	-	400	-	-	400	-
Fehling II sol.	300	300	300	300	300	300

3. Mix well and measure absorbance at 600 nm (the color is stable for 60 minutes).

Reagents:

- Accelerator: aqueous solution of caffeine benzoate (caffeine 260 mmol/L, Na-benzoate 520 mmol/L, Na-acetate 914 mmol/L).
- Reagent 1: ascorbic acid solution (227 mmol/L).
- Diazo reagent I: sulphanilic acid (29 mmol/L) solution in HCl (175 mmol/L).
- Diazo reagent II: Na-nitrite solution (72 mmol/L).
- **Diazo reagent:** right before use mix 10 mL of diazo reagent I and 0.25 mL of diazo reagent II.
- Fehling II solution: K, Na-tartrate (1.24 mmol/L) in Na-hydroxide sol. (2.5 mmol/L).

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the method (Scheme of reaction time, graph of two-point calibration)

Measurements

$A_{control\text{-}blank}$=	$A_{sample\text{-}blank}$=	$A_{control\text{-}dir.blr.}$ =
$A_{control\text{-}tot.blr.}$ =	$A_{sample\text{-}dir.blr.}$ =	$A_{sample\text{-}tot.blr.}$ =

Control interval:

Calculations and results:

($\varepsilon_{azobilirubin,\ 600\ nm}$ = 69400 L/mol cm)

Results

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

DETERMINATION OF ALT AND AST ENZYMES ACTIVITY CONCENTRATION IN SERUM

Assist. Dr. Jasna Omersel, MPharm, EuSpLM

Assist. Dr. Irena Prodan Žitnik, MPharm, EuSpLM

LEARNING OUTCOMES

At the end of the practical, student will be able to:

- recall and identify the significance of determining the total enzyme activity of liver damage markers, specifically ALT and AST,
- explain the role of ALT and AST in liver function and the implications of their elevated levels,
- measure the catalytic activity of ALT and AST in human sera using a simplified reference procedure and apply the calculation of De Ritis coefficient,
- evaluate the significance of the results of enzyme activity measurements and De Ritis coefficient calculations, and interpret the results in the context of liver damage and potential clinical applications,
- integrate the knowledge of enzyme activity measurements, De Ritis coefficient calculations, and liver function to discuss the potential clinical implications of the results.

AMINOTRANSFERASES AND THEIR CLINICAL SIGNIFICANCE

Aminotransferases are enzymes that catalyse the conversion of amino acids to α-keto acids by transferring an amino group. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are clinically important and are measured primarily to assess liver damage. L-alanine:2-oxoglutarate aminotransferase (ALT; E.C. 2.6.1.2) catalyses the transfer of an amino group between the amino acids L-alanine and L-glutamate. L-aspartate:2-oxoglutarate

aminotransferase (AST; E.C. 2.6.1.1) catalyses the transfer of an amino group between L-aspartate and L-glutamate (Figure 1). The reactions are reversible, and the equilibrium shifts toward the formation of alanine or aspartate. Both reactions occur in the presence of pyridoxal-5'-phosphate, which acts as a cofactor.

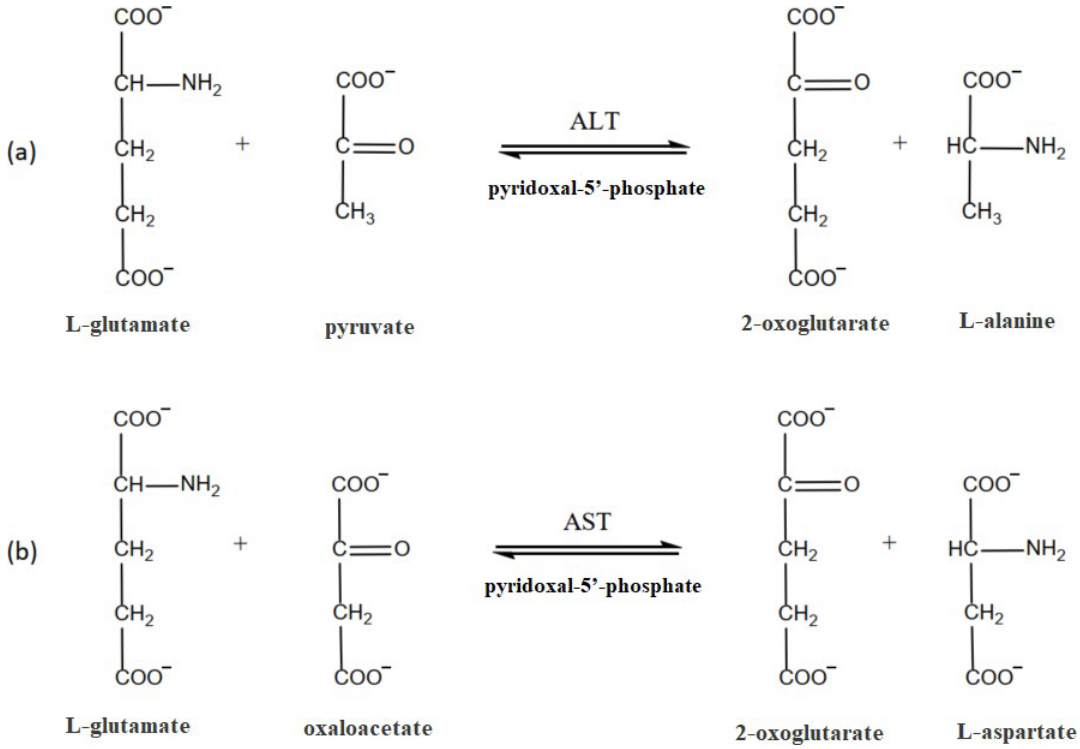

Figure 1. *Reactions catalysed by ALT (a) and AST (b).*

Aminotransferases are present in many different tissues: ALT is found primarily in the liver and in lesser amounts in the kidneys, heart muscle, and skeletal muscle. AST is found primarily in the heart muscle, liver, skeletal muscle, and kidneys, and in lesser amounts in the pancreas, spleen, lungs, and erythrocytes.

CLINICAL SIGNIFICANCE

Liver disease or liver damage is the most common cause of elevated serum activities of ALT and AST. The elevated serum activities are due to the release of both enzymes from damaged hepatocytes and not due to increased synthesis. Their activities in serum may be elevated even before the onset of clinical signs of disease. ALT is a more liver-specific enzyme than AST. In addition, its increased activity lasts longer than that of AST. Measurement of AST is useful and

recommended in cases of abnormal ALT results for calculating the ratio of AST to ALT (De Ritis quotient), a tool that provides useful diagnostic and prognostic information.

Enzymes are located in various places in the cell: membrane-bound, in the cytoplasm, in the mitochondria. AST has both mitochondrial and cytosolic isoenzymes, ALT only cytoplasmic. As such, they can be released upon cell injury and appear in the plasma relatively quickly. The AST isoenzymes are encoded by different genes but are structurally quite similar. The usefulness of measuring AST and ALT in diagnostics is limited by the concentration gradient (plasma vs. cell compartment) after cell injury and the half-life. The half-life of ALT in serum is 47±10 hours, cytoplasmic AST 17±5 hours and mitochondrial AST 87 hours. Several mechanisms appear to be involved in the release of enzymes from hepatocytes. Mild cell injury appears to allow leakage of cytoplasmic enzymes from cells but minimal release of isoenzymes from mitochondria. Mild inflammation of the liver, such as a mild attack of viral hepatitis, is likely to increase only the permeability of the liver cell membrane, allowing cytoplasmic enzymes to leak into the blood. Mitochondrial membrane disruption may be observed in severe viral attack or in cases of cell death (ischemia, trauma, heat...), allowing both cytoplasmic and mitochondrial enzymes to be detected in the blood.

Under conditions associated with acute necrosis of liver tissue, the activities of both enzymes can be up to 100 times normal. As a rule, AST and ALT 10-40-fold elevations above upper reference limits (URL) are observed in viral hepatitis and liver necrosis caused by intoxication or ischemia. Peak transaminase activity occurs between days 7 and 12 after the onset of disease, after which it gradually begins to decline. An increase in ALT for more than 6 months in patients with previous acute viral hepatitis indicates the development of a chronic form of the disease. Periodic follow-up of ALT over 1-2 years is recommended. An increase of more than 85-fold is observed in toxic hepatitis (e.g., acetaminophen intoxication).

A slight increase (up to 10 x URL) in the activity of ALT and AST may be due to nonalcoholic fatty liver disease, liver cirrhosis, liver cancer, and obstructive jaundice; pregnancy-related liver disease also increases ALT and AST (< 20 x URL).

In patients with persistently elevated ALT and AST levels that are not due to viral hepatitis and drug or alcohol use, we should also consider less common causes of chronic liver injury, such as hemochromatosis, Wilson disease, autoimmune hepatitis, primary biliary cirrhosis, celiac disease, sclerosing cholangitis, and α1-antitrypsin deficiency.

Serum AST activity is also increased in liver metastasis, after acute myocardial infarction and in heart disease, progressive muscular dystrophy, pulmonary embolism, crushed muscle injury, acute pancreatitis and haemolytic anaemia.

As mentioned earlier, De Ritis quotient is a useful tool for the differential diagnosis of liver disease:

- AST/ ALT < 1 for mild hepatocyte injury and acute hepatitis, and AST /ALT > 1 for severe hepatocyte injury, liver necrosis, liver cancer, and cirrhosis.
- AST/ALT > 2 is suggestive for and AST/ALT ≥ 3 is indicative for alcoholic liver cirrhosis.

		ALT	AST
Reference values:	M	<0.74 µkat/L	<0.58 µkat/L
	F	<0.56 µkat/L	<0.52 µkat/L

A circadian variation has been observed in ALT activity, and serum concentrations of both aminotransferases tend to increase in the winter for ~ 6%. Increased values of AST are observed in neonates and children under 3 years.

MEASUREMENT OF CATALYTIC ACTIVITY OF ALT AND AST

The amount of ALT and AST is determined by measuring the concentration of their catalytic activity in serum, expressed in µkat/L or IU/L.

1 kat (katal) is the amount of an enzyme that catalyses the reaction of 1 mol of substrate per second under certain conditions, and 1 IU (international unit) is the amount of enzyme that catalyses the reaction of 1 µmol of substrate per minute under certain conditions. Here we use µkat/L (according to the International System of Units).

Laboratories should follow the procedures (reference methods) recommended by the International Federation of Clinical Chemistry (IFCC) and use the reagents, commercial standards and controls that allow traceability and commutability from reference to routine method.

In this laboratory practical we will learn two methods: one for ALT and one for AST catalytic activity determination. The detection limit of the method used is 0.027 µkat/L for ALT and 0.018 µkat/L for AST. The methods for both analytes are linear up to 8.3 µkat/L. For higher enzyme

values, we have to dilute the samples with distilled water in a ratio of 1:10 and repeat the analysis.

Interferences:

- The samples should not be hemolyzed. The presence of hemolysis greatly increases the AST value, the effect on the ALT value is smaller.
- Bilirubin has no effect on the results.
- Severe lipemia (concentration of triglycerides > 5 mmol/L) may falsely increase the readings of both enzymes. When interpreting the results, we must also consider the possible intake of hepatotoxic drugs or drugs that can cause cholestasis.

Biological sample

Catalytic activity concentration of ALT and AST is measured in serum or plasma. As anticoagulant we can use heparin, EDTA, citrates and oxalates. Samples are stable for 2-3 days at room temperature and up to 7 days at 4˚C. For longer storage of samples, freezing at -20˚C (AST) or -80˚C (ALT) is recommended.

DETERMINATION OF CATALYTIC ACTIVITY CONCENTRATION OF ALT IN SERUM

Principle

ALT catalyses the transfer of an amino group from L-alanine to 2-oxoglutarate in the presence of pyridoxal phosphate. In the reaction pyruvate and L-glutamate are formed. Formed pyruvate then enters the second, indicator reaction, in which it is reduced to lactate. The reaction is catalysed by lactate dehydrogenase (LDH). In the same reaction an equivalent amount of NADH is oxidized to NAD^+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the ALT activity in the sample.

Reactions:

$$L\text{-}alanine + 2\text{-}oxoglutarate \xrightarrow{ALT} pyruvate + L\text{-}glutamate$$

$$pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-}lactate + NAD^+$$

DETERMINATION OF CATALYTIC ACTIVITY CONCENTRATION OF ALT IN SERUM

Principle

AST catalyses the transfer of an amino group from L-aspartate to 2-oxoglutarate in the presence of pyridoxal phosphate. In the reaction oxaloacetate and L-glutamate are formed. Formed oxaloacetate then enters the second, indicator reaction, in which it is reduced to malate. The reaction is catalysed by malate dehydrogenase (MDH). In the same reaction an equivalent amount of NADH is oxidized to NAD^+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the AST activity in the sample.

Reactions:

$$L\text{-}aspartate + 2\text{-}oxoglutarate \xrightarrow{AST} oxaloacetate + L\text{-}glutamate$$

$$oxaloacetate + NADH + H^+ \xrightarrow{MDH} malate + NAD^+$$

For detailed procedure requirements and working conditions look in to the original manufacturer´s protocol.

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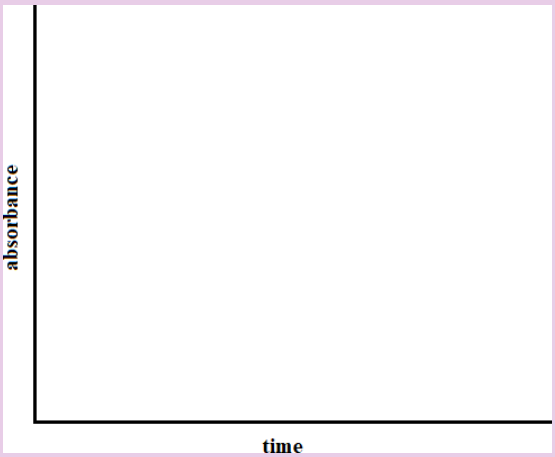
1. Tietz Textbook of Laboratory Medicine. 7th Ed. Editors: Rifai N., W.K. Chiu R., Young I., D.Burnham C., Wittwer CT. Elsevier, 2023.
2. Schumann G, *et al.*: IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37˚C. Part 4. Reference procedure for the measurement of catalytic concentration of alanine aminotransferase. Clin Chem Lab Med 2002; 40:718-724.
3. Schumann G, *et al.*: IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37˚C. Part 5. Reference procedure for the measurement of catalytic concentration of aspartate aminotransferase. Clin Chem Lab Med 2002; 40:725-733.

TEST YOUR KNOWLEDGE

1. Sketch the graph how the absorbance will change over time while measuring ALT catalytic activity.



2. Write down the equation that you will use to calculate the catalytic activity concentration of AST and ALT in your samples.
3. Consider what can cause falsely low catalytic activity concentrations of serum AST.
4. AST catalytic activity of 195 IU/L was measured in the patient's serum. What is this value in µkat/L? Comment on the measured AST activity using the reference values. The patient is male and 65 years old. Explain possible causes for the values obtained.
5. The catalytic activity concentration of ALT was also measured for the same patient's serum. The concentration was 1,2 µkat/L. Clinically interpret both measured results.

LAB REPORT: DETERMINATION OF ALT AND AST ENZYMES ACTIVITY CONCENTRATION IN SERUM

Protocol for catalytic activity concentration of AST and ALT:

1. Warm up the reagents, cuvettes and spectrophotometer to 37 ˚C before the analysis.
2. Prepare the working reagent:
    a. Calculate the amount of the working reagent needed for the whole group.
    b. First add 1 volume of reagent B to 4 volumes of reagent A.
    c. Then add 0.1 volume of reagent C to 10 volumes of mixture A+B.

Warning! Read the labels for the reagents carefully to avoid interchanging reagents between the AST and ALT analyses.

3. Pipette (in mL) into cuvette:

	Sample	**Control**
Sample (serum)	0.05	—
Control sample	—	0.05
Working reagent	1.0	1.0

4. Mix, insert into termostated spectrophotometer and measure the absorbance at 340 nm at timepoints 0, 1, 2, 3 and 4 minutes.

Note: *The procedure is the same for both enzymes. The difference is only in the composition of the working reagent.*

Reagents:

For ALT:

- Reagent A: TRIS buffer (150 mmol/L), L-alanine (750 mmol/L), LDH (>1350 U/L); pH 7.3
- Reagent B: NADH (1.3 mmol/L), 2-oxoglutarate (75 mmol/L), NaOH (148 mmol/L), Na azide (9.5 g/L)
- Reagent C: pyridoxal phosphate (10 mmol/L)

For AST

- Reagent A: TRIS buffer (121 mmol/L), L-aspartate (362 mmol/L), MDH (>460 U/L), LDH (>66 U/L), NaOH (255 mmol/L); pH 7.8
- Reagent B: NADH (1.3 mmol/L), 2-oxogluatarate (75 mmol/L), NaOH (148 mmol/L), Na azide (9.5 g/L)
- Reagent C: pyridoxal phosphate (10 mmol/L)

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the method (scheme of reaction time, graph of two-point calibration)

Measurements

AST

	0'	*1'*	*2'*	*3'*	*4'*	ΔA_1	ΔA_2	ΔA_3	ΔA_4	$\bar{\Delta A}$
Sample										
Control										

ALT

	0'	*1'*	*2'*	*3'*	*4'*	ΔA_1	ΔA_2	ΔA_3	ΔA_4	$\bar{\Delta A}$
Sample										
Control										

Calculations

(ε_{340} = 6220 L/(cm x mol))

Results

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

DETERMINATION OF ALKALINE PHOSPHATASE ISOENZYMES IN HUMAN SERUM

Assist. Dr. Jasna Omersel, MPharm, EuSpLM

LEARNING OUTCOMES

After practical course students will:

- recognize the significance of determining total enzyme activity of alkaline phosphatase,
- understand how the presence or absence of specific isoenzymes in serum reflects disorders related to bone and liver-bile metabolism,
- learn methods for detecting and quantifying ALP isoenzymes, reinforcing their role as diagnostic tools in medical laboratories,
- gain hands-on experience with electrophoretic separation techniques for isolating ALP isoenzymes from serum samples.

ALKALINE PHOSPHATASE AND ITS CLINICAL SIGNIFICANCE

Enzymes are not only effective catalysts for biological reactions, but can also be extremely important diagnostic markers. More than 20 enzymes are determined in medical laboratories for the detection of organ function, disease diagnosis and prognosis, or monitoring therapeutic efficacy.

ALKALINE PHOSPHATASE

Alkaline phosphatases (EC 3.1.3.1.; orthophosphoric- monoester phosphohydrolase; ALP) are membrane-bound glycoprotein enzymes, a class of hydrolases that act in alkaline medium.

ALP hydrolyzes various monophosphate esters, releasing inorganic phosphate. Its exact metabolic function in the human body is not yet clear, but it is thought to be associated with lipid transport in the intestine and with the calcification process in bone. It may also be involved in protein phosphorylation, cell growth, apoptosis and cell migration during embryonic development.

Mammalian ALP contains two zinc atoms (Zn^{2+}) and one magnesium atom (Mg^{2+}) in the active site, which are essential for enzymatic activity contribute substantially to the conformation of the ALP monomer and indirectly regulate subunit–subunit interactions (Figure 1).

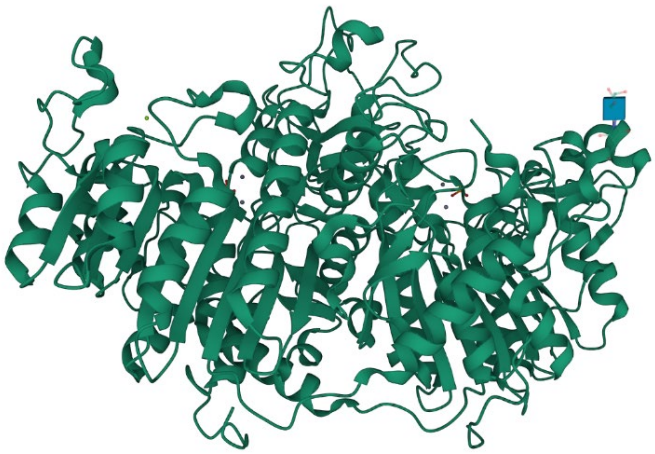


Figure 2: *Crystal structure of a dimeric form of ALP, with visible ions and carbohydrate moiety. (PDB ID: 1EW2)*

Some metabolic processes are controlled by enzymes in different molecular forms called isoenzymes. **Isoenzymes** have similar but not identical amino acid sequences in their structure. The formation of isoenzymes may be encoded by independent genes expressed in different tissues. They differ in their kinetics, physicochemical properties, regulatory properties, and location in the organism. So, isozymes are species-specific forms of enzymes that are produced in different tissues and organs but catalyse the same reactions. They may also differ in the nature of post-translational modifications (**enzyme isoforms**), but still catalyse the same biochemical reaction.

In addition to ALP, there are other enzymes with their isoenzymes that exist in the organism, for example, creatine kinase (CK) and lactate dehydrogenase (LDH).

CLINICAL SIGNIFICANCE OF DETERMINATION OF ALP AND ITS ISOENZYMES

The catalytic activity of the total serum ALP reflects the activity of all isoenzymes present. In healthy adults, the bone and liver isoenzymes account for approximately equal proportions of the total activity of ALP in serum, but the ratio between them changes according to age and sex, during pregnancy, the placental isoenzyme is also present in serum. The increase in the activity of ALP may be physiological or a consequence of bone and/or hepatobiliary diseases. Therefore, determination of the total activity of ALP in the serum of patients has little diagnostic value; it is important to identify specific isoenzymes, which then determine the reason for the increased activity of ALP. Isoenzyme identification results are always interpreted together with the total catalytic activity of ALP.

The clinical utility of ALP isoenzymes is important for the detection and therapy of:

- disorders of increased bone metabolism (e.g. Paget's disease),
- hepatobiliary disorders,
- disorders of placental, renal and intestinal mucosal function.

Of particular importance for clinical diagnostics is the determination of ectopic isoenzymes from ALP, which are derived from tumour tissues.

Reference values

It is recommended that each laboratory establishes its own reference values for the local population for the method used to measure enzyme activity. Reference values should be established using samples from supposedly healthy populations, e.g., excluding individuals with bone, endocrine, and chronic diseases, pregnant women, and menopausal women.

