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Laboratory notes

GHIDURI ȘI ÎNDRUMĂTOARE DE LABORATOR

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L1. QUALITY CONTROL IN THE CLINICAL LABORATORY

LEARNING OBJECTIVES

Define the three phases of the laboratory testing process: preanalytical, analytical, and postanalytical.

Identify the quality control measures applicable at each phase of laboratory testing.

Calculate and interpret precision parameters: mean, standard deviation, and coefficient of variation (CV).

Evaluate the accuracy of an analytical method using bias calculation, linear regression, and the Student t-test.

Perform and interpret a manual glucose determination using the glucose oxidase method.

KEY POINTS

Quality control encompasses all activities that ensure test results are accurate, precise, and reliable throughout the entire testing process.

The preanalytical phase is responsible for the majority of laboratory errors; patient preparation, sample collection, and transport must be carefully controlled.

Precision (measured by CV) reflects reproducibility; accuracy (measured by bias) reflects closeness to the true value. Both must meet defined acceptance criteria.

Method evaluation requires assessment of precision, accuracy, analytical range, interference, and comparison with a reference method.

Acceptable glucose determination performance targets: CV ≤ 2.8%; bias as close to zero as possible.

GENERAL ASPECTS

The clinical laboratory is where tests are carried out on clinical specimens to gain information about the patients' health. The results (in the form of an analysis bulletin) can assist the physician in screening, diagnosing or monitoring the patient's disease or disability.

The most requested laboratory analyses include determining the presence or the concentration of a certain compound (known as analyte or marker) in a clinical specimen (biological sample). To achieve this, it is essential to select an appropriate analytical method, as the method's performance directly impacts the accuracy of the results. However, analytical methods can sometimes be affected by variability and errors. Therefore, it is crucial to regularly verify that the chosen method maintains its performance over time.

Quality control involves a set of measures and procedures designed to ensure consistent quality over time and to minimize errors. In the context of laboratory testing, quality control ensures the analytical method remains accurate and consistently delivers reliable results.

Analysing a biological sample involves three main steps, and quality control must be addressed at each stage to ensure accurate and reliable results:

1. Preanalytical phase (step)
2. Analytical phase (step)
3. Postanalytical phase (step)

I. PREANALYTICAL STEP

The preanalytical phase encompasses all activities that must be completed before the biological sample is analysed. At this step, quality control involves minimising the errors before, during, and after sample collection.

Important Considerations:

- *Thorough Documentation*: Every step of the process must be carefully documented in appropriate registries to ensure traceability and accountability.
- *Informed Patients*: The patient needs to be well-informed regarding the procedures that are about to be used.
- *Trained Personnel*: The medical or laboratory staff must be adequately trained regarding the procedures involved, ensuring proper execution and minimizing errors.

Quality control:

- Before sample collection
  - *Dietary Restrictions*: Inform the patients about any dietary restrictions that should be followed before sample collection (e.g., fasting requirements).
  - *Self-Collection of Samples*: Educate the patient on how and when to collect biological samples such as faeces, urine, or sputum. Provide clear instructions on where and how to store the samples before bringing them to the laboratory.
  - *Patient Identification*: Confirm patient identity using a photo ID.
  - *Data Accuracy*: Check personal information, diagnosis, requested tests, and ensure the prescribing doctor's signature is present.
  - *Informed Consent*: Explain the manoeuvres to be used for sample collection. Use both verbal explanations and written materials to ensure comprehension. Ensure the patient is comfortable and signs a consent form before proceeding.

- During sample collection
  - *Proper Positioning*: Place the patient on an adjustable chair in either a clinostatic (lying down) or orthostatic (standing/sitting) position, depending on the sample type.
  - *Requirements*: Identify the type and quantity of the biological sample needed. Select the appropriate type and number of collection containers.
  - *Labelling*: Properly label containers with the patient's details and the requested tests.
- After sample collection
  - *Transport.* The samples should be promptly (ideally within 4 hours) transported from the collection site to the clinical laboratory. Whenever necessary, ensure special transport conditions, such as the use of special containers or protection from heat or light.
  - *Primary Processing*. At arrival in the clinical laboratory, confirm the integrity of the collection containers. Register the samples in the laboratory database, including patient data and the date of collection. If necessary, perform initial processing, such as fractioning whole blood by centrifugation to separate plasma.
  - *Error sources*. Note any interferences, such as haemolysis, lipemia, or jaundice, that may negatively affect the results of the analysis. Decide whether the sample is acceptable to be used for further analyses.
  - *Storage*. For delayed analyses or long-term use, store samples under the appropriate conditions: room temperature, refrigerator or freezer.

II. ANALYTICAL STEP

This step involves the actual analysis of the biomarker (analyte) in the biological sample: The analysis can be either qualitative (identifies the presence of the analyte) or quantitative (measures the concentration of the analyte).

Important considerations:

- *Selection of Analytical Methods*: Multiple methods are usually available for analysing the same biomarker. The laboratory staff must carefully select the most suitable method based on both practicality and reliability.
  - *Practicality Characteristics*. These factors determine whether the method is feasible and can be implemented effectively in the laboratory. This includes equipment, workload, specimen handling, run size, personnel skill, cost per test, methods of standardization and quality control, space needs, safety precautions and procedures that are required for a specific method.
  - *Reliability characteristics*: These factors evaluate the analytical performance and overall reliability of the method. This includes precision, accuracy, analytical sensitivity, analytical specificity, recovery, interference, blank readings, sample interaction, reagent stability and linear range (Table 1, Table 2, Figure 1).