Reference values of ALP are highly dependent on gender, age, hormonal stage (pregnancy, menopause, puberty), and medications.

- In adult subjects aged 20-50 years, the values of total activity are almost independent of age and gender - there is hardly any difference in the activity of bone and liver isoenzymes between men and women

- With increasing age, total activity ALP increases slightly, as does the activity of bone and liver isoenzymes. After the age of 50, the total activity ALP increases more in women compared to men due to menopause.
- In infants and peripubertal children, the total activity of ALP is up to three times higher than in adults, which is due to the lively process of bone remodeling (leakage of the isoenzyme ALP from osteoblasts during growth). In children and elderly people, the result of ALP can be quickly misinterpreted simply by using the wrong reference values.

Reference values:	M	0.72 -1.92 µkat/L
	F	0.55 - 1.64 µkat/L

ISOENZYMES OF ALKALINE PHOSPHATASE

ALP isoenzymes are coded by at least 3 different structural genes, widely expressed in human tissues:

1) Tissue nonspecific gene (gene symbol: ALPL) is expressed in variety of tissues like bone, liver and kidneys and codes for liver, bone, and kidney isoenzymes (L/B/K ALP)
2) Placental gene (gene symbol: ALPP) codes for placental isoenzyme
3) Intestinal gene (gene symbol: ALPI) codes for intestinal isoenzyme

In the human body, ALP isoenzymes are present as dimeric molecules in body fluids (blood, bile, breast milk, urine) and organs (bones, liver, placenta, kidneys…). The following ALP isoenzymes and isoforms are known:

- liver isoenzyme (L1, L2)
- bone isoenzyme (B)
- kidney isoenzyme
- intestinal isoenzyme (I1, I2, I3)
- placental isoenzyme (P1, P2)
- ectopic/eutopic isoenzyme

Intestinal, placental, and ectopic ALP isoenzymes are encoded by tissue-specific genes located on chromosome 2q 24-37. In vitro, 90-98% homology has been demonstrated between these

isoenzymes. Studies on molecular models have shown that these isoenzymes have a large proportion of hydrophobic chains in their structure, whereas liver, bone, and kidney isoenzymes have more polar chains. This is reflected in slight differences in the activity of each isoenzyme toward the substrate. Differences in the type and rate of glycosylation affect their migration in the electric field and form the basis for their separation for diagnostic purposes.

Bone isoenzyme

Bone ALP is a sialoglycoprotein produced by osteoblasts. It is one of the markers of bone remodeling process. Bone molecule ALP is bound to osteoblast membrane by carboxy-glycan phosphatidyl inositol. After the action of specific phospholipases, it is released into the bloodstream, where its half-life is 1-2 days, which affects the low daily and biological variability. Like many other glycoproteins, ALP is removed from circulation after metabolism in the liver.

Bone ALP is a thermally unstable isoenzyme. When serum is heated to 56˚C, it is inactivated after 15 minutes. It has the same mobility on the cellulose acetate and polyacrylamide gel as the liver isoenzyme. Separation of bone and liver isoenzyme is successful after treatment of the serum with neuraminidase or wheat germ lectin. The bone isoenzyme is the most glycosylated isoform (N- and O-glycosylation).

Catalytic activity of bone ALP is a useful diagnostic marker for monitoring the changes in bone remodelling in postmenopausal osteoporosis, kidney osteodystrophy, Paget's disease, and bone metastasis - bone fraction increases.

Liver isoenzyme

Liver ALP is a sialoglycoprotein bound to the apical membrane of hepatocytes, the basal membrane of sinusoids, and the lateral membrane connecting sinusoids. It is released from the membrane-bound forms by the action of bile acids and specific phospholipases.

L1 is elevated in cholestasis, cirrhosis, viral hepatitis, and other biliary and hepatic pathologies. It is also elevated in malignancies with liver metastases and cancer of the digestive tract.

L2 (macrohepatic and fast hepatic fraction) is present only in low concentrations in healthy individuals. An increase occurs after damaged bile ducts after cholestasis and biliary disease, and in malignancies (breast, liver, lung, prostate, digestive tract) with liver metastases.

Determination of the total activity of ALP and the presence of L1 or L2 is clinically useful to distinguish cholestasis from alcoholic cirrhosis. In cholestasis, the activity of both enzymes is increased; in cirrhosis, the increase in L2 is small.

Kidney isoenzyme

Kidney isoenzyme is encoded by tissuenonspecific gene. It has similar electomobility properties as liver and bone isoenzymes; it is rarely seen as a specific fraction on the elpherogram.

Intestinal isoenzymes

The intestinal isoenzyme is a dimeric molecule, glycoprotein, bound to the membrane of the enterocyte. Bile acids and specific phospholipases cause the release of the dimeric molecule into the bloodstream. It is removed from circulation by binding to mannose-fucose receptors in the liver. This shortens its half-life to 6 hours compared to the liver and bone isoenzyme, whose circulating half-life is 16 to 60 hours

Intestinal ALP activity in serum may increase after a meal, ALP should be measured preferentially in fasting state.With normal ALP activity, intestinal isoenzymes are found more frequently in serum samples from people with blood type 0 or B, including in fasting samples.

Increased activity can be found in some diseases such as hepatic chirosis, diabetes, and chronic renal failure.

Several isoforms of the intestinal isoenzyme have been discovered due to post-translational modifications of the molecule: I1, I2, I3. The intestinal fractions are not affected by lectin.

Placental isoenzyme

Placental isoenzyme (90% of fraction P1 and 10% of fraction P2) is bound to the plasma membranes of trophoblasts in the placenta. It is present in the serum of a pregnant woman from the 12th week of gestation and remains elevated up to one month after delivery. An increase in total activity to two to three times the upper reference limit is observed. Placental

ALP is also re-expressed in some malignancies (ovarian cancer, seminomas, sarcomas, pancreatic and gastric cancers) and can be detected in smokers' sera (release from the lungs).

It is a genetically highly polymorphic isoenzyme (18 different allelic variants), but also shares some of the same antigenic determinants as the intestinal isoenzyme. It is the most thermally stable ALP isoenzyme. Its activity is decreased but still present even when serum is heated to 65˚C for 6.5 minutes.

Atypical isoenzymes

The ectopic isoenzymes are most distinguished from other isoenzymes by their physical and chemical properties. They are found in the serum of patients with certain types of tumours. These tumours may increase the synthesis of isoenzymes normally present in tissues (eutopic isoenzymes) (placental Regan isoform) or secrete isoenzymes not normally present in tissues (ectopic isoenzymes). Ectopic isoenzymes have the same mobility in the electrophoretic field as placental isoenzymes (e.g., the Regan isoform) and are therefore difficult to identify.

As we will learn, ALP isoenzymes can be seperated by elecrophoresis. After separation of the isoenzymes on electrophoretic gels, two additional fractions can be seen:

First, the immunoglobulin complex or macro-alkaline ALP, which is rarely found. After separation, it remains in the vicinity of the application point. Second, ALP within a lipoprotein complex (ALP -X), which is indicative of biliary obstructive disease. It is a form of intestinal isoenzyme. After separation, it also remains in the vicinity of the application point.

DETERMINATION OF ALP ISOENZYMES

In medical laboratories, total serum activity ALP is determined by the IFCC enzymatic colorimetric method. If the total activity is above the reference values, electrophoretic separation and its qualitative/quantitative determination on agarose gels is performed. Bone isoenzyme activity can also be determined immunochemically by the ELISA method. Quantification of the ALP isoenzymes helps identify the tissues responsible for the elevation and can guide the clinician in the differential diagnosis of a particular disease.

There are many different methods available for the determination of ALP isoenzymes: electrophoretic separation, isoelectric focusing, thermal inactivation, lectin precipitation,

immunochemical methods. Which method is the most appropriate depends on the physicochemical or immunochemical properties of the individual isoenzymes:

- electrophoretic mobility of fractions that we want to determine, separate
- thermal stability and isoenzyme resistance to detergents
- sensitivity to the inhibitors (amino acids, reaction buffers in reagents)
- binding affinity for lectin molecule
- type and extent of posttranslational modifications (glycosylation, phosphorylation).

ELECTROPHORETIC SEPARATION OF ALP ISOENZYMES

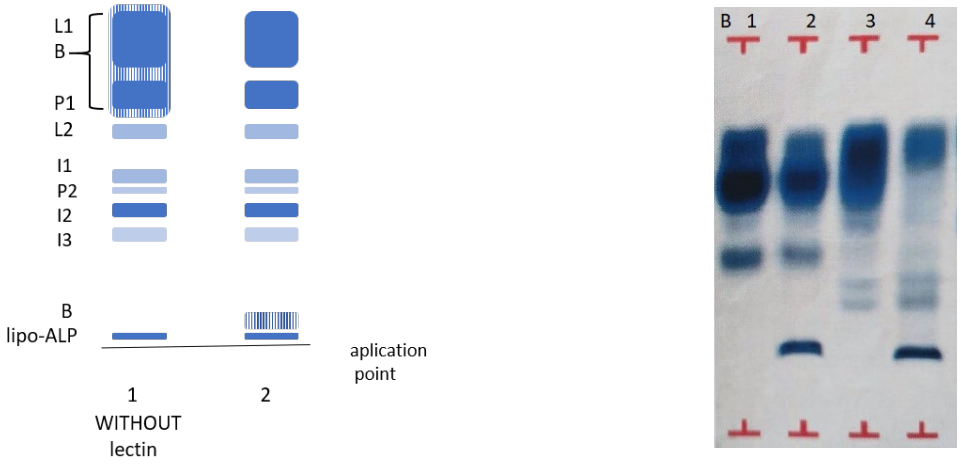
Kits used in medical laboratories are designed for the identification and quantification of ALP isoenzymes in human serum. The procedure is performed with a semi-automated multiparameter instrument. Agarose gels are used to separate the serum samples. Automated steps include: Sample application, electrophoretic migration, incubation with substrate, blotting, rising and drying of the gel. Steps to be performed manually include: Handling the gels and samples, applying the reagents, and setting up the instrument or run. The dried gels are visually examined and can be scanned with a densitometer to obtain the relative quantification of each isoenzyme expressed as a percentage of the total activity ALP.

The ALP isoenzymes are separated on gels based on their physicochemical properties. The differences in the motility in the electric field are mainly due to the differences in posttranslational modifications (glycosylation) and thus the differences in molecular size and charge. Because of the similar physicochemical properties, the separability of liver and bone isoenzyme is poor. We improve their separation by treatment of sample with neuraminidase or wheat germ lectin before the electrophoresis.

Neuraminidase alters the net charge of the isoenzyme. As a result, the travel speed of the bone isoenzyme in the electric field changes, allowing its separation from the liver isoenzyme. Wheat germ lectin exhibits a strong affinity for sialic acid residues and binds preferentially to the bone fraction, which is the most sialated of all. The isoenzyme in complex with the lectin has a higher molecular weight and lower mobility, resulting in only a slight shift toward the cathode.

The mobility of the individual isoenzymes of ALP in the electric field varies depending on the type of gel chosen:

- agarose gel: (anode +) **L1 + B + P1 ⇒ L2 ⇒ I1 ⇒ P2 ⇒ I2 ⇒ I3** (cathode -)
- cellulose acetate: (anode +) **L2 ⇒ L1 + B ⇒ P ⇒ I** (cathode -)



A B

Figure 2: *Electrophoretic migration pattern of ALP isoenzymes on agarose gel (A), diagnostic method. Elpherogram of two patients sera (B). The effect of lectin treatment on electrophoretic separation of ALP isoenzymes is visible when comparing line 2 to line 1 (Sample 1) and line 3 to line 4 (Sample 2)*

Principle of the method

Electrophoretic separation of serum proteins and staining of the cellulose acetate plate with a specific substrate for ALP give us qualitative information about the presence of ALP isoenzymes in the sample. Electrophoresis is performed in barbital buffer at alkaline pH and can be done in different media (agarose or polyacrylamide gel, cellulose acetate). The separated isoenzymes are visualised with a specific chromogenic substrate (diazonium salt and α-naphthyl phosphate) in basic solution:

$$\alpha\text{-}naphthyl\ phosphate\ +\ diazonium\ salt\ \xrightarrow{ALP}\ coloured\ diazo\ copound + PO_4^{3-}$$

Preanalytical influences and interferences:

- Citrates, EDTA and oxalates inhibit ALP enzyme activity.
- Sample preparation after blood collection can greatly affect the result of the analysis. The most reliable measurement is on the day of blood collection. ALP Activity increases by up to 2% within 6 hours for fresh samples stored at 25˚C. After thawing

and warming frozen samples, ALP activity increases significantly (up to 30%) due to the release of ALP from lipoprotein complexes. Therefore, it is recommended to gradually warm the serum sample to room temperature (18-24 hours) to allow ALP to develop its full activity.

- More than 250 active substances increase the overall activity of ALP, especially those that are hepatotoxic or induce cholestasis. There are also some substances that reduce the ALP activity: clofibrate, oral contraceptives (oestrogen).
- Intestinal ALP activity in serum may be increased after a meal, so ALP total activity should be measured preferentially in fasting sera (8-12 hours after the last meal).
- Haemoglobin, bilirubin and lipids do not interfere with the electrophoretic determination of ALP isoenzymes.
- When using the method of isoenzyme separation by lectin, the robustness of the method is questionable due to possible variation between lectin series, stability of the lectin, and repeatability of quantification of precipitation. Lectin precipitates 90% of bone ALP and < 5% of liver ALP.

Biological sample

- fresh, non-haemolysed serum or heparinised plasma
- If the analysis is not performed within 4 to 6 hours after the blood collection, the sample should be frozen at –20˚C unil the analysis.

TEST YOUR KNOWLEDGE

1. Think about and explain what is neuraminidase's mechanism of action that allows the separation of bone and liver isoenzyme.
2. Explain the function of the reagents and working conditions during electrophoretic separation of activity determination:
    - barbital buffer, pH=8.6
    - preparation of substrate right before use
    - incubation of the elpherogram with the substrate at 37˚C
    - aminomethyl propanol buffer (AMP), pH=10.4.

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LAB REPORT: DETERMINATION OF ALP ISOENZYMES IN SERUM

Protocol:

Reagent preparation

1. Cellulose acetate plate (TITAN III cellulose acetate plates, 60x76 mm, Helena Bioscience)
2. Barbital buffer, pH=8.6, 1L (Electra® B_1 Buffer Barbital/Sodium Barbital/Na Azide, Helena Bioscience)
3. Lectin solution: Add 50 µL of dH_2O and the tip of spatula of lectin (Sigma-Aldrich) into the test tube. Mix by tapping.
4. Substrate solution: dissolve 3 mg of α-naphthyl phosphate (Sigma-Aldrich) into 1 mL of aminomethyl propanol buffer (0.42 mol/L, pH = 10.4) and then add 3 mg of diazonium salt (Variamin blue B).

Sample preparation

Serum samples should be analysed fresh or thawed slowly to room temperature before analysis. Mix well before use.

Preparation of sample with lectin: 10 µL of lectin solution + 10 µL of serum sample, mix by pipetting, incubate at room temperature for 10 minutes.

Procedure

1. Slowly soak the cellulose acetate plate in the barbital buffer for 12-15 minutes (activation of cellulose acetate plate).
2. Pour 100 mL of barbital buffer into the outer compartments of the electrophoresis tank.
3. Pipete 10 µL of serum samples into the wells of the sample holder.
4. Gently blot the cellulose acetate plate between two sheets of filter paper to remove the excess buffer. Then transfer the samples from the sample holder to the cellulose acetate plate using a special "comb" applicator. Lightly mark the application site with

a pencil and cut the corner of the cellulose acetate plate at sample number 1.Place the plate on the edge of the tank so that it touches the moistened filter paper and the part with the applied samples is at the negative pole. Then cover the tank and start electrophoresis. Electrophoresis is performed at 180 V for 15 minutes.

5. At the end of electrophoresis switch off the power supply, remove the cellulose acetate plate and put it in the plastic chamber.
6. Pour freshly prepared substrate solution on the elpherogram, cover the camber with plastic wrap and put it in the incubator. Incubate for 17 minutes at 37˚C.
7. After the incubation, rinse the elpherogram under running water and qualitatively evaluate the presence of individual ALP isoenzymes.

Warnings! Reagents used to prepare substrates may cause respiratory irritation if inhaled. Substrate solution is irritating to the skin, so wearing gloves during preparation is mandatory.

Independent theoretical work in groups:

Divide into groups of 3–4 students. Each group will get an article. Read the article and do a mind mapping for a 5-minute long group presentation based on given points of reference.

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Principle and reaction of ALP with substrate solution:

Conditions and observations:

Electrophoretic separation of isoenzymes

- Sketch the appliance and electrophoresis chamber:

- Sample application:

- Electrophoresis settings and conditions:

Preparation of substrate solution and incubation

- Substrate solution:

Preparation of plastic chamber and incubation with substrate:

Results

Sketch elpherogram and evaluate the results

	Sample tag	Total catalytic activity (µkat/L)	Present isoenzymes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

DETERMINATION OF TOTAL, HDL AND LDL CHOLESTEROL AND TRIGLYCERIDES IN SERUM

Assist. Lara Smrdel, mag. lab. biomed.

LEARNING OUTCOMES

After successfully completing this laboratory practical, students will be able to:

- define lipoproteins and differentiate between total cholesterol, LDL cholesterol and HDL cholesterol,
- describe the pathways of lipoprotein metabolism,
- list the analytes that are important for determining the lipid profile in serum samples,
- explain the clinical significance of determining the total lipid profile,
- use commercial kits for the measurement of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides in serum samples,
- identify which analytes are essential for investigating the risk of developing ischemic cardiovascular disease,
- apply the acquired knowledge for a comprehensive clinical interpretation of the calculated and measured data and relate it to the patient status.

LIPOPROTEINS AND THEIR METABOLISM

Lipoproteins are spherical macromolecular particles (10-1000 nm) composed of lipids and specific proteins, apolipoproteins. Hydrophobic lipids (triglycerides and cholesterol esters) are located in the centre of the particle, while more polar amphiphilic lipids such as phospholipids and free cholesterol are located on the surface (Figure 1). Lipoproteins differ

not only in their physical and chemical properties (Figure 2), but also in their physiological and pathophysiological roles.

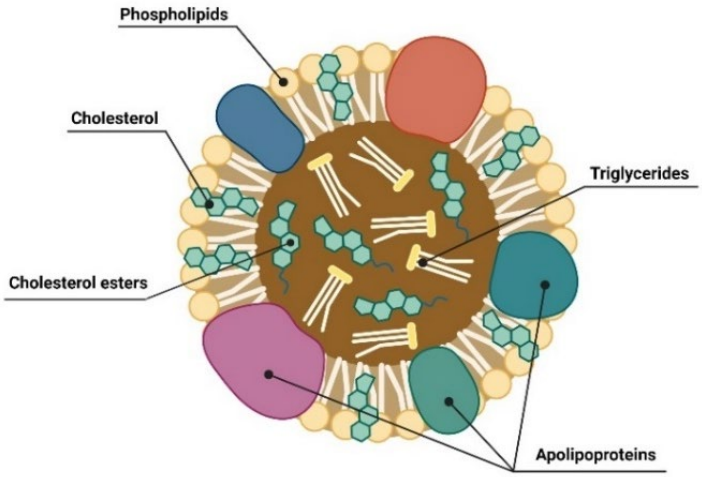

Figure 1. *Schematic representation of lipoprotein particle structure. Created by biorender.com.*

Apolipoproteins are the protein components of lipoproteins. Their main functions are to maintain the structural integrity of lipoprotein complexes, modulate enzyme activity, and function as ligands for specific cell receptors. Each class of lipoproteins contains several different apolipoproteins. Apo A-I and -II are the major proteins in HDL particles. Apo A-I plays a key role in lipid metabolism and is a cofactor of the enzyme lecithin-cholesterol acyltransferase (LCAT). Apo C-I, -II, -III and E are present in varying proportions in all lipoproteins except LDL. Apo C-II is synthesised in hepatocytes and is cofactor of lipoprotein lipase (LPL), which hydrolyses lipoprotein triglycerides. Apo B is the major protein on VLDL, LDL and chylomicrons. It exists in two isoforms, Apo B-100, which is a ligand for the LDL receptor, and Apo B-48, which is found exclusively on chylomicrons.

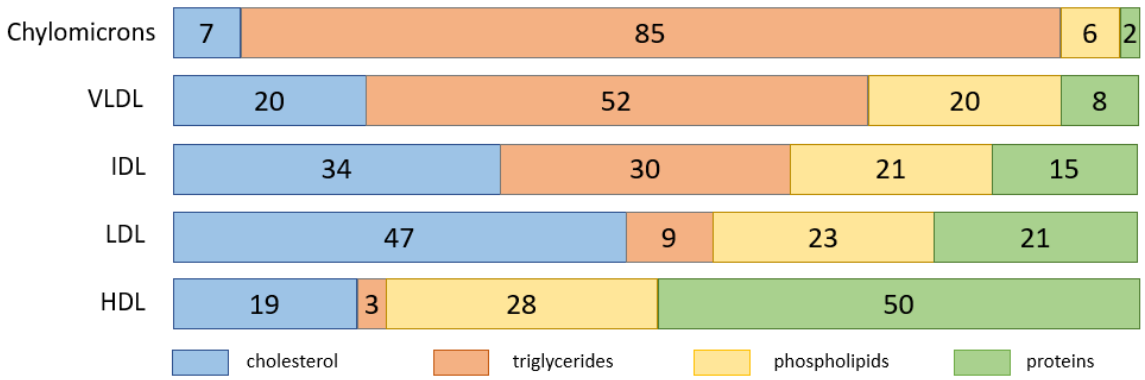

Figure 2. *Structure of different lipoprotein particles such as chylomicrons, VLDL, IDL, LDL, and HDL. All lipoproteins are composed of four main building particles (cholesterol, triglycerides, phospholipids, and proteins). Therefore, differences in size of various lipoprotein particles can be explained due to different proportions between each building particle.*

LIPOPROTEIN CLASSIFICATION

Lipoproteins contain different proportions of lipids and proteins and consequently have different physicochemical properties (Table 1), which are reflected in different physiological and pathophysiological functions. Originally, the different lipoproteins were separated into 4 main fractions by ultracentrifugation:

Chylomicrons: They are the largest particles with the lowest density. Chylomicrons contain the apolipoprotein Apo B-48 and are synthesized in the digestive system. Their main function is to transport dietary lipids from the gastrointestinal tract to the liver and peripheral cells.

VLDL: They are synthesized in the liver and contain Apo B-100, Apo E and Apo C-I, -II, -III. VLDL are rich in triglycerides and are the main transporters of endogenous triglycerides from the liver to peripheral tissues. A diet high in carbohydrates, saturated fatty acids, and trans fatty acids increases hepatic synthesis of triglycerides and, consequently, synthesis of VLDL particles.

LDL: They come in different sizes and with different compositions. They primarily contain Apo B-100, by means of which they enter the cell via the LDL receptor, being regulated by a negative feedback loop. Due to their small size, LDL particles can enter the extracellular space of the cell wall, where they are oxidized and contribute to the formation of atherosclerotic plaques.

HDL: They are the smallest and densest lipoprotein particles synthesized in both the liver and gastrointestinal tract. HDL can exist in either discoid or globular form. The discoid form of HDL is most effective in removing excess cholesterol from peripheral cells. When discoid HDL takes up additional lipids, it transforms into a globular form. The removal of cholesterol from cells is called reverse cholesterol transport.

Table 1: *Lipoproteins physicochemical properties.*

Properties	Lipoproteins			
	Chylomicrons	**VLDL**	**LDL**	**HDL**
Density [kg/L]	0.93 – 0.96	0.96 – 1.006	1.019 – 1.063	1,063 – 1,21
Diameter [nm]	80 – 1200	30 – 80	18 – 30	5 – 12
Main Apolipoproteins	Apo A-I	Apo B-100	Apo B-100	Apo A-I, -II
	Apo B-48	Apo C-I, -II, -III		
	Apo C-I, -II, -III	Apo E		

LIPOPROTEIN METABOLISM

Lipoprotein metabolism can be divided into four main pathways: exogenous pathway, endogenous pathway, intracellular cholesterol transport and reverse cholesterol transport.

Exogenous metabolic pathway

The main role of the exogenous metabolic pathway is the transport of lipids from the gastrointestinal tract to the liver and peripheral cells (Figure 3). Chylomicrons play an important role in this process. Chylomicrons are formed in enterocytes by the association of Apo B-48 with triglycerides and other lipids. After entering the bloodstream, Apo C-II and E are added, causing activation of the enzyme lipoprotein lipase (LPL), which hydrolyses some of the triglycerides on the chylomicrons into free fatty acids. These free fatty acids bind to albumin and enter muscle cells as a source of energy or adipocytes as stored energy source. After hydrolysis, the chylomicron remnants enter the liver, where the remaining triglycerides are used as an energy source or as an energy storage, or are combined with Apo B-100 and excreted as part of VLDL particles.

Endogenous metabolic pathway

The main function of the endogenous pathway is to transport of hepatic lipids from hepatocytes to peripheral cells, where they are used as an energy source (Figure 3). The endogenous pathway involves lipoproteins containing Apo B-100 and Apo E - mainly VLDL. Hepatic lipids are lipids that are either synthesized in the liver or enter the liver via an exogenous metabolic pathway. After VLDL enters the bloodstream from the liver, Apo C is added, which activates LPL on endothelial cells. Therefore, the enzyme LPL hydrolyses the triglycerides on the VLDL particles and converts them into free fatty acids, which enter the peripheral cells as an energy source. Lipolysis converts VLDL to VLDL remnants, which are returned to the liver where conversion to either IDL or LDL particles occurs. LDL particles take up cholesterol esters with HDL and return to the liver via the LDL receptor, where the cholesterol is used for the re-secretion of lipoproteins or the formation of bile acids.

Intracellular cholesterol transport

Intracellular cholesterol transport consists of several different homeostatic mechanisms by which cells regulate cholesterol concentration. Although cholesterol is an important component of cell membranes, excess cholesterol can alter the physicochemical properties of membranes and become toxic to cells. Lipoprotein particles enter lysosomes via specific lipoprotein receptors, where they are degraded. Apolipoproteins are converted into smaller peptides and amino acids, and cholesterol esters are converted into free cholesterol by acid lysosomal lipase. Free cholesterol is used in the cell for membrane biogenesis, and it can be stored in lipid droplets or excreted from the cell by reverse transport. In addition, there are specific regulatory mechanisms in cells by which the expression of genes involved in cholesterol biosynthesis (e. g., HMG-CoA) is reduced at high cholesterol concentrations. Similarly, high concentrations of cholesterol in cells inhibit the expression of LDL receptors. Hepatocytes represent a special case, as cholesterol can be further converted to bile acids or excreted directly in bile.

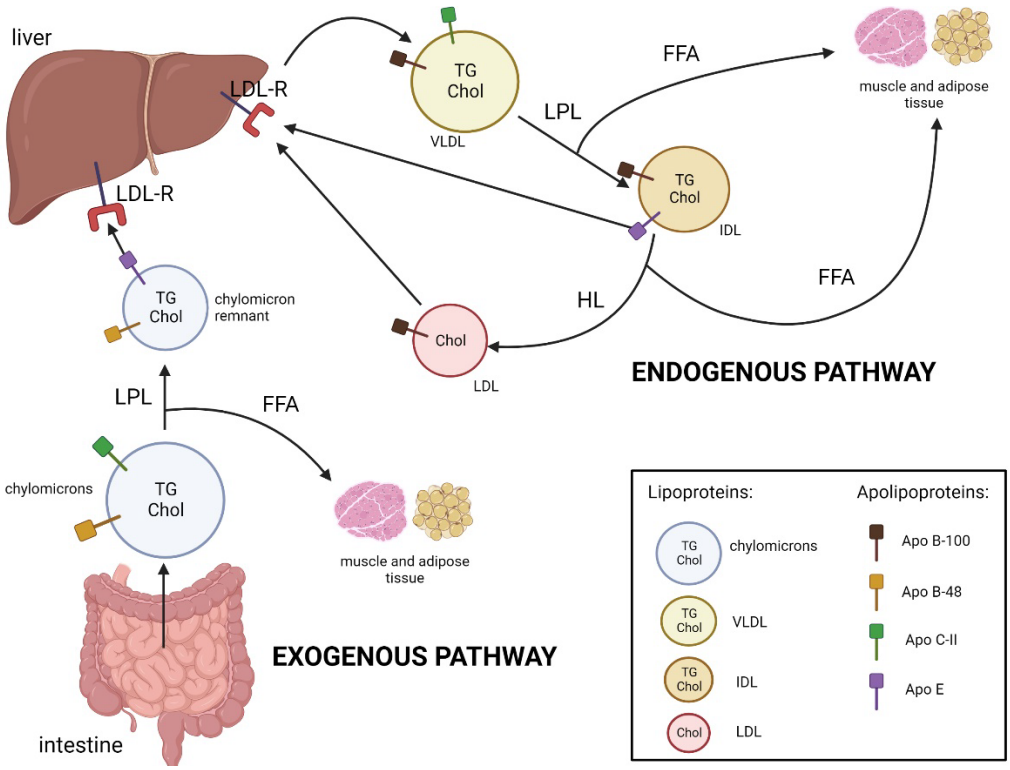

Figure 3. *Endogenous and exogenous metabolic pathway. Lipoproteins: chylomicrons, VLDL, IDL, and LDL; Apolipoproteins: Apo B-100, Apo B-48, Apo C-II, and Apo E; Abbreviations: LPL – lipoprotein lipase, FFA – free fatty acids, TG – triglycerides, Chol – cholesterol, LDL-R – LDL receptor, HL – hepatic lipase. Created by biorender.com.*

Reverse cholesterol transport

Most peripheral cells are unable to metabolise cholesterol, so it can accumulate in cells and become toxic. The role of reverse cholesterol transport is to remove the excess cholesterol from the peripheral cells and transport it back to the liver. This is done by HDL particles. The cholesterol leaves the cell via different pathways and binds to HDL particles. The LCAT enzyme then converts the cholesterol on HDL to a cholesterol ester, which allows transfer to LDL particles and enters the liver via the LDL receptor.

CLINICAL RELEVANCE OF LIPOPROTEINS

Disturbed lipoprotein balance in plasma is the main risk factor for the development of atherosclerosis and cardiovascular complications such as stroke and myocardial infarction. Disorders of lipoprotein balance are grouped under the term dyslipidaemias. Depending on their origin were divided into primary and secondary dyslipidaemias. Primary dyslipidaemias are the result of hereditary abnormalities, according to 2019 guidelines are named the genetic

disorders of lipoprotein metabolism (mentioned in Table 2). Secondary dyslipidaemias could be described as the result of other factors or diseases, such as a diet rich in carbohydrates and saturated fat, alcoholism, obesity, diabetes, kidney and liver disease, and certain medications (more factors mentioned in Table 3).

Table 2: *Genetic disorders of lipoprotein metabolism.*

Genetic disorders of lipoprotein metabolism	
	Effects on lipoproteins
Heterozygous Familial hypercholesterolemia	↑ LDL-Chol
Homozygous Familial hypercholesterolemia	↑↑ LDL-Chol
Familial combined hyperlipidaemia	↑ LDL-Chol, ↑ VLDL-Chol, ↑ ApoB
Familial dysbetalipoproteinaemia	↑↑ IDL-Chol, ↑Chylomicron remnants
Familial lipoprotein lipase deficiency (familial chylomicron syndrome)	↑↑ VLDL-Chol, ↑↑Chylomicrons
Tangier disease (analphalipoproteinaemia)	↓↓ HDL-Chol
Familial LCAT deficiency	↓ HDL-Chol

In the last few years the Guidelines for the management of dyslipidaemias have changed due to personalised medicine approach. Therefore, the risk assesment has shifted from population-based towards more personalized prediction, where omics, genetics, and imaging play an important role. The main approach similar, although the ESC, AHA/ACC/MS, and CCS Guidelines have some differences in result interpretation, definition of reference values, and treatment recommendations. The assesment of the dyslipidaemias risk factors is still based on the principle that increased LDL-Chol should be considered as primary target. To detect dyslipidaemias in the blood, a lipid profile is obtained, which usually consist of total, HDL, LDL cholesterol, and triglycerides measurements. Tables 2 and 3 show differences in the individual analyte values in some dyslipidaemias.

When assessing the risk of developing cardiovascular disease, in addition to elevated concentrations of total cholesterol and LDL cholesterol, low concentrations of HDL cholesterol

and other factors such as gender, age, lifestyle, family history, and the presence of other diseases are important.

Table 3: *Some examples of indiviuals` lifestyle influence on lipoprotein concentrations (previously known as secondary dyslipidaemias).*

		Total Cholesterol	LDL	HDL	Triglycerides
Nutrition	**Saturated fatty acids**		↑		
	Carbohydrates				↑
	Ethanol			↑	↑
Diabetes	**Type I**		↑	↓	↑
	Type II			↓	↑
Kidney diseases	**Nephrotic syndrome**		↑		
	Chronic renal failure				↑
Liver diseases	**Acute hepatitis**		↑		
	Cholestasis				
	Cirrhosis	↓	↓	↓	↓
Medications	**β-blockers**		↑		↑
	glucocorticoids		↑		↑

Care must be taken when interpreting results of the lipid profile, as lipid concentrations change over the years. At birth, the typical cholesterol concentration is about 1.71 mmol/L and is evenly distributed between LDL and HDL particles. Triglyceride concentration is very low at about 0.41 mmol/L. In the first months of life, lipoprotein concentrations increase rapidly, and LDL becomes the major carrier of plasma cholesterol. After puberty, the concentrations of triglycerides and LDL cholesterol increase in both genders, whereas the concentration of HDL cholesterol decreases in males. The concentrations of the other lipids then increase continuously and are usually higher in males than in females.

Cholesterol values considered as risk factors for CVDs differ in individuals according to genetical, epigenetical, and environmetional factors. Moreover, from laboratory perspective is risk assessment based on the LDL-Chol concentrations, that have higher treashold in healthy

individuals than in patients receiving a treatment. The reference values in healthy individuals are <1.8 mmol/L, 1.8 – 3.0 mmol/L, and >3.0 mmol/L for low, moderate, and high risk, respectively. In addition to required lower LDL-Chol concentrations, have patients on therapy more strict criteria, including non-HDL cholesterol, ApoB and triglycerides concentrations.

Table 4: *2019 ESC/EAS Guidelines for the management of dyslipidaemias: Treatment goal concentrations for individuals.*

	LDL-Chol [mmol/L]	non-HDL Chol [mmol/L]	ApoB [mg/dL]	Triglycerides [mmol/L]
Very high risk	< 1.4	< 2.2	< 65	
High risk	< 1.8	< 2.6	< 80	< 1.7 means lower risk factor
Moderate risk	< 2.6	< 3.4	< 100	
Low risk	< 3.0			

METHODS FOR CHOLESTEROL AND TRIGLYCERIDES MEASUREMENT

TOTAL CHOLESTEROL

Measurement of total cholesterol is based on a three-step enzymatic reaction, also known as the CHOD-PAP method. In the first step, the enzyme cholesterol esterase (COE) hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second step of the reaction, the enzyme cholesterol oxidase (COD) then oxidizes free cholesterol to cholesten-3-one, producing hydrogen peroxide. In the final step, hydrogen peroxide reacts with hydroxybenzoate and 4-aminoantipyrine in the presence of the enzyme peroxidase (POD). A coloured complex is formed, which is detected with a spectrophotometer by measuring the absorbance at 510 nm. The intensity of the coloration is proportional to the concentration of total cholesterol in the sample.

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{cholesterol} + H_2O + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholesten-3-on} + H_2O_2$$

$$H_2O_2 + \text{hydroxy benzoate} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{coloured complex}$$

The reaction can be interfered with by coloured compounds and compounds that compete in the oxidation reaction, such as bilirubin, ascorbic acid and haemoglobin.

HDL CHOLESTEROL

The working process for determining the concentration of HDL cholesterol consists of two phases. In the first phase of the process, LDL, VLDL and chylomicrons are removed by precipitation. Polyanionic compounds such as dextran sulphate/$MgCl_2$ and phospho-tungstic acid/$MgCl_2$ or polyethylene glycol (PEG 6000) can be used for precipitation. In the second phase, the concentration of HDL cholesterol in the supernatant is determined using the CHOD-PAP method.

1. PRECIPITATION reaction:

$$\text{lipoproteins} + \text{precipitation reagent} \longrightarrow \textbf{HDL (supernatant)} + \text{VLDL, IDL, LDL (precipitant)}$$

2. CHOD-PAP method:

$$\textbf{cholesterol esters (supernatant)} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{cholesterol} + H_2O + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholesten-3-on} + H_2O_2$$

$$H_2O_2 + \text{hydroxy benzoate} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{coloured complex}$$

As with total cholesterol, bilirubin, ascorbic acid and haemoglobin can interfere with the reaction.

LDL CHOLESTEROL

LDL cholesterol concentration is determined indirectly by first determining the concentrations of total cholesterol, HDL cholesterol, and triglycerides. LDL cholesterol is then calculated using the Friedewald equation, which assumes that total cholesterol represents VLDL, LDL, and HDL

cholesterol combined. The factor (0.46 x triglycerides) is used as an estimate of VLDL cholesterol. Therefore, deduction of HDL, VLDL cholesterol and triglycerides is forseen in the Friedewald equation.

FRIEDEWALD equation:

$$\boldsymbol{LDL\ Chol = Total\ Chol - HDL\ Chol - 0,46 \times Triglycerides}$$

There are also direct methods for measuring LDL cholesterol based on selective precipitation of LDL particles with polycyclic anions at low pH. The supernatant is discarded and the concentration of LDL cholesterol is determined in the precipitate after prior solubilization.

TRIGLYCERIDES

The concentration of triglycerides is determined by a four-step enzymatic reaction. In the first step, the enzyme lipoprotein lipase (LPL) hydrolyses triglycerides into glycerol and fatty acids. In the second step, the enzyme glycerol kinase (GK) phosphorylates glycerol to glycerol-3-phosphate in the presence of ATP, which is then oxidised to dihydroxyacetone phosphate in the third step in the presence of glycerol-phosphate-oxidase (GPO), producing hydrogen peroxide. Finally, hydrogen peroxide reacts with ethylsulfopropyl toluidine (ESPT) and 5-aminophenazone in the presence of peroxidase (POD), forming a coloured product. The absorbance of the resulting complex is measured at 550 nm.

$$\text{triglycerides} + 3H_2O \xrightarrow{\text{lipoprotein lipase}} \text{glycerol} + 3\text{RCOOH}$$

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerophosphate} + \text{ADP}$$

$$\text{glycerophosphate} + O_2 \xrightarrow{\text{glycerophosphate oxidase}} \text{dihydroxy acetone} + H_2O_2$$

$$H_2O_2 + \text{ESPT} + \text{4-aminophenasone} \xrightarrow{\text{peroxidase}} \text{coloured complex}$$

Endogenous glycerol can cause a false increase in triglyceride concentration up to 0.11 mmol/L. Under conditions where the endogenous glycerol concentration is greatly increased, such as liver disease, diabetes, and certain medications, the influence of endogenous glycerol can be eliminated by a parallel reaction without lipase. Thus, only the concentration of endogenous glycerol is measured, which is then subtracted from the concentration in the primary sample.

The reaction is disturbed by reducing substances such as bilirubin, ascorbic acid and haemoglobin.

Biological samples

Lipid profile is determined in a serum or plasma sample with EDTA anticoagulant. High concentrations of triglycerides interfere with the analysis, so samples must be collected on an empty stomach, at least 12 hours after the last meal. Samples are stable for 1 day at 4°C. The presence of higher concentrations of chylomicrons in serum is observed as a creamy layer on the surface of the sample, while high concentrations of triglycerides are observed as a turbid serum sample.

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LAB REPORT: DETERMINATION OF TOTAL, HDL AND LDL CHOLESTEROL AND TRIGLYCERIDES IN SERUM

Protocol for determination of total cholesterol in serum:

1. Pipet in eprouvettes as described below [written in mL]:

	Blank	**Standard**	**Sample**	**Control**
dH_2O	0,01	-	-	-
Standard Sample	-	0,01	-	-
Serum Sample	-	-	0,01	-
Control Sample	-	-	-	0,01
Reagent 1	1,00	1,00	1,00	1,00

2. Mix and incubate 10 minutes on 37°C. After that measure absorbance at 510 nm.

Reagents:

Reagent 1: Pipes buffer (pH 6,7) 100 mmol/L, hydroxybenzoate 10 mmol/L, 4-aminophenazone 0,5 mmol/L, COE 300 U/L, COD 100 U/L, POD 200 U/L, Sodium-aside 15 mmol/L

Protocol for determination of HDL-Chol in serum:

PRECIPITATION reaction:

1. Pipet in centrifuge tubes as described below [written in mL]:

	Sample	**Control**
Serum Sample	0,25	-
Control Sample	-	0,25
Precipitation Reagent	0,25	0,25

2. Mix and incubate 10 minutes on room temperature, during incubation mix few times. After that centrifuge 10 minutes at 3000 – 3500 rpm. Supernatant must be clear!

CHOD-PAP method:

1. Pipet in 10 mL eprouvettes as described below [written in mL]:

	Blank	**Standard**	**Sample**	**Control**
dH_2O	0,02	-	-	-
Standard Sample	-	0,02	-	-
Serum Sample Supernatant	-	-	0,02	-
Control Sample Supernatant	-	-	-	0,02
Reagent 1	1,00	1,00	1,00	1,00

2. Mix and incubate 10 minutes on 37°C. After that measure absorbance at 510 nm in 10 – 30 minutes timeframe.

Reagents:

Reagent 1: Pipes buffer (pH 6,7) 100 mmol/L, hydroxybenzoate 10 mmol/L, 4-aminophenazone 0,5 mmol/L, COE 300 U/L, COD 100 U/L, POD 200 U/L, Sodium-aside 15 mmol/L

Precipitation reagent: PEG 6000 20 g/dL

Protocol for determination of triglycerides in serum:

1. Pipet in eprouvettes as described below [written in mL]:

	Blank	**Standard**	**Sample**	**Control**
dH_2O	0,01	-	-	-
Standard Sample	-	0,01	-	-
Serum Sample	-	-	0,01	-
Control Sample	-	-	-	0,01
Reagent 1	1,00	1,00	1,00	1,00

2. Mix and incubate 10 minutes on 37°C. After that measure absorbance at 550 nm.

Reagents:

Reagent 1: Pipes buffer (pH 6,7) 20 mmol/L, ESPT 2mmol/L, ATP 1 mmol/L, Mg^{2+} 0,6 mmol/L, 4-aminophenazone 0,8 mmol/L, LPL 350 U/L, GK 40 U/L, GPO 4000 U/L, POD 800 U/L, Na-aside 15 mmol/L

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of methods (scheme of reactions):

Total cholesterol:

HDL-Chol:

Triglycerides:

Measurements:

Total cholesterol

Control: Standard:

Serum:

HDL-Chol

Control: Standard:

Serum:

Triglycerides

Control: Standard:

Serum:

a) Known concentration values or concentration ranges:

Control [range]: Standard:

b) Reference values used in clinical practice for lipide concentration measurement (UKC LJ, KIKKB):

	Total cholesterol [mmol/L]	LDL-Chol [mmol/L]	HDL-Chol [mmol/L]	Triglycerides [mmol/L]
Desired values	<5.2	<3.4	>1.3 F >1.0 M	<1.7
Moderate risk values	5.2–6.5	3.4–4.0	/	1.7–5.0
High risk values	>6.5	>4.0	/	>5.0

Calculations:

Total Cholesterol Concentration

HDL-Chol Concentration

LDL-Chol Concentration

Calculation Formula [written in mmol/L]:

Triglycerides Concentration

Results:

Total Cholesterol Concentration

Control: Serum:

HDL-Chol Concentration

Control: Serum:

Triglycerides Concentration

Control: Serum:

LDL-Chol Concentration

Serum:

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

DETERMINATION OF SERUM GLUCOSE USING THE GLUCOSE OXIDASE METHOD AND GLYCATED HEMOGLOBIN BY ION-EXCHANGE CHROMATOGRAPHY

Assist. Dr. Tijana Markovič, MPharm

LEARNING OUTCOMES

By the end of this practical the student will be able to:

- list the key parameters used for the diagnosis and monitoring of diabetes,
- describe the biochemical basis of glucose metabolism and glycated haemoglobin formation,
- distinguish between normal and pathological glucose and glycated haemoglobin levels in blood samples,
- summarize the clinical significance of serum glucose and glycated haemoglobin measurements,
- explain the principles of glucose and glycated haemoglobin detection methods,
- apply the obtained knowledge to perform serum glucose measurement and glycated haemoglobin determination in whole blood samples,
- use the newly obtained knowledge to interpret clinical results and assess the glycaemic status of patients.