Table 1. Characteristics of analytical methods

Characteristic	Description
Analytical range	Is the range of concentration or other quantity in the specimen over which the method is applicable without modification. Should be wide enough to include most (95%) of the expected clinical specimens without dilution.
Analytical sensitivity	Is a measure of the ability of an analytical method to detect small quantities of the measured component. Sensitivity is related to precision, therefore: (1) when the concern is performance at concentrations above the detection limit, it is best to determine the precision at the concentration of interest, and (2) when the concern is performance at very low concentrations, then it is useful to determine the detection limit.

Detection limit	Is the smallest single result which, with stated probability (95%), can be distinguished from a suitable blank. The limit may be a concentration or an amount and defines the point at which the analysis becomes just feasible.
Blank readings	Are responses observed by the measurement procedure due to reagent and sample constituents, exclusive of the desired analyte.
Analytical specificity	Is the ability of an analytical method to determine solely the component it wants to measure.
Interference	Is the effect of a component, which does NOT by itself produce a reading, on the accuracy of measurement of another component relates to specificity.
Recovery	Is the ability of an analytical method to correctly measure pure analyte when added to the samples routinely analysed. It is related to accuracy, and it tests whether the analytical method can measure the desired substance in the presence of all of the other materials appearing in the matrix of the real specimen.
Precision	Is the closeness of laboratory results to one another.
Accuracy	Is the closeness of a laboratory result to the true value.

low accuracy due to low precision | low accuracy due to low trueness | high accuracy due to high precision and trueness

Figure 1. Precision and accuracy

Table 2. Differences between precision and accuracy

	Precision	*Accuracy*
Represents	the closeness of laboratory results to one another	the closeness of a laboratory result to the true value
Refers to the	CONSISTENCY of the results	CORRECTNESS of the results
Measures	Statistical variability	Statistical bias
Degree of	Reproducibility	Conformity
Determined by	performing a replication experiment in which the same sample material is analysed several times (10-20 times) and is expressed as	comparing to standard or calibrator (substance of known composition)
Represented as	standard deviation (SD) and coefficient of variation (CV)	Bias (displacement)
Dependency	independent of accuracy (precise values may or may not be accurate)	dependent on precision (accurate values must be precise in most cases)
Origin	Random errors	Systematic errors

Quality control:

- Minimization of the Risk of Errors (for each determination):
  - *Proper Handling of Reagents*. Use reagents that are at the correct concentration, stored under appropriate conditions (e.g., temperature, light exposure), free from contamination, within their expiration dates.
  - *Suitable Equipment and Space*. Ensure the laboratory environment is clean and equipped with necessary tools, including protective gear like lab coats and gloves, dedicated, contamination-free workspaces.
  - *Functional Instruments*. Regularly check and maintain instruments to ensure calibration of machines for accurate measurements, proper functioning of pipettes and other tools, cleanliness of vessels and containers used in analysis.
  - *Trained Personnel*. All laboratory staff should be adequately trained in the chosen analytical methods and follow standard operating procedures (SOPs).
- Regular Monitoring of Precision and Accuracy (on daily basis):
  - *Measure of Standards and Control Products*: Employ standards or control materials (e.g., serum, plasma, blood, microbial strains). Test standards at various concentrations, below, above, and within the reference interval (normal range). Conduct 10-20 individual tests of a standard at the same concentration.
  - *Evaluate Results*: Compare individual test values to the actual concentration of the standard. Determine statistical parameters for precision, such as mean, standard deviation, and coefficient of variation (CV).

III. POSTANALYTICAL STEP

The postanalytical phase covers the period between completing the testing and interpreting the results. The primary output of this phase is the creation of an analysis bulletin, which serves as the official laboratory report.

Important considerations:

- The analysis bulletin must include:
  - *Patient's Data*: Full name, ID, or any other unique identifier. Age and sex (for proper interpretation of results).
  - *Laboratory Information*: Laboratory's name and contact information. Identification of the person who performed the analysis.
  - *Testing Details*: The analytical method and the instrument used.
  - *Results*: The measured values of each marker or analyte.
  - *Reference Interval (Normal Range)*: The expected range for each marker, considering factors such as sex and age.

Quality control:

- *Reference Interval Validation*: Confirm that the stated reference intervals are appropriate, considering variations due to sex, age, pregnancy, etc.
- *Use of Correct Units of Measurement*: Ensure that each marker is reported with its appropriate unit of measurement (e.g., mg/dL, mmol/L). Consistency in units is crucial to avoid misinterpretation by healthcare providers.

STEPS IN EVALUATING AN ANALYTICAL METHOD

1. Obtain the reagents, kit, instruments, and all necessary materials and supplies. Test the method on standards and biological samples for several days to become familiar with the procedure. The samples must contain different quantities of the analyte along with possible interfering substances (bilirubin, haemolysis, high turbidity). Any frozen specimens must be thawed and mixed completely before use.

2. Determine the within-run precision by analysing 10-20 aliquots of a control material or a single clinical specimen. Alternatively, perform duplicate analyses on a variety of clinical specimens. Select control materials that have concentrations corresponding to the different decision-level concentrations of interest. Each set of determinations will have different values because of the errors produced during the procedure. To determine the precision, we must calculate the mean, the standard deviation (SD) and the coefficient of variation (CV) for each data set.

Mean	*Standard deviation (SD or S)*	*Coefficient of variation (CV)*
$\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n}$ X_i-individual values; n-number of values; Σ-summation of the individual values	$S = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \overline{X})^2}{n-1}}$ n-1 represents the degrees of freedom (df)	$CV = \frac{S}{\overline{X}} \cdot 100$
is a measure of the average of the values	is the measure of dispersion (variation) of the individual values from the mean	is another useful way to express the variability of results
is calculated by adding the values together and dividing by the total number of values	is calculated as the square root of the variance	is a mathematical expression to cite the SD as a percentage of the mean

It has been experimentally determined that the distribution of the values follows a Gaussian