DIABETES AND ITS DIAGNOSTIC CRITERIA

Diabetes is a condition of **chronic hyperglycaemia** resulting from **insulin deficiency or resistance** to its action**.** Chronic hyperglycemia results in the **excretion of glucose into the**

urine when the blood glucose concentration exceeds 10 mmol/L. Due to the osmotic effect, larger amounts of water are excreted (urine is diluted and more abundant than normal), resulting in increased **thirst**. Glucose also causes the glycation of immunoglobulins, which increases susceptibility to infections (especially of the urogenital tract). Although blood glucose levels are high in diabetic patients, chronic glucose deficiency is predominant in cells. The cells, therefore, shift the metabolism to the production of energy from fat and protein sources. This results in accelerated **weight loss**, increased **ketone body** formation and **hyperlipidemia**. Increased ketone concentrations can lead to diabetic ketoacidosis coma (in type I diabetes). Diabetic coma rarely occurs as a result of haemoconcentration due to hyperosmolar hyperglycemic nonketotic syndrome (in type II diabetes). Hyperlipidemia increases the possibility of atherosclerosis and, in addition to the glycation of proteins in the basal membrane of the vessels, causes damage to the capillaries in the retina and kidney, which can lead to **blindness** or **renal failure**. The accumulation of sorbitol (a product of the reduction of glucose) in tissues can lead to the opacity of the lens or neuropathy.

CLASSIFICATION OF DIABETES:

Primary forms of diabetes

- **TYPE I DIABETES**

It usually occurs in younger patients, representing 5- 10% of patients with diabetes. The amount of β cells and insulin is reduced, so patients are dependent on insulin. The cause of the disease is the destruction of pancreatic β cells, which is an autoimmune process. Both environmental influences (such as viral infectons or early exposure to certain foods), as well as genetic predisposition (HLA genotypes) contribute to the start of the autoimmune process. Type I diabetes can also be idiopathic.

- **TYPE II DIABETES**

In most cases, it occurs in **elderly overweight** patients but has recently become more common in younger subjects (even in children). Symptoms appear gradually and the disease is usually discovered accidentally in a medical examination. The amount of β cells is normal, and the amount of insulin can be normal, increased or decreased. The cause of the disease is usually the unresponsiveness of insulin receptors (decreased receptor number or receptor activity). The impact of heredity is very strong (more than 90% match in identical twins), and

the increased amount of abdominal fat secreting adipokines that reduce the responsiveness of insulin receptors also contribute to increased risk. Treatment of the disease is with a diet, oral antidiabetics and insulin.

- **GESTATIONAL DIABETES (diabetes in pregnancy)**

It is a consequence of reduced glucose tolerance during pregnancy. The condition usually normalizes postpartum, but some women who have had gestational diabetes develop type II diabetes later in life.

Secondary Diabetes

It occurs as a **result of another primary disease**, i.e. disease which causes: a decrease in insulin concentration (pancreatic diseases), an increase in the concentration of insulin antagonists (acromegaly, Cushing's syndrome, tumours), genetic changes in insulin or genes related to insulin production and its secretion from pancreatic β cells. **Iatrogenic diabetes** due to the use of certain medicines (glucocorticoids, thyroid hormones, β-adrenergic agonists, thiazides) is also a form of secondary diabetes.

There are also two states of **prediabetes**: the state of **(1) impaired fasting glycemia**, in which patients have fasting glucose concentration in the "grey zone" (6.1 - 6.9 mmol/L) and oral glucose tolerance test concentrations below 7.8 mmol/L, and a state of **(2) impaired glucose tolerance**, in which patients have a fasting glucose concentration below 6.1 mmol/L and **oral glucose tolerance test (OGTT)** concentrations between 7.8 and 11.1 mmol/L. For some patients, this condition is transient, while others later develop diabetes.

DIAGNOSTIC CRITERIA FOR DIABETES

Diabetes may be diagnosed based on clinical signs of diabetes and on plasma glucose criteria, either the random plasma glucose, fasting plasma glucose value or the 2-h plasma glucose value during an oral glucose tolerance test (OGTT), or haemoglobin HbA1c criteria.

1. **Clinical signs of diabetes** and **random plasma glucose ≥ 11.1 mmol/l** (random means at any time during the day, regardless of the time of the last meal). Typical signs of diabetes are polyuria, polydipsia and unexplained weight loss, but acute or chronic complications of diabetes may also be present.

or

2. **Fasting plasma glucose ≥ 7.0 mmol/l** (the sample is taken in the morning on an empty stomach without caloric intake for at least 8 hours)

or

3. **Plasma glucose 2 hours after** the start of **OGTT ≥ 11.1 mmol/l.**

or

4. **HbA1c ≥ 6.5%** (according to American Diabetes Association guidelines)

The presence of diabetes should **always be confirmed by another glucose measurement**, which should not be performed on the same day. The repeated measurement of glucose is not required if the patient has significant acute clinical signs of diabetes.

Criteria for interpreting 2-hour OGTT

OGTT should only be performed if fasting glucose levels are between 6.1 - 6.9 mmol/L. The test is performed in the morning between 7 am and 9 am. The subject is instructed to fast for 8-12 hours, then he/she drinks a glucose solution containing 75 g of glucose dissolved in 250 to 300 ml of water (within a 5-minute frame). The blood glucose level is determined after 1 hour and 2 hours after the oral intake of glucose solution:

Table 1. Plasma glucose levels at OGTT.

	Plasma glucose levels at OGTT (mmol/l)	
	0 h	**2h**
Impaired fasting glycemia	6.1 – 7.0	< 7.8
Impaired glucose tolerance	< 6.1	7.8 – 11.1
Diabetes	> 7.0	> 11.1

Criteria for the diagnosis of gestational diabetes

At the early stage of **pregnancy** (within the first examination), one of the prescribed tests for pregnant women is a measurement of plasma glucose concentration. If serum glucose levels are ≥ 7.0 mmol/l (fasting glucose) or ≥ 11.1 mmol/l (random glucose), then the pregnant woman has diabetes. During the 24th to 28th week of pregnancy, pregnant women without

previous diagnosis of diabetes or gestational diabetes during this pregnancy undertake a 2-hour 75 g OGTT. At this stage of pregnancy gestational diabetes is present if at least one diagnostic value is reached or exceeded:

Table 2. Glucose concentration in serum.

Time	Glucose concentration in serum (mmol/l)
0 h (fasting)	5.1
1h	10.0
2h	8.5

METHODS IN LABORATORY DIAGNOSTICS OF DIABETES

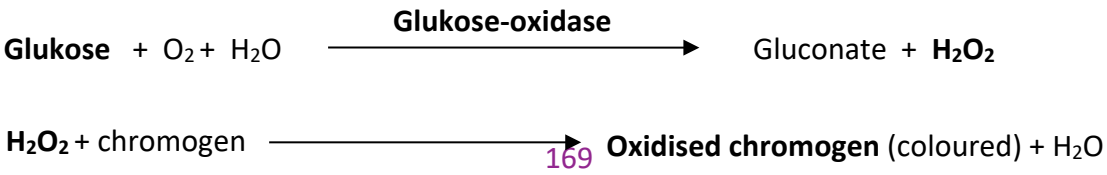
DETERMINATION OF GLUCOSE CONCENTRATION

Methods for glucose determination are divided into **oxidation-reduction** methods and colorimetric methods, which are mostly obsolete, and **enzyme** methods, which are most commonly used today and are the basis for most automated methods. Two important enzymatic methods include (1) the **reference hexokinase method** and (2) the **glucose oxidase method (GOD)**, which is commonly used. The latter will be practically performed in the laboratory practical.

Reference values:	3.6 - 6.1 mmol/L.

Glucose-oxidase method

The first reaction is very specific, which is guaranteed by the enzyme glucose oxidase. The oxidation of glucose produces **hydrogen peroxide**, which is determined in the second step. **Chromogen**, which in our case will be 4-aminoantipyrine (PAP), and phenol are present in the reagent solution. In the presence of peroxidase, the chromogen is oxidized to a coloured compound and we can measure its **absorbance** (in our case at 505 nm).



The **glucose oxidase method** is used on **reagent strips** to determine glucose in urine, in many **automated procedures** and in **glucometers** for glucose self-monitoring (Figure 1).

Figure 1: *Glucometer for blood glucose self-monitoring.*

Interferences:

Chromogenic substances competing for peroxide may produce a false negative reaction (bilirubin, vitamin C, uric acid, glutathione, haemoglobin). A false positive reaction is given by oxidants. The sample in which glucose concentration is determined should not be lipemic (the turbidity of the sample interferes with the determination of the analytes by absorption spectroscopy), icteric or hemolyzed (bilirubin and haemoglobin absorb in a broad spectrum of visible light).

It is important to separate erythrocytes from plasma/serum within one hour of blood collection as glucose concentration decreases 5-7% per hour due to glycolysis. Alternatively, blood can be drawn into tubes containing glycolysis inhibitors (NaF, which inhibits enolase in the late stages of glycolysis; iodoacetate, which inhibits glyceraldehyde-3-phosphate dehydrogenase), but unfortunately this doesn't completely prevent glycolysis. Also, the effect

of glycolysis inhibitors is not immediate, so it is preferable to refrigerate the samples or analyze them immediately.

Some medicines affect serum/plasma glucose levels. Certain medicines increase the glucose concentration (β-adrenergic agonists, caffeine, calcitonin, diuretics, corticosteroids, dopamine, adrenaline, estrogens, lithium, morphine, oral contraceptives, phenytoin, etc.), while others reduce glucose concentration (β-adrenergic antagonists, anabolic steroids, antihistamines, acetylsalicylic acid, ethanol, captopril, indomethacin, MAO inhibitors, nifedipine, etc.).

These preanalytical errors and interferences should be considered when interpreting laboratory results. In addition, *in vitro* interferences are also known because some drugs (mainly non-steroidal anti-inflammatory drugs) are oxidized by H_2O_2.

Biological samples

The sample of choice for glucose determination is **serum or plasma**. Serum and plasma glucose concentrations are not the same, because the addition of an anticoagulant increases the osmolality of the extracellular fluid in the sample taken. Fluid transfer from erythrocytes to extracellular fluid occurs and results in a decrease in plasma glucose levels.

Whole blood glucose concentration is 12-15% lower than serum levels due to the influence of haematocrit. Liquor concentrations represent 40-80% of serum levels, and glucose is normally not detected in urine.

DETERMINATION OF GLYCATED HAEMOGLOBIN

Formation of glycated haemoglobin

Glycated haemoglobin is formed by the non-enzymatically **covalent binding of sugars to the NH_2 group** of the N-terminal valine of the β-chain of HbA (Figure 2). The reaction is dependent on the blood glucose concentration and takes place continuously in the erythrocytes. It reflects the glucose concentration that an erythrocyte has been exposed to during its 120-day life cycle. The first stage of the reaction (the formation of the so-called labile fraction) is rapid and reversible, the Amadori rearrangement, which produces stable HbA1c, proceeds slowly and is irreversible. Unlike glycated haemoglobin, the labile fraction, therefore, does not

reflect long-term glucose concentrations but depends on the current blood glucose concentration.

The percentage of glycated haemoglobin gives an estimate of **blood glucose concentration 2 to 3 months before the test**. This test is particularly suitable for monitoring the success of therapy and assessing the risk of chronic complications of diabetes, since unlike glucose, the proportion of glycated haemoglobin is not dependent on the current state but is a reflection of a long-term process.

Types of haemoglobin

- HbA (non-glycated Hb): 90%
- HbA1 (glycated Hb): 5-7%
- **HbA1a1 (glycation with fructose-1,6-bisphosphate)**
- HbA1a2 (glycation with glucose-6-phosphate)
- HbA1b (glycation with pyruvate)
- **HbA1c (glycation with D-glucose on the N-terminal valine of the β-chain): represents 75 to 80% of glycated Hb**
- HbA2: 2.5%
- HbF: 0.5% (fetal haemoglobin)

There are numerous methods for determining the proportion of glycated haemoglobin based on the separation of Hb molecules according to differences in charge, chemical reactivity or structure. In this exercise we will use **ion-exchange chromatography** based on charge-based separation of Hb molecules.

The reference values differ slightly depending on the method of determination used and are generally expected to be between 4.2 and 6.2%. Values of 7 to 30% are found in untreated diabetic patients and values below 7% are considered as good control of diabetes.

The principle of determination of glycated Hb by ion-exchange chromatography

Glycation causes a decrease in the positive charge of Hb. On **weakly acidic cation exchangers (resins)**, at a **given ionic strength and pH of buffer**, glycated Hb is **less positively charged than free Hb**. **Glycated Hb elutes earlier** than free Hb. HbA1a and HbA1b elute first (these two fractions are discarded), then HbA1c elutes, and HbA stays bound on the column. Before

application to the column, the **erythrocytes must be hemolyzed** to release Hb. Also the labile fraction prior to application must be removed, because it may interfere with the analysis.

Biological sample

The sample of choice for determining glycated haemoglobin is whole blood, and capillary blood can also be used. There is no need for fasting for this test.

DO YOU WANT TO LEARN MORE?

Watch the following video lectures.

- **Diabetes mellitus**
  - https://www.youtube.com/watch?v=2I_F7D__Ixo
  - https://www.youtube.com/watch?v=-B-RVybvffU
- **Types of Diabetes**
  - https://www.youtube.com/watch?v=LgXM_N7e6MA
- **Hypo- and Hyperglycemia: Glucose Metabolism & Diabetic States**
  - https://www.youtube.com/watch?v=UUB4jIg2Hgc
- **Diabetic Case**
  - https://www.youtube.com/watch?v=ewk0u7xAiY8

TEST YOUR KNOWLEDGE

1. What are blood and urine glucose levels in healthy individuals?
2. What laboratory tests would you suggest for diagnosis of diabetes and which ones for monitoring?
3. Write a formula to calculate the percentage of glycated haemoglobin according to the procedure you will perform in the exercise.
4. While watching the video lectures, write down 5 important findings for each clip.

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LAB REPORT: DETERMINATION OF SERUM GLUCOSE USING THE GLUCOSE OXIDASE METHOD AND GLYCATED HEMOGLOBIN BY ION-EXCHANGE CHROMATOGRAPHY

Protocol for plasma glucose concentration:

1. Pipette into a 1.5 mL test tube

	Blank	**Standard**	**Sample**	**Control**
Demineralized water	10	-	-	-
Standard glucose solution	-	10	-	-
Sample (serum)	-	-	10	-
Control sample	-	-	-	10
Working reagent	1000	1000	1000	1000

2. Mix well and incubate for 10 minutes at a temperature of 37°C.
3. Measure the absorbance at 505 nm. The color is stable for 30 minutes.

Reagents:
The working reagent is a solution of 4-aminoantipyrine, GOD, POD and phenol in phosphate buffer with pH 7.4.

Protocol for glycated haemoglobin:

a) Preparation of hemolysate and removal of the labile fraction

1. Pipette 250 µL of lysing reagent into three 10 mL test tubes.
2. Add 50 µL of whole venous blood, standard or controls.
3. Mix well and leave for 5 minutes at room temperature

b) Isolation of glycated hemoglobin

1. Mix the solution with the ion-exchange resin (Glycohemoglobin Lysing Rasin).
2. Pipette 1.5 mL of the solution with the ion-exchange resin into a 13x100 mm glass test tube labeled sample.
3. Repeat the first and second steps with standard and control.

Attention! *Before each pipetting of the solution with the ion-exchange resin, mix the solution well.*

4. Add 50 µL of the hemolysate from point A3 to the resin solution in glass tubes.
5. Carefully insert the separation filters with a tube into the glass test tubes, leaving 1 cm of space between the surface of the solution and the rubber.
6. Mix the reagent mixture on a shaker for 5 min.
7. After mixing, lower the separation filter with the tube to the bottom of the glass test tube.
8. Pour the supernatant inside the tube into the cuvette and measure the absorbance at 415 nm. Deionized water is used as a blank.

c) Measurement of total hemoglobin

1. Pipette 5 mL of deionized water into three glass test tubes.
2. Pipette 20 µL of hemolysate from point A3 (sample, standard, control) into each test tube. 3. Mix well.
4. Measure the absorbance of the sample, standard and control at 415 nm. Deionized water is used as a blank.

Reagents:

Lysis Reagent: 10mM potassium cyanide, detergent; Ion-exchange resin solution: 8mg/ml cation-exchange resin in borate buffer, pH 6.9

Materials:

Separating filter with tube, 10 mL glass test tube, Glass test tubes of dimensions 13x100 mm

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the methods (scheme of reactions):

Measurements:

Glucose concentration

Control: _____ Sample: _____

Standard: _____ Blank: _____

Glycated haemoglobin measurements

HbA1c

Control: _____ Sample: _____

Standard: _____

Total Hb

Control: _____ Sample: _____

Standard: _____

Calculations:

Glucose concentration

Glycated haemoglobin measurements

$$\%HbA1C_{sample} = \frac{HbA1C_{sample}}{Hb(total)_{sample}} \cdot 100\%$$

$$HbA1C_{sample} = \frac{A_{HbA1C_{sample}}}{A_{HbA1C_{st}}} \cdot \%_{HbA1C\ st}$$

$$Hb(total)_{sample} = \frac{A_{Hb(total)_{sample}}}{A_{Hb(total)_{st}}} \cdot \%_{Hb(total)\ st}$$

Results:

Glucose concentration:

Glycated haemoglobin measurements:

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

ANALYSIS OF SERUM PROTEINS BY BIURET REACTION AND MEMBRANE ELECTROPHORESIS

Assist. Dr. Dunja Urbančič, MPharm

LEARNING OUTCOMES

After successfully completing this laboratory practical, student will be able to:

- list basic biochemical properties of proteins and selected methods for their detection,
- state the main sites of synthesis of serum proteins,
- distinguish between the concentration of proteins in different biological samples,
- summarize the causes for hyper- and hypoproteinaemia,
- list the fractions of serum proteins and their approximate percentage,
- describe the biuret method and the role of the components in the reagent,
- describe the electropherogram obtained by electrophoresis on solid carrier in terms of location of fractions relatively to the electrodes and their migration properties,
- apply the obtained knowledge to perform the analysis of serum proteins in the laboratory setting on actual clinical samples,
- use the newly obtained knowledge to interpret clinical results of the patients and provide the most probable diagnosis.

SERUM PROTEINS AND THEIR CLINICAL SIGNIFICANCE

Proteins perform variety of functions in the body. In plasma, they maintain oncotic pressure and acid-base balance, they transport ions and insoluble substances, as enzymes and hormones they regulate metabolism and signalling pathways, they participate in the immune

response as antibodies and components of complement system, they are involved in coagulation processes.

BIOCHEMICAL AND PHYSICAL PROPERTIES OF PROTEINS

Proteins are polymers built of amino acids, that are interconnected by peptide bonds. They are characterized by primary, secondary, tertiary and sometimes quarterly structure. Their structure, function and other properties strongly depend on side chains of amino acids, glycosylation and / or co-factors such as metal ions. When analysing proteins, we exploit the following biochemical and physical properties:

- Size
- Solubility
- Electrostatic charge (isoelectric point)
- Absorption of UV light
- Adsorption to certain material
- Biding with specific antibodies

By considering these properties of proteins in biological samples, we can use several different methods for their detection:

- spectrophotometric methods: biuret method, bicinchonic, Bradford assays for total protein determination) …,
- electrophoresis: PAGE electrophoresis, electrophoresis on solid membrane, capillary electrophoresis …,
- immunochemical methods: turbidimetry (see CRP), flow cytometry (see Flow cytometry), western-blot for analysis of specific protein …
- catalytic activity measurements (see ALT, AST and ALP).

CONCENTRATION OF PROTEINS IN DIFFERENT BODY FLUIDS

So far, more than 300 different proteins have been discovered in the serum. Main sites of their synthesis are liver and B lymphocytes. They all degrade in liver. Altered concentrations of serum proteins thus usually, but not exclusively, indicate the pathological conditions in

these two organs. The most common biological samples in which the concentration of proteins is determined are:

a) **Serum**: the reference value for total serum proteins is 65-80 g/L.

b) **Plasma** also contains coagulation factors, mostly fibrinogen, so the reference values are approximately 3 g/L greater than for serum. In blood clotting, besides fibrinogen, other factors of blood clotting are used, and platelets that are caught in the clot release many different proteins... Therefore, serum and plasma are not distinguished only by the presence of fibrinogen, but also by the presence or the absence of many other proteins.

c) **Urine** contains proteins only in traces. Healthy people usually excrete less than 0.15 g of proteins per day. Higher amounts of proteins can occur with severe physical strain or in kidney damage.

d) **Cerebrospinal fluid** (CSF): 80% of the proteins in the CSF originate from plasma via ultrafiltration and pinocytosis and therefore contain primarily low molecular weight proteins (albumin, transferin, prealbumin). 20% of the proteins are of intrathecal origin (Ig). The reference values are 0.15 - 0.45 g / L. In the event of damage to the hematoencephalic barrier (meningitis, subdural haemorrhage), the concentration of α2-macroglobulin, which is present in CSF at very low concentrations, is increased. Intrathecal synthesis of immunoglobulins (especially IgG) increases with multiple sclerosis.

e) **Transudates** are pathological ultrafiltrates of plasma and contain only low molecular weight proteins (<30 g/L). They occur by filtration through undamaged capillary walls due to increased hydrostatic pressure in the veins (congestive heart failure), reduced oncotic pressure (nephrotic syndrome) or a combination of both (ascites in cirrhosis of the liver).

f) The **exudate** is a serosae fluid resulting from increased capillary permeability due to a local inflammatory process. It also contains high molecular weight proteins (> 30 g/L).

CONDITIONS WITH ALTERED TOTAL SERUM PROTEIN CONCENTRATIONS

Regarding the changes of protein concentrations in blood, we distinguish two states: hyper- and hypoproteinaemia. Here, we describe their common causes.

Hyperproteinaemia (total serum protein > 80 g/L)

a. **Apparent hyperproteinaemia:** only the amount of water is reduced, and the amount of proteins remains the same (haemoconcentration). The cause of this is dehydration due to drinking of insufficient amounts of water, diarrhoea, vomiting, Addison's disease or lack of ADH (diabetes insipidus).

b. **True hyperproteinaemia** is always the result of an increase in the synthesis of antibodies, which may be increased mono- or polyclonally. Since antibodies migrate in gamma fraction (see electrophoretic fractions), we call such increases gammopathies. The cause of **polyclonal gammopathy** may be chronic inflammation (rheumatoid arthritis), chronic infection (syphilis, malaria, leprosy, TBC), liver cirrhosis, various autoimmune diseases, and metastases. The most common cause of **monoclonal gammopathy** is multiple myeloma (malignant plasmocyte transformation), but also occurs in Waldenström's macroglobulinaemia, chronic lymphatic leukemia, and benign monoclonal immunoglobulinemia, which occurs in 1% of elderly people and can develop into multiple myeloma. In the above conditions, Bence-Jones proteins can occur in the urine (but not necessarily); in cases where there are IgM paraproteins, cryoglobulins are present in the blood.

Hypoproteinaemia (total serum proteins <65 g/L)

There are several causes for hypoproteinaemia:

a) Hyperhydration (haemodilution): only the volume of water increases, the amount of proteins remains unchanged. Possible causes: i.v. infusion, water poisoning, and pregnancy.
a) Reduced food protein intake as a result of starvation (anorexia) or inadequate nutrition / protein starvation.
b) Reduced absorption of proteins due to chronic gastrointestinal disorders (diarrhoea) or celiac disease.
c) Reduced protein synthesis:
    - Hepatic disease (hepatitis, poisoning): all protein fractions, except gamma fraction.
    - Reduced synthesis of antibodies (hypogammaglobulinemia): congenital hypogammaglobulinemia, immunosuppressive drugs.

d) Increased metabolism of proteins in hyperthyroidism or diabetes mellitus.
e) Increased loss of proteins through kidneys (nephritis), GIT (enteritis) or skin (severe burns).

FRACTIONS OF SERUM PROTEINS

Oftentimes, in the case of pathological conditions, the total protein concentration in a patient's serum may be within reference values, but the ratio of the individual protein fractions may deviate from normal. The main cause of this is compensation for oncotic pressure. Any deviation from normal oncotic pressure results in altered expression of proteins synthesized in the liver, especially albumin. Therefore, in addition to the total protein concentration in serum, the proportion of individual protein fractions should be determined by electrophoresis. Serum protein electrophoresis is a protein separating method based on their physical and biochemical properties (see details below). Under the introduction of the voltage, serum proteins are separated into six fractions: albumins and four (to five) fractions of globulins: α1-, α2-, β- (sometimes separated as β1 and β2) and γ-globulins. Serum protein electrophoresis has clinical significance for identifying multiple myeloma and other serum protein disorders, and it also helps to identify morphological changes in response to acute and chronic inflammation, various malignancies as well as liver and kidney failure. Below, we describe the role of selected proteins in each fraction.

a) **Albumin** (36.3-49.1 g/L; 55.7%) is the most represented serum protein. It is important in maintaining oncotic pressure, acid-base balance, viscosity of blood and in transport. Its transcription is thightly regulated by changes in oncotic pressure. Hyperalbuminemia is rare (often due to dehydration). Hypoalbuminemia may occur in:

- inflammation (increased permeability of membranes, inflammatory cytokines reduce its synthesis, compensatory reduction due to increased concentration of positive acute-phase inflammatory reaction);
- liver disease (decreased hepatic synthesis);
- kidney and gut diseases;
- severe burns;
- protein malnutrition.

High concentrations of bilirubin can cause a widening of the albumin peak due to its binding to albumin.

b) **α1-globulins** (1.1-3.5 g / L; 3.1%) are the least represented group of serum proteins. The concentration of the α1 fraction increases with inflammation.

- α1-antitrypsin represents 90% of the α1 fraction. It is a protease inhibitor. Congenital lack of α1-antitrypsin causes an increased activity of lung elastases, leading to emphysema. In some cases, liver function changes in emphysema, which can lead to cirrhosis. It's a positive acute phase reactant...
- α1-acid glycoprotein binds progesterone and other steroid hormones. It is a positive acute phase reactant. Its concentration is increased by glucocorticoid hormones, and lowered by estrogens.
- α1-fetoprotein is the main fetal protein. It does not normally occur in adults and is present in pregnant women. In diagnostics it is used as a tumor marker and for prenatal detection of Down's syndrome and nerve damage (spina bifida).

c) **α2-globulins** (6.5-11.7 g / L; 11.3%); their concentration increases with inflammation.

- Haptoglobin binds free hemoglobin. In intravascular hemolysis, its concentration is reduced because it is quickly consumed. Estrogen, liver disease (via estrogen) and ineffective erythropoiesis also decrease its concentration. It's a positive acute phase reactant.
- α2-macroglobulin is a protease inhibitor. It has a very high molecular weight, so it cannot transit into urine. Its concentration is increased in nephrotic syndrome due to compensation in order to maintain oncotic pressure. Its concentrations are also increased under the influence of estrogen and age (children have higher concentrations of α2-macroglobulins), but are reduced in pancreatitis and prostate cancer (PSA binding).
- Ceruloplasmin is a transport protein for copper, but also acts as a ferroxidase. Its concentration is reduced by Wilson's disease (a congenital disorder that accumulates copper in tissues and causes liver, neuromuscular and eye disorders). It's a positive acute phase reactant.

d) **β-globulins** (7.4-12.6 g / L; 11.8%); their concentration increases with inflammation. On some electrophoretic carriers, they can be seen as 2 fractions (β1 and β2).

- Transferrin (β1) is a transport protein for Fe3+ ions. It is used for differential diagnosis of anemia and monitoring of therapy. In hypochromic anemia, its concentration is increased, but its saturation with iron is low. In chronic diseases, its concentration is normal or decreased, and its saturation with iron is high. At high concentrations of iron, the concentration of transferin is normal, and saturation with iron is very high. It's a negative acute phase reactant
- Hemopexin (β1) binds the free heme. It is useful in the diagnosis of haemolytic conditions with concomitant inflammation, as unlike haptoglobin, it is not an acute phase reactant and does not increase in inflammatory conditions and its concentrations decrease hemolysis.
- LDL (β1) is one of the lipoproteins that transports faty acid molecules around the body and its elevated levels are associated with hypercholesterolaemia (see chapter Determination of HDL and LCL cholesterol and triglycerides in serum). As LDL migrates in β fraction (or sometimes in region between α and β), elevated levels of β fraction are seen in serum of patients with hypercholesterolaemia.
- Antibodies traveling in the β region are predominantly IgA monoclonal antibodies. IgM travels between β- and γ-regions, IgE, IgD and Bence-John's proteins travel in the β- or even α-region. The most abundant type of antibodies, IgG, appears in γ region (see below).
- Complement components C3 and C4 play a role in blood clotting and are positive acute phase reactants.
- The β2-microglobulin is part of the MHC I on the cell surface. Because of its low molecular weight, 100% is filtered through the glomeruli and then completely reabsorbed from the tubules back into the blood. It is used to evaluate renal tubular function. In the case of tubular defects, its serum concentrations are reduced and its concentrations are increased in the urine.
- C-reactive protein (CRP) can travel in the β or γ fraction, depending on the carrier used. It is an activator of the classic complement cascade and is an early acute phase

reactant (its concentration increases after 4-8 hours). Its concentration is also increased in patients with a higher risk of cardiovascular disease.

e) **γ-globulins** (5.8-17.4 g/L; 18.1%) are the second most represented fraction of serum proteins. They mainly contain immunoglobulins of IgG class but some other serum proteins such as CRP can be stained and appear on the membrane in this fraction. As this region is presented mainly by immunoglobulins, the changes in this fraction indicate the changes in immune response.

REFLECT YOUR KNOWLEDGE:

Classes of immunoglobulins are:

- IgG (75-80% of Ig in serum, monomer) participates in a secondary immune response and passes through the placenta.
- IgA (10-15% of Ig in serum, dimer) is found in mucous membranes, milk, saliva, sweat and tears.
- IgM (5-10% of Ig in serum, pentamer) participates in the primary immune response and is the first fetal Ig.
- IgE (very low concentrations of Ig in serum, monomer) is increased in allergies and parasitic infections.
- IgD (very low concentrations of Ig in serum, monomer) is a B-cell receptor mainly on the cell surface.

ANALYTICAL METHODS FOR DETERMINATION OF SERUM PROTEINS

As described before, determining total serum protein concentration as well as dissecting the protein fractions is important for clinical diagnosis.

DETERMINATION OF TOTAL SERUM PROTEINS

Several different methods can be used to measure protein concentration: Lawry method, biuret reaction, dicetone method, turbidimetric methods after precipitation of proteins in e.g.

sufosalicylic acid etc. In this practical we will use biuret reaction to determine total concentration of serum proteins.

Principle of biuret reatcion: In a strongly alkaline medium, peptide bonds of proteins form complexes with cupric ions, resulting in a blue-violet coloring of the solution. The absorbance of the formed complex is measured at 540 nm.

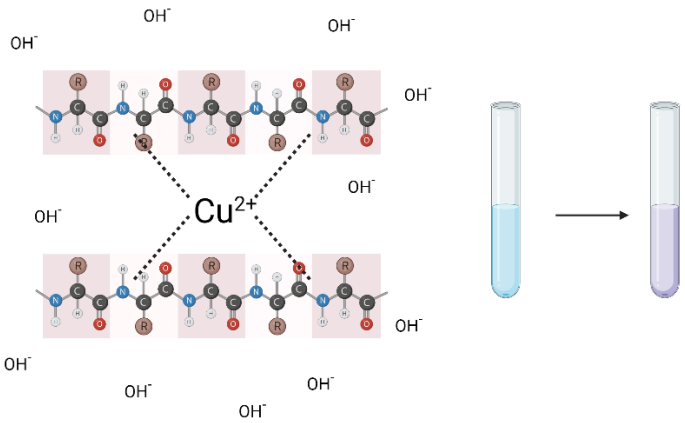


Figure 1. *Schematic presenation of coordination bond formation between Cu(II) ions and four peptide bonds in biuret reaction. Created with biorender.com.*

Interferences:

- High ammonia concentration (genetic predisposition, liver diseases …)
- Sample:
    - Haemolysis
    - Lipemia
    - Icterus (high bilirubin levels)
- Drugs – pre-analytical:
    - False negative: certain sex hormones, corticosteroids, insulin, adrenalin…)
    - False elevated: sulfasalazine, allopurinol, oestrogens

SERUM PROTEIN ELECTROPHORESIS

Electrophoresis is a separation method based on the migration of charged macromolecules (proteins) in an electric field. Nowadays capillary electrophoresis is most commonly used method to determine fractions of serum proteins. However, electrophoresis of serum

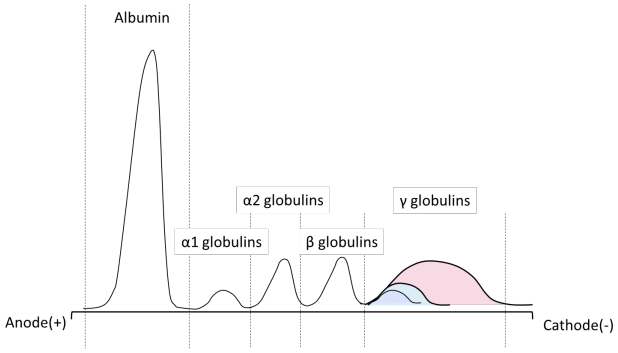
proteins can also be performed on several different carriers. Among them, the most commonly used are

- agarose gel (charge-based separation; 6 fractions visible),
- acetate cellulose (separation based on charge, 5-6 fractions visible),
- paper (charge-based separation, obsolete),
- starch gel (separation based on charge and size, 25 fractions visible).
- polyacrylamide gel (separation based on charge and size, 25 fractions visible).

Serum proteins are negatively charged at pH = 8-10 and therefore travel in an electric field from a negatively charged cathode to a positively charged anode. The rate of protein migration on acetate cellulose depends on the size of its net negative charge, which is determined by the isoelectric point (Ip) of the individual protein. Proteins with lower (more acidic) Ip migrate faster. Albumin is the fastest (Ip = 4.7) and γ globulins are the slowest (Ip = 7.2).

After electrophoretic separation the are proteins visualized by staining. For the detection of proteins on the carrier we can use the following staining dyes:

- Ponceau S in trichloroacetic acid is an anionic dye, which binds to the -NH3 + group of proteins. The trichloroacetic acid creates the acidic environment that is essential for the formation of the -NH3 + functional groups, while at the same time denaturing the proteins and fixing them on the carrier. The color is about 10 times less sensitive than the Coomassie Brilliant Blue R250.
- Coomassie Blue R250 is a very sensitive dye with which we can detect less than 0.2 µg of proteins.



Fraction **g/L** **%**

Albumins	41,0–48,6	55,8–66,1
α1 globulins	2,1–3,6	2,9–4,9
α2 globulins	5,2–8,7	7,1–11,8
β globulins	6,2–9,6	8,4–13,1
γ globulins	8,2–13, 8	11,1–18,8

Figure 2: *Densitogram and reference values of individual fractions of serum proteins.*

After staining, the proportions of individual protein fractions are measured by **densitometer** and their concentrations are calculated. By this we can plot densitogram or so called proteinogram. It consists of five to six peaks representing individual protein fractions. The first peak is the largest and represents albumins, and is followed by α1-, α2-, β- (sometimes separated as β1 and β2) and γ-globulins. Fractions obtained after electrophoresis and reference values of individual fractions and their proportion to total serum proteins are presented in Figure 2.

Biological samples for protein electrophoresis

The sample of choice is the serum. Plasma is less suitable due to strong fibrinogen bands in the β region. Urine and CSF must be concentrated (ultrafiltration, dialysis, etc.) before analysis due to low protein content.

CLINICAL SIGNIFICANCE OF SERUM PROTEIN ELECTROPHORESIS

Serum protein electrophoresis is primarily of clinical importance in the detection of multiple myeloma and other serum protein disorders (e.g. α1-antitripsin deficiency), otherwise electrophoresis can identify morphological patterns with respect to acute or chronic inflammation, liver and kidney disease. Below we highlight the most characteristic electropherograms for selected pathological states.

ACUTE INFLAMMATION

Acute inflammation can be a result of acute infection, tissue or organ injury, severe necrosis, burns, heart attack, tumour disintegration, or after surgery. Electrophoretic fractions of serum proteins containing acute phase reactants are thus increased. We observe elevated α1 (α1-antitrypsin, α1- acid glycoprotein), α2 (haptoglobin, ceruloplasmin), and β (complement components C3 and C4, fibrinogen, C-reactive protein) fractions (Figure 3). There may be a compensatory reduction in albumin and transferin. γ fraction is within normal range, however, if the inflammation persists for days, it becomes elevated. Upon C-reactive protein rise, we can observe slightly elevated region between β and γ fractions.

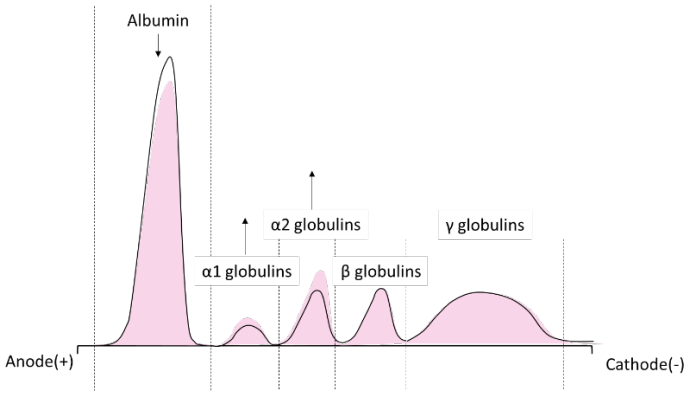


Figure 3: *Electropherogram representing changes in fractions in acute inflammation.*

CHRONIC INFLAMMATION

Chronic inflammation refers to late or long-lasting inflammatory conditions. The pathological states characterized by chronic inflammation are: chronic liver diseases (autoimmune or viral hepatitis, alcoholism), autoimmune disease (rheumatoid arthritis, SLE, IBD), chronic viral (varicella zoster, infectious mononucleosis, chickenpox …) or bacterial (osteomyelitis, endocarditis, tuberculosis, bacteraemia) infections, malignancies, allergies. In chronic inflammation specific immune response overcome unspecific one and is therefore characterized with polyclonal production of antibodies. As a result, γ-fraction is greatly increased and is polyclonal (Figure 4). This state is thus referred as **polyclonal hypergammaglobulinemia**. The fractions containing acute phase reactants (α1, α2) can also be increased. The albumin concentration is normal or reduced due to preserving total plasma protein concentration in range.

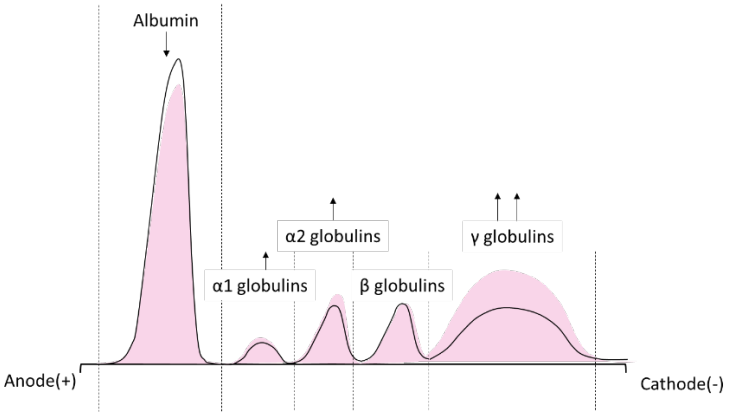


Figure 4: *Electropherogram representing changess in fractions in chronic inflammation.*

LIVER CIRRHOSIS

Cirrhosis is an end-stage pathological liver condition in which the liver function is impaired due to formation of scars throughout the liver tissue. Due to impaired synthetic function of the liver, the proteins that are synthesized in liver are reduced on proteinogram. Therefore, fractions of albumin, α1-, α2-, and β-globulins are decreased (Figure 5). As a compensatory mechanism in preservation of oncotic pressure, antibodies are excessively synthesized in B lymphocytes. The synthesis of these antibodies is unspecific and results in increase of all immunoglobulin classes (IgG, IgM and IgA). The production of antibodies is polyclonal. In liver cirrhosis, we thus observe polyclonal hypergammaglobulinemia. On account of more extensive formation of IgA antibodies, that migrate faster compared to IgM and IgG immunoglobulins, a typical β-γ bridge can be observed on proteinogram. This is a distinct characteristic, which distinguishes liver cirrhosis from other chronic inflammatory conditions.

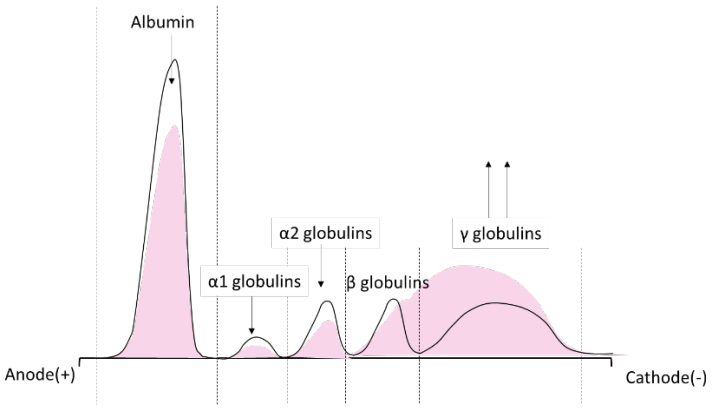


Figure 5: *Electropherogram representing changes in fractions in liver cirrhosis.*

NEPHROTIC SYNDROME

Nephrotic syndrome is a kidney disorder that causes the body to pass too much protein in urine. Causes include a number of kidney diseases that result in the damage of the glomeruli. The loss of proteins in plasma depend on the degree of damage. In lighter damages of glomeruli, only smaller amount of proteins is lost and accounts mainly on albumin. However, with the progression of the syndrome, also IgG antibodies and certain proteins in β fraction (β1 lipoprotein) are excreted via urine, resulting in additional decrease of β and γ fractions in the proteinogram. In severe cases of nephrotic syndrome, albumin, α1-, β-, and γ-globulins are decreased (Figure 6). The body tries to compensate the extensive protein loss, and therefore activates synthesis of protein in the liver. All small proteins excrete via damaged glomeruli, except α2 macroglobulin as its high molecular mass disables him to pass the glomerular basal membrane. On the proteinogram of nephrotic syndrome, we thus observe an elevation of α2 fraction.

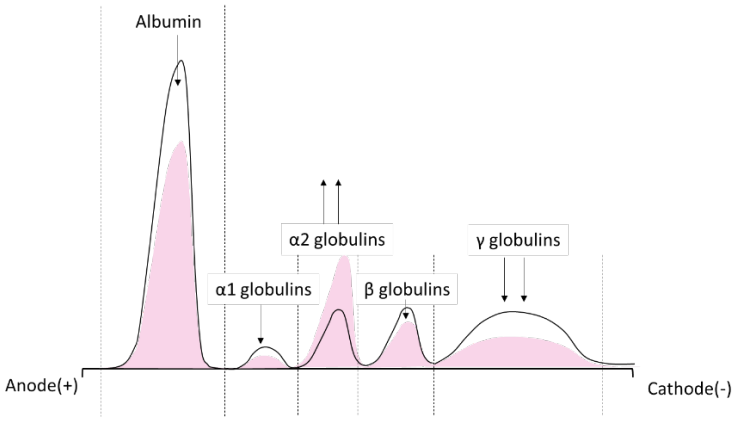


Figure 6: *Electropherogram representing changes in fractions in nephrotic syndrom.*

HYPOGAMMAGLOBULINEMIA

Hypogammaglobulinemia is a state with lower production of immunoglobulins. There are two types of hypogammaglobulinemia: congenital and acquired. Congenital hypogammaglobulinemia can be caused by genetic diseases (CVID, X-linked agammaglobulinemia, some IgA deficiencies) or can be transient, occurs only in infants and is associated with delayed immunoglobulin production. Acquired or secondary hypogammaglobulinemia is a result of decreased function of immune system due to one of the various primary causes: chronic infections (HIV, EBV, tuberculosis…), malignancies (CLL, multiple myeloma, solid tumours…), autoimmune diseases, immunosuppressive drugs,

disorders in biochemical homeostasis (malnutrition, diabetes …) etc. On the proteinogram of a patient with hypogammaglobulinemia, γ-fraction is reduced (Figure 7). If γ-fraction is completely absent, we talk about agammaglobulinemia.

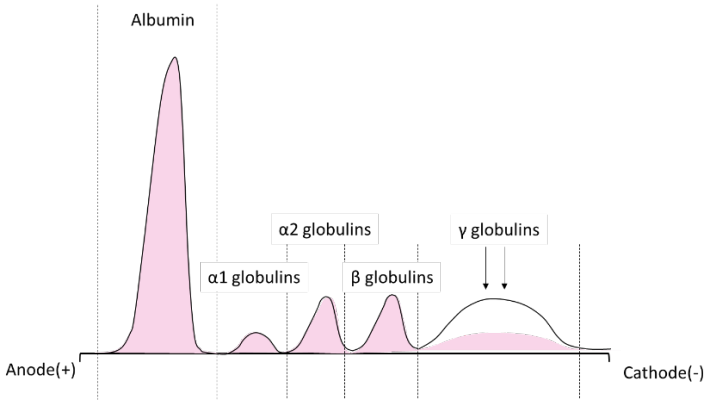


Figure 7: *Electropherogram representing changes in fractions in hypogemmaglobulinaemia.*

MONOCLONAL GAMMOPATHY

As opposed to polyclonal gammopathy that occurs in inflammation, monoclonal gammopathy is characterized by excessive production of antibodies derived from one clone of B lymphocytes. Monoclonal gammopathy results from malignant or potentially malignant clonal process. It occurs in patients with multiple myeloma, Waldenstöm's macroglobulinemia (IgM paraproteinemia), solitary plasmacytoma, indolent plasmacytoma, plasma cell leukaemia, or premalignant monoclonal gammopathy of undetermined significance.

On proteinogram of a patient with monoclonal gammopathy, we observe a narrow peak – monoclonal spike in the area of γ-globulins. γ-globulins are usually (but not necessarily) elevated (Figure 8). Other fractions, especially albumin, may be compensatory decreased. If the clonal process occurs in B lymphocytes producing IgM or IgA class of immunoglobulins, the monoclonal spike can occur earlier in the proteinogram (towards or in the β fraction).

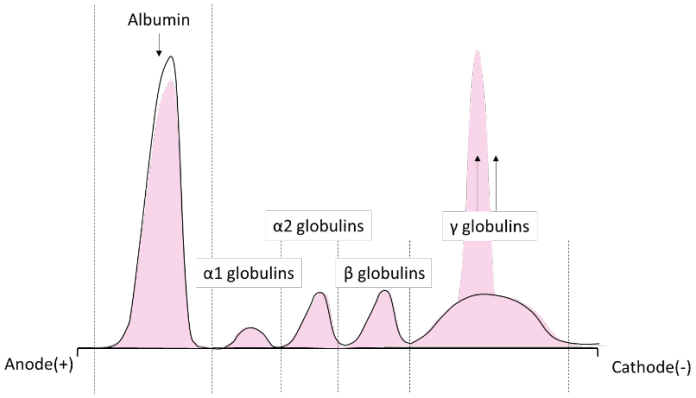


Figure 8: *Electropherogram representing changes in fractions in monoclonal gemmopathy.*

TEST YOUR KNOWLEDGE

1. Reflect on and investigate:
    a. What is isoelectric point?
    b. Which wavelengths protein absorb and why?
2. Describe the composition of the reagent for the biuret method and explain the role of all ingredients!
3. Indicate what may interfere with the determination of the overall serum protein concentration with the biuret method!

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LAB REPORT: ANALYSIS OF SERUM PROTEINS BY BIURET REACTION AND MEMBRANE ELECTROPHORESIS

Protocol for total serum protein concentration:

1. Pipette into 10 mL tubes (the volumes are in mL):

	r-Blank	Standard	Blank-St	Sample	Blank-Spl	Control	Blank-Ctrl
Demin. water	0.05		2.5		2.5		2.5
Standard		0.05	0.05				
Sample				0.05	0.05		
Control						0.05	0.05
Biuret reagent	2.5	2.5		2.5		2.5	

2. Mix well and measure the absorbance at 540 nm after 30-60 minutes.

Reagents:
Biuret reagent: $CuSO_4$ x $5H_2O$ (6 g/L) in alkaline solution (0,2 M NaOH) with K,Na-tartrate x 4 H_2O (28 g/L) and KI (0,5 %).

Protocol for electrophoresis of serum proteins on agarose gel:

A) Reagents and equipment:

1. Agarose gel: must be stored horizontally, between 15-25°C. Uncover the gel just before the use.
2. Diluent solution (ready to use): working electrophoresis buffer + Bromphenol blue and other non-reactive ingredients. Store between 15-25°C.
3. Electrophoresis buffer (concentrated solution): Non-barbital buffer and other important ingredients. Store between 15-25°C. To prepare a working solution dilute the content of the bottle according to the instructions on the bottle. If crystals appear, place the bottle in the warm water to dissolve the crystals.
4. Staining solution: acetic acid free concentrated Amido Black solution. Store between 15-25°C. To prepare a working solution dilute the content of the bottle according to the instructions on the bottle.
5. Destaining solution: 2 % citric acid solution. To prepare a working solution dilute the content of the bottle according to the instructions on the bottle.
6. Gel blotter strips: thin filter paper strips to blot the gel in the application area. Blot for 5 s only.
7. Power supply, electrophoresis chamber, plastic baths for staining/destaining.

B) Electrophoresis procedure:

1. Dilute fresh serum sample with protein diluent solution 1:6 (e.g. 10 μL serum + 50 μL diluent solution).
2. Fill the electrophoretic chamber with adequate volume of electrophoresis buffer (2x100 mL on each side of the chamber)
3. Take the agarose gel out of the packing just before use, uncover from the plastic plate and put it on the backside of the plate in horizontal position. Use the needle or pencil tip to punch through the little hole in the plastic holder. The gel will fall out easier.
4. Take one blot paper strip, apply it on the desired sample position and blot the gel for 5 seconds with the paper strip. This will prevent serum samples from spilling on the gel.
5. Take the plastic sample template strip and place it to the desired sample position on the gel.
6. Rub the template with the forefinger so that it gets in contact with the gel surface.

7. Use appropriate pipette to apply 5 mL of diluted serum samples in the corresponding slit and let them absorb into the gel for 2 min.
8. Blot the excess of the samples with gel blotter strip, gently remove both the sample template and the gel blotter strip and discard them
9. Carefully pick up the gel and transfer it to the electrophoresis chamber with the active side DOWN, and so that the place of application of the samples will be on the CATHODE SIDE (-).
10. Cover the electrophoresis chamber and connect it to the power supply. Run 10 min at 100 V.
11. Turn off the power supply. Carefully take out the gel.
12. Dry the gel completely (the proteins will be fixed in agarose) with hot air (less than 90°C), approximately 10 min.
13. Stain the gel with diluted protein staining solution for 5 min. Use a small plastic bath.
14. Transfer the gel to diluted destaining solution and shake vigorously. Repeat the process 3 times, for 5 min, each time with fresh solution, until the background is discolored.
15. Dry gel with hot air, clean well the backside of the film and evaluate the results by naked eye or densitometer (520-600 nm filters). In a healthy control serum, you are expected to see 6 main protein fractions.

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

Conditions:

Principle of the methods (scheme of reactions):

Measurements:

Total serum proteins:

control:

control blank:

standard:

standard blank:

serum sample:

serum sample blank:

other:

given values:

Control interval:

standard concentration:

Densitogram and values:

Calculations:

Results:

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

POINT OF CARE TESTING (POCT)

Assist. Dr. Jasna Omersel, MPharm, EuSpLM

LEARNING OUTCOMES

In the practical course you will learn the theory of different principles and methods used in POCT rapid tests and perform glucose measurement in capillary blood and some other analytes (drugs, C-reactive protein, viral particles, ...). At the end of the practical student will be able to:

- recall the importance of POCT as part of laboratory activity and explain significance of POCT in laboratory settings,
- describe the basic principles of rapid tests based on glucometers and immunochromatography,
- summarize the key concepts of glucometer-based and immunochromatography-based rapid tests,
- demonstrate the ability to independently perform capillary blood puncture,
- apply and independently perform POCT tests to obtain reliable result,
- critically evaluate and clinically interpret the results of POCT tests.

POINT OF CARE TESTING

Point-of-care testing (POCT) (also near-patient testing, bed-site testing) is defined as medical diagnostic testing performed outside of a laboratory setting by healthcare professionals who are not necessarily laboratory personnel. The goal of the tests is to obtain real-time, laboratory-quality diagnostic results within a few minutes (usually between 5 and 20 minutes) to help physicians make decisions about diagnosis, treatment, or hospitalization. The tests can be performed inside or outside the hospital

a) intensive care unit, operating room, delivery room, diabetic and anticoagulant therapy clinic, ...
b) emergency ward, resident physicians, sports medicine, pharmacy, phorensic drug screening.

All technological advances of the last century in laboratory testing and analytical techniques have led to a vibrant POCT market. Today, POCT devices and principles are used to measure numerous clinical parameters (Table 1). The key drivers of market growth are: Upward trend in infectious disease incidence, increasing demand for rapid and accurate diagnostics, increasing preference for home care (self-testing/self-monitoring), increasing number of highly equipped laboratories and services in North America, technological advancements, increasing investment in research and development. Challenges include primarily the high cost of product development and R&D investment in some regions of the world.

The cost of POCT analysis is typically higher than conventional laboratory testing, so the effectiveness of POCT testing must be calculated on a case-by-case basis, considering organizational, diagnostic, and patient advantages and disadvantages. Some outcomes or examples of improved clinical outcomes with point-of-care testing include (Price_1286):

a) faster decision making, earlier initiation of treatment (overdose, drug abuse, myocardial infarction)
b) improved treatment adherence, lower incidence of complications (diabetes)
c) faster optimization of treatment (anticoagulation therapy)
d) patient satisfaction, recognition of condition (pregnancy, ability to travel, ...).

POCT generally provides faster results than conventional laboratories. Nevertheless, POCT analyses are expected to provide credible and accurate results. Therefore, it is also important to ensure the quality of the results, internal quality control (control samples) and, wherever possible, participation in external quality assessment (comparison of results with the reference and other laboratories). Nevertheless, sometimes it is not possible to achieve the same accuracy with POCT instruments as with laboratory analyzers. The reasons may be: type of analyte, lack of control tests, improper storage ...

Table 1. *POCT – key laboratory parameters that can be measured.*

Clinical application	Analyte
Acid-base status, blood gas analysis	pH, pCO_2, pO_2 also combined with electrolytes Na+, K+
Electrolytes	Na^+, K^+, Cl^-, ionized Ca^+ and Mg_2^+
Metabolites	cholesterol, HDL-cholesterol, triglycerides, creatinine, urea, uric acid, bilirubin, lactate, ammonia
Enzymes	α-amylase, alkaline phosphatase, creatine kinase, aspartate amino-transferase, alanine aminotransferase, γ-glutamyl transferase
Hematology	partial thromboplastin time, thromboplastin time, D-dimer, platelet function, bleeding time
Haemoglobin fractions	CO-oximetry
Cardiac markers	troponin T and I, myoglobin, creatine kinase, pro-BNP
Diabetes mellitus status	blood and urine glucose, HbA_{1c}
Acute phase protein	C-reactive protein
Allergy diagnostics	allergen specific IgE
TDM and drugs screening	alcohol, amphetamine, barbiturate, cannabinoids, cocaine, opiates, …
Infectious diseases	HIV, infectious mononucleosis, *Chlamidia tachomatis*, influenza type A and B, SARS CoV-2, streptococcus A and B, …
Fertility	human chorionic gonadotropin, luteinizing hormone, sperm count in ejaculate, …
Urine diagnostics	test strips with multiple parameters (pH, proteins, glucose, ketones, blood, nitrite, leucocytes, …)
Stool diagnostics	occult blood
Molecular genetics	SARS-CoV-2, pharmacogenetics for improving the treatment efficacy (CYP2D6, CYP2C19)
Self-monitoring	urine protein and glucose levels, blood glucose, thromboplastin time (INR evaluation)

Like all tests, rapid POCT tests are subject to preanalytical, analytical, and postanalytical errors, many of which may be preventable through organizational measures. Although POCT tests generally do not require extensive sample preparation, the correct method of sample collection is of particular importance. Biological samples most commonly used in POCT are: Capillary blood, urine, swabs. The procedures for obtaining these samples are less or non-

invasive to the patient, which increases patient compliance and also facilitates the use of rapid tests for near patient monitoring or use of rapid tests for self-monitoring (e.g., blood glucose monitoring in diabetics or in pregnancy). As with all laboratory tests, false positive and false negative results can occur with POCT - depending on the analyte or test principle. All results must be interpreted carefully in conjunction with the patient's clinical picture.

There are different types of POCT devices that can be broadly classified into three groups: a) devices that are relatively simple to use (blood glucose meters, pregnancy tests), b) devices with moderate complexity (e.g., use of cartridges, need for trained medical professionals in clinical settings), c) devices/small analyzers with high complexity that require some sample preparation prior to testing and medical professionals (benchtop blood gas analyzers, molecular-based POCT devices). Classification may also be as follows:

Type I: a: Qualitative POCT methods

b: Unit-use POCT systems

Type II: Benchtop POCT analyzers

Type III: Viscoelastic coagulation analyzers

Type IV: Continuous POCT measurements methods

Type V: Molecular biological POCT analyzers

Type VI: Direct-to-consumer testing (DCT)

POCT technology uses a variety of analytical methods: electrochemical methods, mass change methods, optical methods, chromatographic methods. Thus, POCT tests/devices evolved from simple dry chemistry lateral flow test strips to complex unit instruments with multiple detection options. Today, many POCT instruments incorporate sensors as analytical systems. The results are not read by the human eye but are detected by a transducer and converted into an electrical signal. This principle is referred to as chemosensor or biosensor technology.

Conventional tests (in medical laboratories) and POCT methods may only be marketed if the product or device bears a CE mark. This confirms conformity with Regulation (EU) 2017/746. In addition, there are various legal acts in the EU states that regulate the field of in vitro

medical devices. EU members have also committed to follow EU legal acts and standards (e.g. ISO 15.189) or national guidelines for POCT testing. Their goal is to ensure high quality testing and safety for patients.

CAPILLARY BLOOD SAMPLING

There are many suitable biological samples for POCT: Capillary blood, urine, saliva and other mucous (buccal, nasal, nasopharyngeal, rectal, or wound swabs), stool. Collection of blood samples from the fingertip, heel, or earlobe (rare) is of particular importance in paediatrics, geriatrics, and adults for blood gas analysis, glucose and lactate measurements, and POCT. Why? This biological sample is called capillary blood and is relatively easy to obtain, but we are limited to quantities ~ 0,5 mL. It is a mixture of arterial, venous and capillary blood as well as interstitial and intracellular fluids. The relative composition depends on the blood flow to the puncture site. Warming of the puncture site (e.g., with a warm water compress) results in arterialization of the capillary blood. This is important if the sample is to be further analysed for pH or blood gas determination. There are few studies that look at the difference in values of biochemical parameters. For most parameters, the difference is less than 5%. This lowers the clinical significance of the difference and encourages professionals to use this biological sample in the situations described in Table 2. However, there are some exclusion criteria/contraindications for capillary blood collection: when quantities <0.5 mL of blood are needed (e.g. blood cultures), use in coagulation analysis, inflammation and patients in shock, hypoperfusion or inflammation of the puncture site.

Table 2. *Indications of the capillary blood collection.*

Sample needed is < 0.5 mL
Venous blood sample is not possible (for geriatric patients, severe burns, and overweight patients).
Paediatrics (volumes larger than 1 mL could pose a risk of anaemia or cardiac arrest).
Patients on anticoagulant therapy (capillary blood collection reduces the risk of bleeding).
Self-monitoring patients (diabetics and patients on anticoagulant therapy), POCT analysis.
Patients with anxiety, pain, or a fear of venous blood collection.

The proper technique of capillary blood collection will be demonstrated to you in the laboratory practical. The capillary blood collection procedure is relatively simple. National (Table 3) or worldwide guidelines must be followed to minimize preanalytical errors and ensure the best quality biological specimens with little risk to the patient.

Table 3. *Capillary blood collection: sequence of the procedure.*

Step	Activity
1	Preparation of supplies for capillary blood sampling
2	Hand washing and desinfection
3	Approaching the patient
4	Identifying patients, informing about the procedure
5	Positioning the patient
6	Checking fasting state, taking of medication and potential latex allergy
7	Inspecting the request form and types of the analysis
8	Selecting the microcollection tube, lancet
9	Selecting the skin puncture site
10	Warming up the puncture site
11	Desinfecting puncturing site
12	Inform the patient when starting the puncture
13	Perfororming skin pucture
14	Disposal of incision device into safety container
15	Elimination of the first drop of capillary blood sample
16	Capillary blood collection; order of draw in capillary blood collection
17	Filling, closure and mixing of capillary tube of microcontainer for capillary blood collection
18	Bandaging the skin after capillary sampling
19	Repeting patient identification, labeling of the samples
20	Gloves removal
21	Inspect the samples, if the collection was unsuccessful, repeat the sampling
22	Making note about the sample type

Prior to sampling, personnel must prepare all materials (Figure 1) and decide on the most appropriate puncture device and site during the patient visit (Figure 2). Puncture devices, or lancets for short, are small plastic cylinders or small cassettes with a needle or blade. The lancet needle or blade is used to poke a small hole in the skin to obtain capillary blood. After puncture, the blood is collected using a capillary tube or directly in a sample tube.

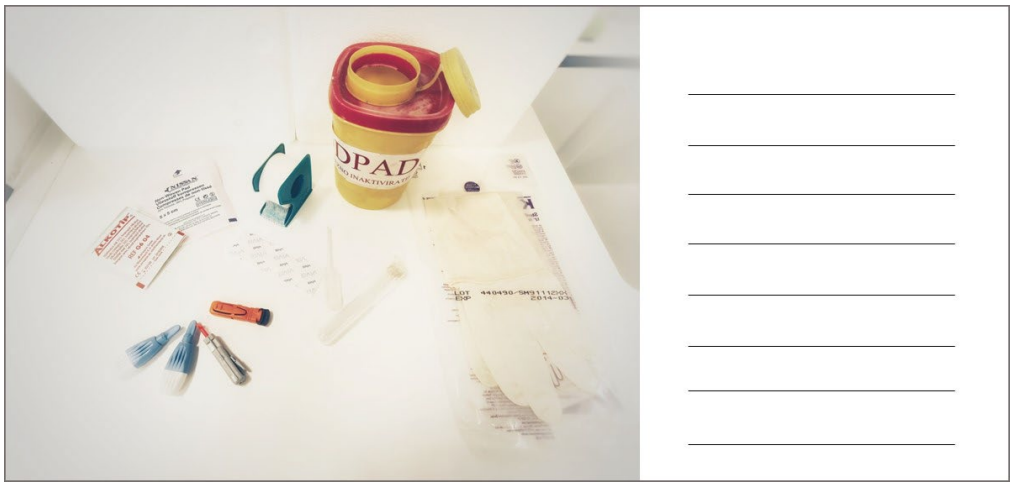


Figure 1. *Materials needed for the capillary blood collection. Name the materials needed!*

Figure 2. *Possible puncture site. Mark the appropriate puncturing position on the palm, foot and ear.*

The puncture site and depth depend on the age of the patient (Table 4). Special care should be taken when puncturing infants and young children. In neonates, the finger capillaries are only 0.35-1.60 mm below the skin. A heel prick at a depth of 2.4 mm can already damage bone and cause inflammation. There are many lancets on the market, usually divided into standard open needle/blade lancets and pip lancets (safety lancets), which were later improved to be safer to dispose of and eliminate the pain and anxiety associated with normal lancets.

Table 4. *Recommended puncturing site and depth.*

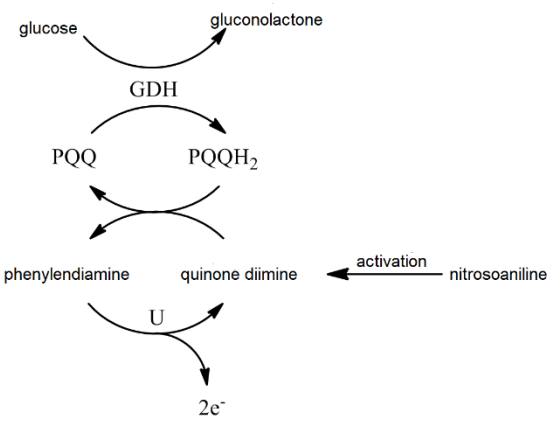
	Recommended puncturing site	**Recommended penetration depth**
Premature infants (up to 3 kg)	heel	0.85 mm
Infants (< 6 months)	heel	2.0 mm
Babies (6 months – 8 years)	finger	1.5 mm
Children (> 8 years, adults)	finger	2.4 mm

To ensure that capillary blood sample represents the best arterious-venous mixture any preanalytical errors should be minimized or avoided since they might affect the further laboratory analysis. These errors include:

a) **Use of first droop after puncture might contaminate the sample** with interstitial fluid, tissue factors, platelets etc. accelerating clotting. For most tests, you should wipe away the first drop of the blood with a dry pad.
b) **Contamination with infusion solutions (e.g. glucose).** Do not puncture at the site where infusion solutions might be in contact with skin.
c) **Sugar remnants on the skin (e.g. fructose from fruit on the finger tips).** Always wipe the puncturing site with alcohol pad and let it dry.
d) **Contamination with alcohol from alcohol pad.** After cleaning the puncturing site always wait until the skin dries.
e) **Sample coagulation.** Always follow the instructions about the correct mixturing of the sample with the anticoagulants in the specific tube. If there are more than too tubes to be collected or when the blood flow is to slow think about taking the venous blood collection
f) **Hemolysis.** Avoid aggressive skin squeezing at the puncture site, dry the alcohol remnants.

GLUCOMETER

At practical you will get to now one of the possible glucometers - Accu-Check® Guide. It is designed for fast and accurate glucose measurement in capillary blood. In addition to fingertip, samples can also be taken from the palm, forearm or upper arm, but only after consultation with the physician. The skin puncture is performed using a special lancing device with possible presetting of the puncture depth and a variety of safety mechanisms to protect the patent from possible infection. In an electrochemical assay principle, glucose dehydrogenase reacts enzymatically with glucose in a sample, releasing an electron that reacts with the mediator. The modified mediator is then electrochemically converted to its initial state by applying a voltage difference between the electrodes, resulting in a small electric current that is detected by the glucometer. The electric current is then proportional to the glucose concentration in the patient's sample. The use of the electrochemical principle of testing instead of the photometric principle (first generation of glucometers) increased the sensitivity and specificity of the tests. The system uses special and compatible test strips that offer an easy-to-use dosing range and accurate results with only a small drop of the sample (only 0.6 L). After effective blood application, the system automatically begins testing. The glucometer can be easily connected to the mySugr mobile app. In the mySugr app, you can log important therapy data such as blood glucose, meals, activity, insulin, ...log. It also calculates the estimated HbA1c value.



GDH: enzyme glucose-dehydrogenase

PQQ: coenzyme pyronolactone

U: voltage

Figure 3. *Electrochemical reaction in glucometer Accu-Check® Guide.*

Specifications (Accu-Check® Guide, Roche):

Sample volume: 0.6 µL

Sample type: fresh capillary blood

Measurement time: 5 seconds

Detection range: 1.1 do 33.3 mmol/L

Storage conditions for test strips: - 2 °C do 32 °C

Storage conditions for test strips: -25 °C do 70 °C

Acceptable range of humidity: 10 % do 90 %

Internal memory capacity: 720 results of blood glucose and 32 results with date and time.

Connectivity: Bluetooth wireless technology to communicate and transfer information and data to other devices

Lancing device: Accu-Check® FastClix or other certified lancet

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1. Point-of-Care Testing: Principles and Clinical Application. Editors: Luppa P., Junker R. 2nd Edition, Springer, 2018.
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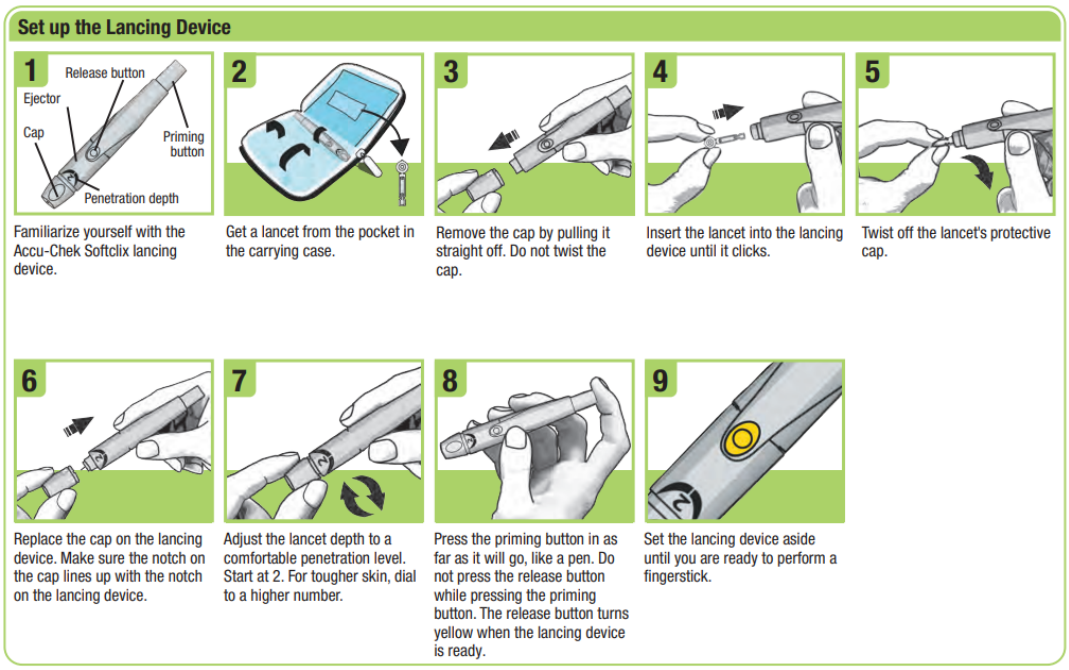
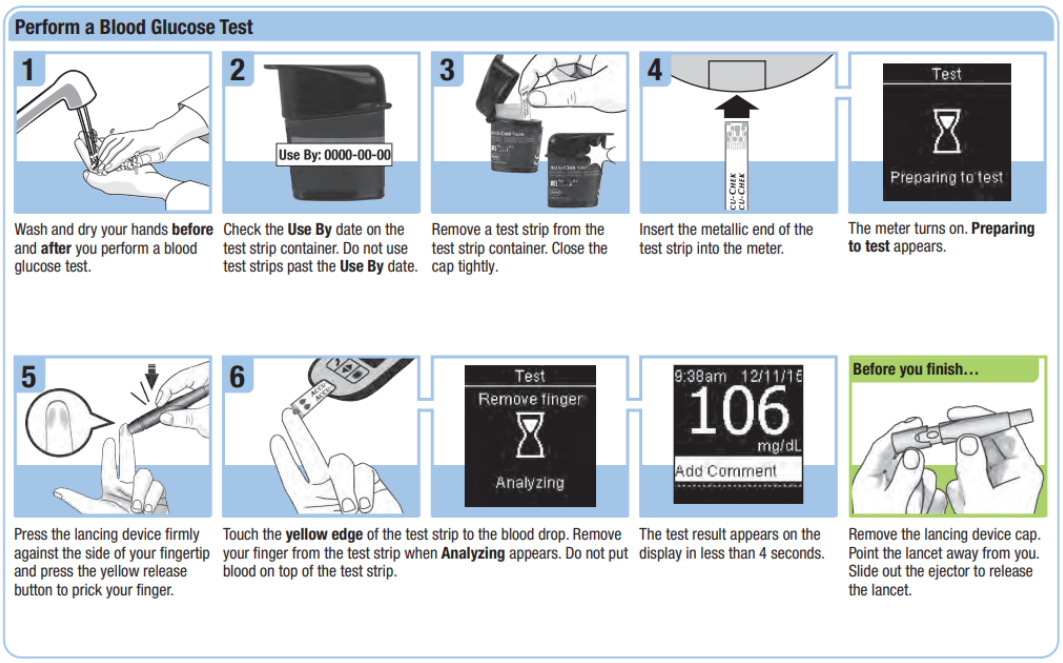
4. AccuCheck Guide Meter. Dostopno na: https://www.accu-chek.si/. Datum dostopa: 10.11.2024

LAB REPORT: POCT ANALYSIS

Protocol for glucose measurement:

1. Familiarise yourself with the glucometer and lancing device.
2. Prepare yourself or your colleague for capillary blood collection.
3. Follow the capillary blood collection protocol (Table 3) to obtain a drop of blood.
4. Immediately follow the procedure for glucose analysis with Accu-Check® Guide:





Protocol for rapid chromatographic tests

At practical you will get two of the POCT tests available on the market. Tests use immunochromatographic principle for the detection of the analyte in the sample.

1. Read the original manufacturer's instructions carefully. Think about each test and make brief notes:
   a) What is the purpose and significance of the analyte being tested?
   b) What biological sample is used and how is it prepared?
   c) What is the principle of the test? Make a sketch of the immunochemical reaction of the molecules on the test strip.
   d) Are there any important interferences that lead to false negative/positive results?
   e) How are the results reported, what is the reference range?
   f) Is there a control sample or internal control and what is it used for?
2. After discussion with the teacher, perform the tests and comment on your results.

Results and comments

Description of the sample, the material and the equipment:

Conditions:

Principle of the methods (scheme of reactions):

Glucometer

POCT rapid chromatographic test

Measurements:

A. POCT: glucometer measurements

Sample number:

Results:

Analytical evaluation (controls, samples):

Clinical interpretation:

B. POCT: _____

Sample number:

Results:

Analytical evaluation (controls, samples):

Clinical interpretation:

C. POCT: _____

Sample number:

Results:

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

MONITORING OF CARBAMAZEPINE IN SERUM BY ENZYME MULTIPLIED IMMUNOASSAY TECHNIQUE

Assist. Prof. Dr. Alenka Šmid, MPharm

Assist. Dr. Dunja Urbančič, MPharm

LEARNING OUTCOMES

After successfully completing this laboratory practical, student will be able to:

- list in which cases therapeutic drug monitoring (TDM) is suggested,
- explain what kind of biological sample should be collected for TDM and when,
- state at least 5 types of data that should be collected from the patient together with the biological sample,
- describe the principle of immunochemical method enzyme multiplied immunoassay technique,
- apply the obtained knowledge to determine carbamazepine concentration in the laboratory setting on actual clinical samples,
- use the newly obtained knowledge to interpret clinical results of the patients and provide the most probable diagnosis.

THERAPEUTIC DRUG MONITORING

Monitoring drug concentrations during treatment allows optimization of drug dosing, which in turn increases drug efficacy, reduces the risk of toxic or adverse events, increases patient compliance, and consequently improves quality of life. Plasma concentrations of drugs are monitored when:

- the drug has a narrow therapeutic index (small difference between therapeutic and toxic plasma concentrations)
- there is an increased risk of death due to dosing that is too high or too low
- there is a poor correlation between the dosage and the plasma concentration of a drug
- there are large interindividual differences in the pharmacokinetics of a drug (e.g., due to genetic variability, concurrent diseases, age, body mass, or gender)
- the clinical outcomes of a drug cannot be accurately measured (e.g., for prophylactic treatment)
- the plasma concentration of a drug is unstable in the patient due to physiological or pathological conditions (e.g., impaired hepatic or renal function)
- there is a risk of interaction between concomitantly prescribed drugs or foods
- we suspect poor drug compliance.

SAMPLE COLLECTION FOR THERAPEUTIC DRUG MONITORING

The most commonly used biological samples in clinical biochemistry for TDM are serum, plasma, whole blood, and saliva. The timing of sample collection is the most important part of TDM. The best time window for sampling is when the system is in a state of dynamic equilibrium, i.e., when the amount of drug entering the system is equal to the amount of drug exiting the system. The time required to reach dynamic equilibrium depends on the half-life of the drug ($t_{1/2}$); usually it takes between five and seven $t_{1/2}$. The rule of thumb is that we collect the sample right before the next dose.

If we assume that the patient has reached toxic plasma concentrations, we do not wait for the drug to enter the dynamic equilibrium, but rather perform TDM immediately. Waiting to reach the dynamic equilibrium is also not of importance for drugs with long $t_{1/2}$ (e.g., phenobarbital, phenytoin), because the variation in plasma concentration between two applications is minimal.

To obtain the most reliable results, sampling for TDM should be done at appropriate intervals:

- When determining the minimum plasma concentration (c_{min}), sampling should be done immediately before the next application of the drug. The minimum plasma concentration is determined when
    - we assess o whether the dosage is high enough to reach the minimum effective concentration of a drug, or
    - a drug accumulates in the body.
- When determining the maximum plasma concentration (c_{max}), sampling should be done after the distribution of the drug is complete. The determination of the maximum plasma concentration is important for the evaluation of the toxicity of a drug - the plasma concentration must not exceed the minimum toxic concentration (MTC).

As many variables affect the plasma concentration of a drug, we must also collect a patient's demographic and other therapeutic data (Table 1) to adjust the dose as efficiently as possible.

Table 1. *Patient's data that should be collected together with the biological sample*

Basic data of a patient	Therapeutic data of a patient
• name, gender, age, body mass • pathological conditions • liver and kidney function • personal characteristics (e.g. previous poor response to treatment) • genetic markers	• drug • dosage • duration of the treatment • date and time of the last drug application • concomitant treatment

ANALYTICAL METHODS USED IN TDM

Various immunochemical and chromatographic methods can be used to measure a wide range of drugs in biological samples (Table 2). Which method we use depends on the drug we want to analyse, the equipment we have, and the expertise of the staff.

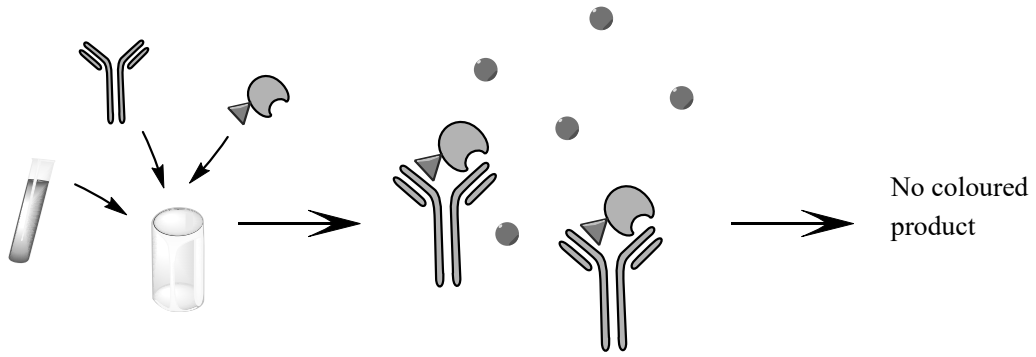
Table 2. *Immunochemical and chromatographic methods in TDM.*

Immunochemical methods	Chromatographic methods
• **FPIA**; *Fluorescence Polarisation Immunoassay* • **EMIT**; *Enzyme-Multiplied Immunoassay Technique* • Dry chemistry tests	• Gas chromatography (GC) • High-performance liquid chromatography (HPLC) • Mass spectrometry coupled liquid chromatograaphy (LC-MS)

EMIT (ENZYME-MULTIPLIED IMMUNOASSAY TECHNIQUE):

The enzyme-multiplied immunoassay technique (EMIT®) is a simple, rapid, and homogeneous method based on competition between the drug present in the sample and the chemically labeled drug in a reagent. A chemically labeled drug is in a complex with an enzyme (e.g., glucose-6-phosphate dehydrogenase). Antibodies specific for this drug-enzyme complex bind to the complex and reduce enzyme activity. Introduction of a biological sample containing the same drug releases the enzyme-labeled drug from the antibody, increasing enzyme activity. Enzyme activity correlates with drug concentration in the sample and can be measured as the change in absorbance resulting from the conversion of a substrate to the coloured product.

2A – no drug in a sample



2B – drug present in a sample

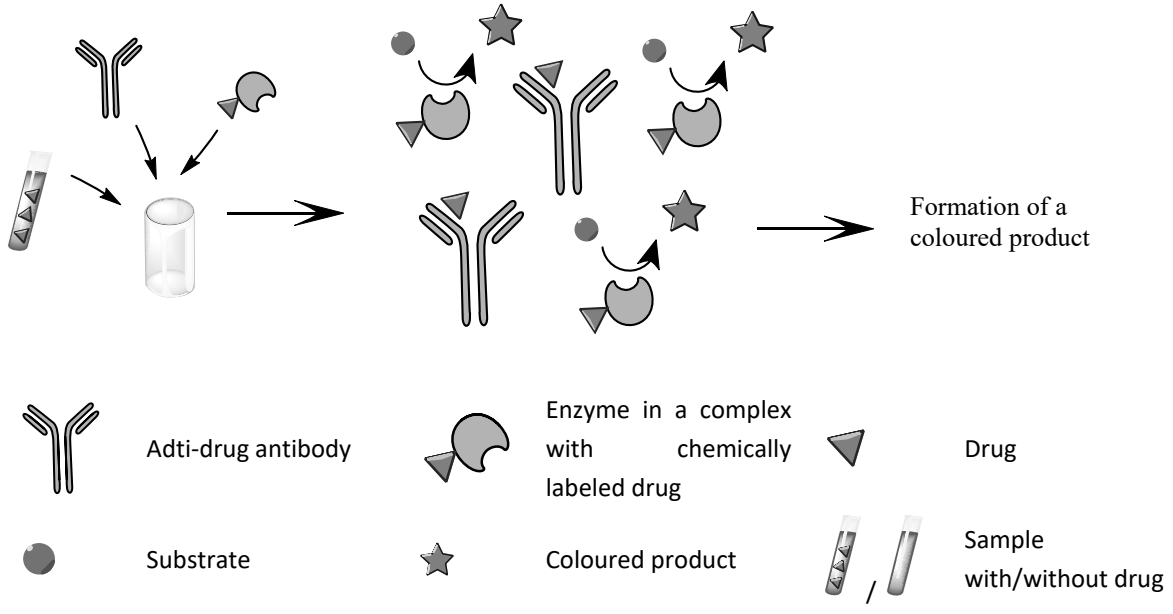


Figure 1. *Schematic representation of the method EMIT for analysis of a drug in a biological sample. Created with biorender.com.*

TEST YOUR KNOWLEDGE

1. Reflect on:
   - ADME proceses after intravenous and (per-)oral application of a drug.
   - Plasma concentration curve after drug administration.
2. What types of methods can be used to measure the drug concentration?
3. You would like to introduce a new drug to the treatment of a patient. However, the data on TDM for this drug is unclear. What factors would you take into consideration when deciding on the usefulness of a TDM for this drug?

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LAB REPORT: MONITORING OF CARBAMAZEMIPE IN SERUM BY EMIT

Protocol for automatic measurement of carbamazepine concentration by EMIT method:

1. Insert the original reagent mix for carbamazepine determination into the analyzer.
2. Pipette 200 µL of the control and samples into the beakers/tubes on the calibration plate.
3. In the "ORDER SAMPLES" dialog box, enter the number or the label of the sample, tick off the desired test (carbamazemine, sample blank) and place the tube into the assigned location.
4. Separately, prepare a test tube with 200 µL of distilled water and put it into position 1.
5. Place the control, the standard and reagents in designated, well-defined locations in the reagent rotor.
6. Read the result.

***Note**, the detailed description and the usage of the automatic anaylser can be found in the chapter Determination of creatinin in serum by kinetic Jaffe reaction and in the manual you can find at the lab practicals.*

Results and comments

Sample number: _____

Description of the sample, the material and the equipment

- Drug:_____
- Therapeutic range:_____
- Reagnet mix _____

    - Reagent 1 (composition):

    - Reagent 2 (composition):

- Control: _____

Conditions:

Principle of the methods (scheme of reactions):

Results:

	Control sample interval	Concentration
Control 1		
Control 2		
Control 3		
Plasma sample		

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date:

THIOPURINE S-METHYLTRANSFERASE GENOTYPING USING HYDROLYSIS PROBES

Assist. Dr. Dunja Urbančič, MPharm

LEARNING OUTCOMES

Upon completion of this laboreatory practical students will be able to:

- list the applications for molecular diagnostic tests,
- describe the principles of pharmacogenetics and explain its advantages and disadvantages,
- illustrate genotype and activity correlation for an enzyme thiopurine S-methyltransferase and its involvement in thiopurine treatment,
- describe polymerase chain reaction in detail (reagents used, conditions applied),
- explain the principle of genotyping using hydrolysis probes,
- apply the knowledge to perform genotyping of thiopurine S-methyltransferase single nucleotide polymorphisms in the laboratory setting,
- interpret results of genotyping and explain their implication on clinical case.

MOLECULAR DIAGNOSTICS AND PHARMACOGENETICS

Within the field of molecular diagnostics, the nucleic acids changes can be analysed at DNA and RNA level. At the DNA level, changes in the number and structure of individual genes (point variations, deletions and insertions) can be determined. At the RNA level, we primarily detect differences in the expression of an individual genes. A general term for a change in a

DNA nucleotide sequence is genetic variation. More specific terms for genetic variations are single nucleotide polymorphisms (SNPs) and other types of mutations such as deletions, insertions, variable number of tandem repeats etc.

Based on DNA/RNA analyses, molecular diagnostics enables to:

- discover the cause of monogenic diseases,
- discover disease predispositions, disease carriers or prenatal pathological conditions,
- detect somatic mutations in cancer (diagnosis, prognosis),
- discover the causative agents of infectious diseases,
- evaluate the treatment efficacy in cancer and infectious diseases,
- determine predisposition to drug response (which is the subject of pharmacogenetics),
- support the gene therapy (determining the site of retroviral integration),
- establish identity (in forensic medicine).

PHARMACOGENETICS

An individual's response to a drug depends on a number of factors that influence the pharmacodynamics and pharmacokinetics of the drug and can be broadly divided into genetic, physiological, and environmental (Table 1).

Table 1. *Main factors influencing the response to drugs.*

Factor	Type of the influenced response
Genetic	
Therapeutic targets	Efficacy (pharmacodynamics)
Metabolic enzymes	Metabolism (pharmacokinetics)
Transporters	Distribution (pharmacokinetics)
Targets in the pathways for side effects	Toxicity
Indirect	Efficacy, pharmacokinetics and toxicity
Physiological	
Age, gender, disease state, pregnancy, physical activity, circadian rhythm, starvation	Efficacy, pharmacokinetics and toxicity
Environmental	
Chemicals from the environment, concomitant use of other drugs, smoking, alcohol consumption, dietary habits	Efficacy, pharmacokinetics and toxicity

Pharmacogenetics is a part of molecular diagnostics that studies individual differences in drug response that is a direct consequence of differences in the genome. The goal of pharmacogenetics is to predict an individual's response to a drug based on known genetic characteristics (i.e., genotype). Clinical pharmacogenetic testing is used to support therapeutic drug monitoring (TDM; see next chapter) to adjust drug dosing or drug selection.

Acquired and inherited genetic variation, either on the level of SNPs or gene expression, influence drug response in patients through changed pharmacokinetics (enzymes and proteins involved LADME processes) or pharmacodynamics (biological targets). If genetic variant lies in a gene encoding drug metabolizing enzyme, the variant may affect enzyme activity. Considering this genetic variability, the patients are usually categorized into the following prediction phenotypes: normal (or extensive) metabolizer, ultra-fast metabolizer and poor metabolizer (Figure 1) These individuals will respond to the drug differently and they will need different doses for safe and efficient treatment. If a genetic variant affects the expression or structure of a biological target (e.g. receptor), the response to the drug can be preserved or absent. In both cases, a patient may experience more side effects of the drug compared to the wild-type individual.

The most studied pharmacogenetic targets (genes) are those encoding proteins involved in drug metabolism (e.g., cytochrome P450 2C9, thiopurine S-methyltransferase, UDP-glucuronyltransferase 1A1) and drug target proteins (e.g., HER2, KRAS, VKORC1).

In contrast to classical TDM, which is performed after initiation of therapy, pharmacogenetic testing can be performed before the start of a treatment. Compared with classical TDM, pharmacogenetics has many advantages:

- it is not necessary to wait for the state of dynamic equilibrium,
- it is less invasive (isolation of DNA from saliva, swab of oral mucosa),
- genetic information of an enzyme allows to predict the response to several drugs metabolized by this enzyme,
- it allows a mechanistic, not only descriptive explanations,
- the genotype is quite constant throughout the life of a person and does not depend on physiological and disease conditions.

Please note, that pharmacogenetic information is not the only parameter that influeces drug response (Table 1). One of the limitations of pharmacogenetics is, that pharmacogenetic testig is not applicable for many drugs that are currently on the market. Therefore, therapeutic drug monitoring is sometimes necessary to assure safty dosing of the applied medicine.

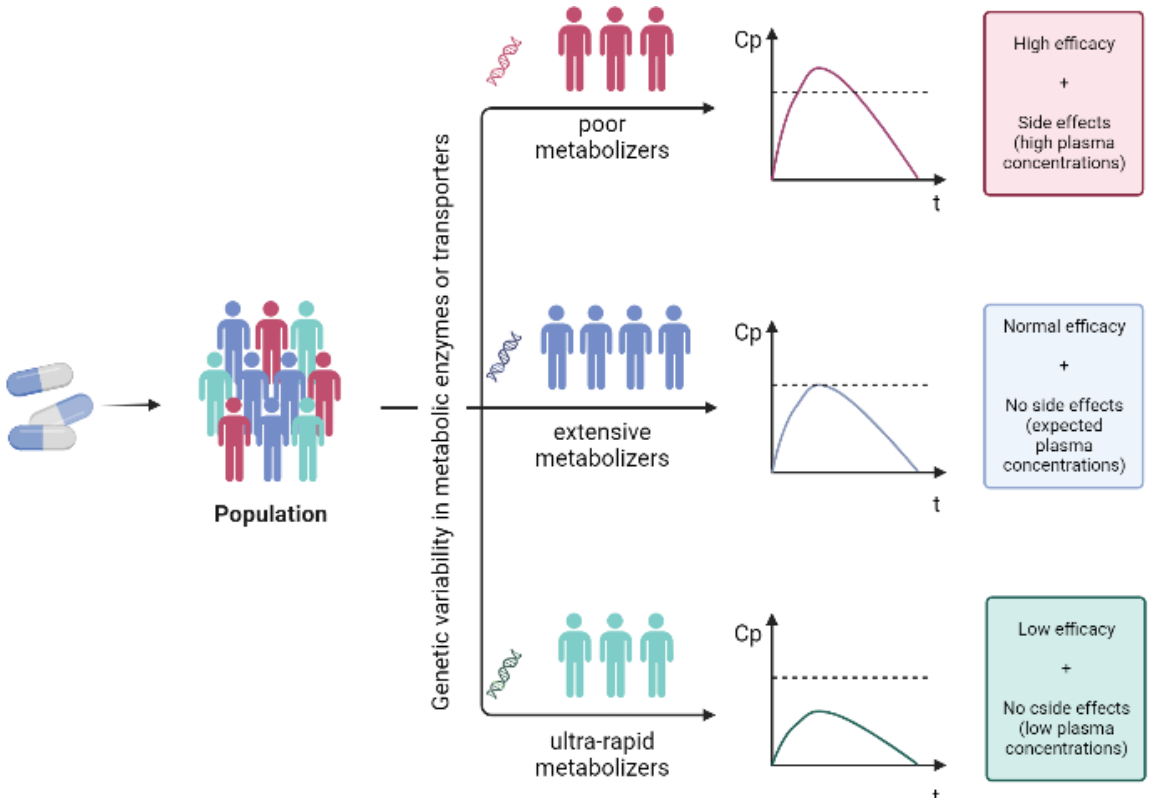

Figure 1. *Schematic presentation of effects that genetic variability in metabolizing enzymes can have on response and disposition of a drug. Created with biorender.com.*

Pharmacogenetic example: thiopurine S-methyltransferase

Thiopurine S-methyltransferase (TPMT) is an enzyme that catalyses methylation of cytostatic and immunosuppressive drugs, thiopurines. Thiopurines (6-mercaptopurine, azathioprine, and 6-thioguanine) are prodrugs that require activation to thioguanine nucleotides, which are active metabolites that exert cytostatic and immunosuppressive effects. One of the major deactivating enzymes of thiopurines is TPMT. A patient with low TPMT activity treated with thiopurines produces a greater amount of active thioguanine nucleotides, the target effect of thiopurines is higher, but he also has a higher risk of severe side effects of thiopurines than a patient with normal TPMT activity (Figure 2).

TPMT activity is highly dependent on genetic polymorphisms in the gene encoding TPMT. The most common variant allele is called *TPMT*3A*. *TPMT*3A* is a haplotype, which means it is composed of two SNPs, namely *TPMT*3B* and *TPMT*3C*. Allele *TPMT*3B*, contains the transversion 460G>A, which means that in this allele gunanine (G) on locus 460 of *TPMT* transcript is swaped for adenin (A). Allele *TPMT*3C* contains transversion 719A>G which means that gunanine (G) on locus 719 of *TPMT* transcript is swaped for adenin (A).

A patient with two *TPMT*3A* alleles has very low enzyme activity. Please keep in mind that human somatic cells have two sets of chromosomes, which means that each gene has two "versions" called alleles. A patient with one variant *TPMT*3A* and one normal (wild-type, *TPMT*1*) allele has intermediate TPMT activity, whereas a patient with two wild-type alleles has normal TPMT activity. Because of lower TPMT activity, patients with one or two *TPMT*3A* alleles have a higher risk of developing severe side effects if treated with thiopurines (Figure 2). Therefore, thiopurine dose for these patients must be significantly reduced.

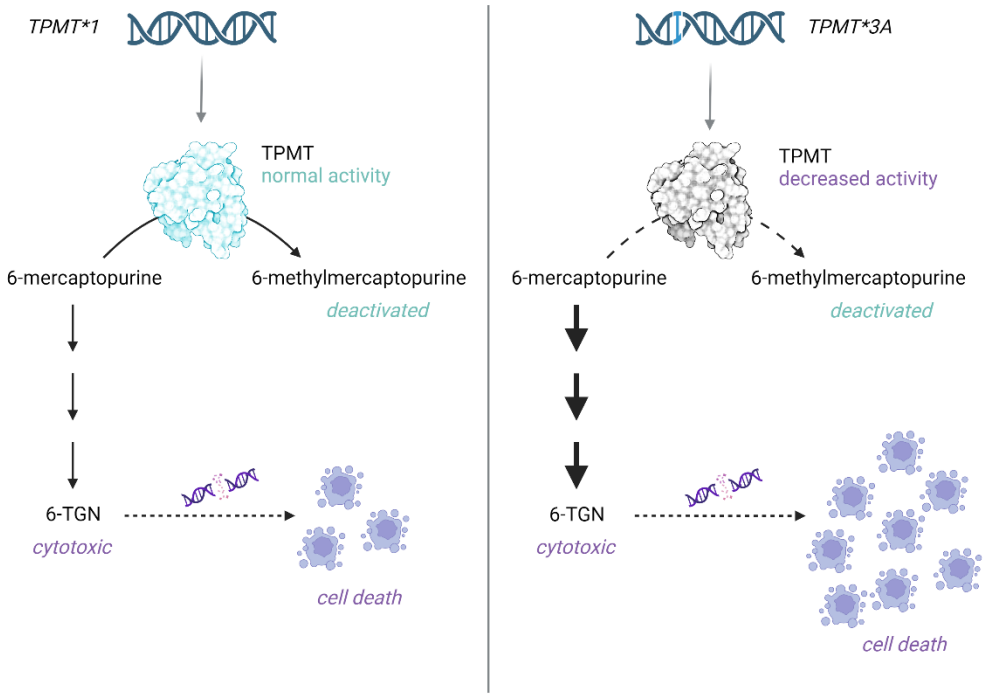

Figure 2. *Pharmacogenetics of thiopurine S-methyltransferase (TPMT). Decreased TPMT activity as a consequence of genetic polymorphisms in TPMT gene results in more extensive formation of active thioprine metabolites and more severe cell death. Created with biorender.com.*

POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is an *in vitro* method for the synthesis of nucleic acids. The reaction occurs if appropriate components and conditions are applied. The reaction components are:

- Taq DNA polymerase: a thermostable enzyme that enables the formation of phosphodiester bond between nucleotides in DNA.
- Magnesium ions: a cofactor for DNA polymerase.
- Deoxynucleotides (dNTPs): building blocks of DNA.
- Matrix DNA: isolated DNA from a biological sample, that represents a template for newly formed PCR product.
- Buffer: appropriate ionic strength and pH (around 8) for optimal conditions for PCR.

PCR proceeds in a cyclic way, in which each cycle consists of three phases (Figure 3):

1. Denaturation of DNA (96° C),
2. Annealing of primers (50-60° C; usually 55°C),
3. Extension, i.e. synthesis of the complementary chain with thermostable DNA polymerase (72° C).

Usually, 30 to 60 cycles are performed within one PCR reaction. The number of copies theoretically doubles with each cycle, however, due to limited amounts of reactants and capacity of DNA polymerase it eventually slows down. The kinetics of PCR reaction thus follows sigmoid curve. PCR is performed in polypropylene tubes or thin-walled microtiter plates with a volume of 0.2 or 0.5 ml. The volumes of the reaction mixtures usually reach 5 to 100 µl. PCR takes place in a cycling thermostat.

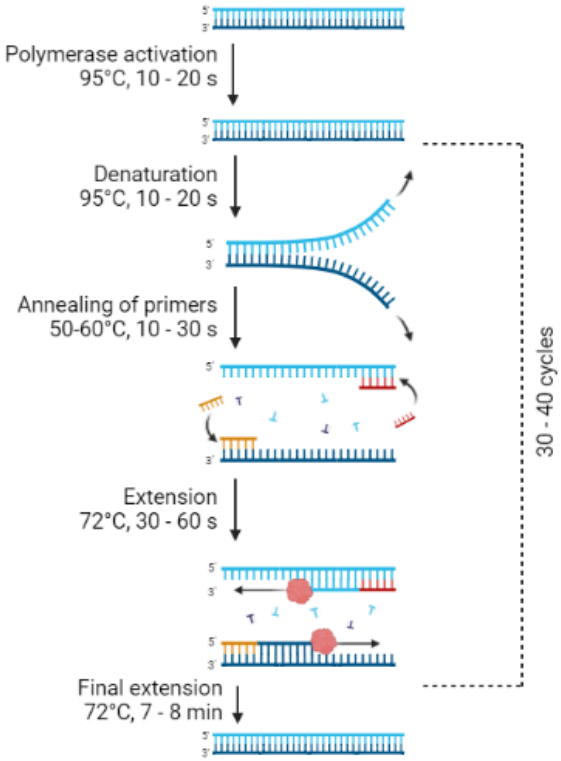

***Figure 3**. PCR principle and phases. Created with biorender.com.*

The successful completion of the reaction can be verified by electrophoresis on an agarose gel, in which we can evaluate if the desired PCR fragment was specifically formed. Another way of monitoring the reaction is by adding the fluorescent DNA-detecting dye into the reaction mixture and detecting the emerging fluorescence during the course of the reaction – in real time. Because of the ability to determine the concentration of the DNA in the sample, the later form of detection is also called quantitative PCR (qPCR).

Experimental samples

As with other analysis in Clinical Chemistry, also molecular diagnostic testing requires the use of control samples. Control samples in PCR consist of

1. positive control(s): a sample with DNA that we successfully amplified for a specific fragment in the past, and
2. negative control: a mixture in which the DNA sample is replaced by water; it allows detection of possible contamination of the reagents.

GENOTYPING

Numerous methods are available to determine genetic variations, including restriction fragment length polymorphism (RFLP) analysis, allele-specific PCR, genotyping using hydrolysis probes, single-stranded DNA conformation polymorphism (SSCP) analysis, microarrays, sequencing, and others. In this practical, we will perform a practical example of single nucleotide polymorphism (SNP) determination by genotyping using **hydrolysis probes**.

Genotyping using hydrolysis probes

Genotyping using hydrolysis probes is based on qPCR. In this method, the reaction mixture contains:

- sample with patient's DNA,
- a thermostable (Taq) DNA polymerase with 5'-exonuclease activity,
- a suitable buffer system containing magnesium ions and dNTPs,
- primers and hydrolysing probes specific for genetic polymorphism of interest.

Hydrolysis probes, also called TaqMan probes, are short oligonucleotides that anneal onto the DNA, specifically on the site that contains SNP, and is located between the two primers. Probes are designed to have a fluorophore attached to the 5' end and a quencher attached to the 3' end of the oligonucleotide (Figure 4). As long as the fluorophore and quencher are in close proximity, the quencher inhibits fluorescence emitted by the fluorophore and the fluorescence signal cannot be detected. In the annealing step of PCR, both probe and primers anneal to the target DNA. During the extension, Taq polymerase reaches the probe, and on account of its 5'-exonuclease activity degrades the probe. The fluorophore-quencher distance is extended and thus allows the emission of fluorescence. The increased fluorescence is then used to determine the quantification cycle in each reaction. If the probe does not bind to the target DNA during PCR, Taq polymerase cannot degrade it and the fluorescence from the fluorophore is not detected.

Interpretation of results after genotyping using hydrolysis probes

SNP detection using hydrolysis probes requires two probes with different fluorescent reporters. This allows for differentiatiation of homozygous and heterozygous samples.

In SNP genotyping the first probe, labelled with fluorescent dye 1 detects the first allele sequence (e.g. nonvariant allele), while the second probe, labelled with fluorescent dye 2, detects the second allele sequence (e.g. variant allele). If the individual is homozygous for nonvariant allele, we will detect mostly fluorescence of the dye 1 (Figure 4). If the sample is homozygous for variant allele, the plot will mainly show signal from dye 2. If the sample is heterozygous, however, there should be roughly equal signal for each dye.

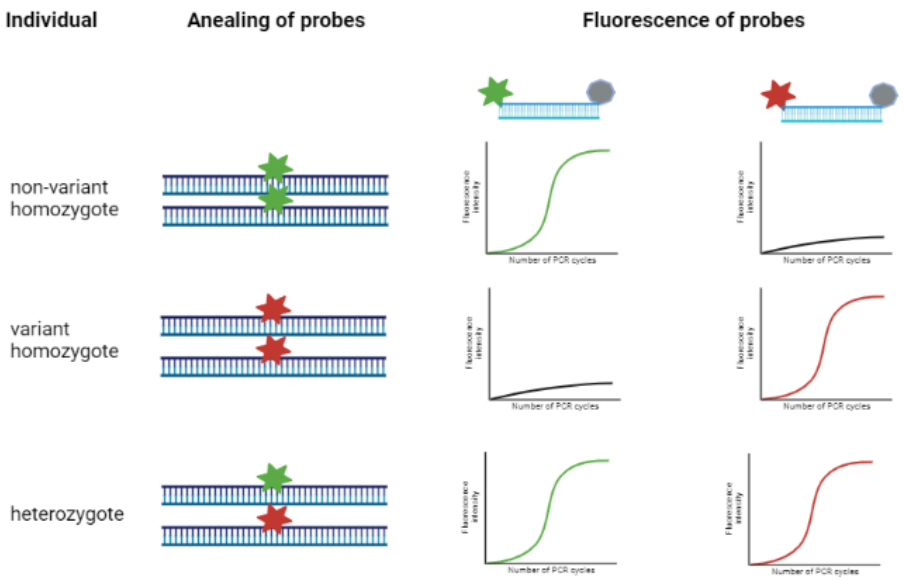

Figure 4. *Shematic presentation of results after genotyping with hydrolysis probes on how to distinguish individuals with different genotype. Created with biorender.com.*

Usually, polymorphisms are studied in a larger group of individuals or patients. After analysis, the results can be presented in the form of an "allelic discrimination plot". **Allelic discrimination** is a technique for distinguishing between genotypes, mutations, or polymorphisms by comparing fluorescence signals obtained with allele-specific probes. The plot shows the fluorescence intensity of each dye on the axes. In this type of diagram, three groups of individuals can be distinguished: non-variant homozygotes, heterozygotes and variant homozygotes (Figure 5).

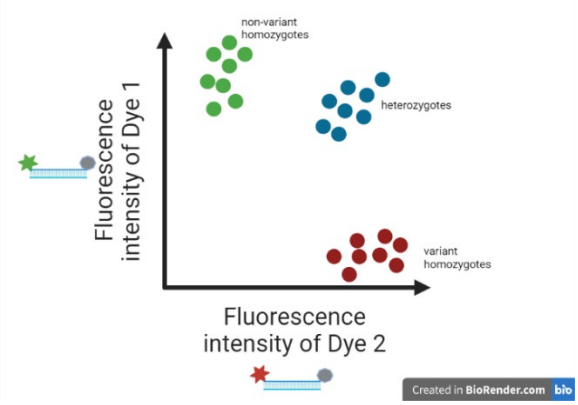

Figure 5. *Allelic discrimination after genotyping of patients with hydrolysis probes. Created with biorender.com.*

LABORATORY CONDITIONS AND INTERFERENCES IN PCR

Analysis environment

To avoid contamination and interferences during the reaction, separated facilities for pre- and post-PCR should be used. Short DNA fragments form previous PCRs can inhibit currently performing PCR. The reaction mixture should be prepared in the cleanest environment, while the test tubes containing the final PCR products should be centrifuged and opened in a separate laboratory. The pipette tips and PCR tubes are sterilized before work, and latex or nitrile gloves are worn at all times during lab work and changed if needed.

Interferences

The successful performance of PCR is also influenced by contaminating compounds or residual solvents in the sample that may inhibit PCR. Inhibitors of the reaction can act either by interaction with DNA or by interaction with DNA polymerase. PCR inhibitors may originate from the biological sample, from which the DNA was isolated, or from the reagents used during DNA extraction. Some most common inhibitors of PCR are listed in Table 2.

Table 2. *Most commonly present inhibitors of PCR.*

Inhibitors originating from biological material		**Inhibitors originating from reagents**
Inhibitor	**Biological sample**	**Inhibitor**
bile salts, complex polysaccharides	stool	Ionic detergents (e.g. SDS)
haem, haemoglobin, lactoferrin, IgG	blood	ethanol in isopropanol
melanin and eumelanin	skin, hair	phenol
myoglobin	muscle tissue	guanidine salts
potassium ions	milk, bones	
urea	urine	

Biological samples

A whole blood sample is often used for DNA isolation. Instead of whole blood, samples from other tissues (e.g., muscle, liver, heart, brain tissue, or bone marrow) can be used for DNA extraction. DNA can also be isolated from swabs (e.g., buccal or nasal mucosa) or other body fluids (e.g., cerebrospinal fluid, urine).

TEST YOUR KNOWLEDGE

1. How can SNP in the gene of a metabolizing enzyme of a drug affect the drug response?
2. Describe the role of each reagent used in PCR!
3. Explain in which cases and why we detect fluorescence of the hydrolysis probe in a patient's sample?
4. How many different genotypes can be used to determine SNPs at a specific locus of the target gene if two different hydrolysis probes are used? What is the number of different genotypes if we use three different hydrolysis probes (each probe is conjugated with different dye)?

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LAB REPORT: THIOPURINE S-METHYLTRANSFERASE GENOTYPING USING HYDROLYSIS PROBES

Protocol for genotyping for haemochromatosis with hydrolysis probes:

*In this exercise, you will prepare and examine DNA samples to determine the presence of two SNPs: TPMT*3B and TPMT*3C. The information on the probes and their labelles can be found in Table 3.*

***Table 3:** The information on hydrolysis probes for TPMT*3B and TPMT*3C determination.*

SNP	Rs number	Non-variant allele and dye	Variant allele and dye
TPMT*3B	rs1800460	G – FAM	A – VIC
TPMT*3C	rs1142345	G – VIC	A – FAM

Samples:

- The DNA sample of concentration 5 ng/µl was isolated from venous whole blood collected with EDTA.

Warning! The mixture of probes, DNA and MasterMix should be stored on ice. The probes must be protected from light.

1. Prepare a master mix for all samples in the group. The table below shows volumes for each component of the reaction. The total reaction volume should be 10 µl.

a) Calculate the volume of each component needed for the analysis of all samples in the group. Each sample will be run in duplicates. In addition to the samples, we will prepare three controls - one for each genotype; these will also be run in duplicates. Write the volumes down into the table:

Component	1X volume (µl)	Volume for _____ x reactions (µl)
Ultra pure water	2.75	
2X Master mix containing buffer, Taq-polymerase, Mg2+, dNTP	5.00	
40X Probes with primers	0.25	
DNA sample	2.00	//////////////////

b) Mix the calculated amounts of each component (without the sample) in a 1.5 ml test tube.

c) Briefly centrifuge the test tube containing the reagent mixture for 2 minutes at 1900 rpm.

2. Prepare a pipetting scheme for a 96-well plate (96 WP):

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

3. Fill the corresponding wells on the microtiter plate with 8 µl of the reagent mixture.
4. Add 2 µl of each sample and control in duplicate to the wells.
5. Cover the plate with a transparent foil.
6. Centrifuge the plate for 2 minutes at 1900 rpm.
7. Perform the PCR according to the program:

Activation of polymerase	95°C, 10 min	
Denaturation of DNA	95°C, 15 s	40 cycles
Annealing of oligonucleotides and amplification	60°C, 60 s	

8. Fill in the template for samples in the analyzer.
9. Read the results.

Results and comments

Sample number: _____

Description of the sample, the material and the equipment:

Conditions:

Principle of the methods (scheme of reactions):

Measurements / Results:

Analytical evaluation (controls, samples):

Clinical interpretation:

Signature: Date: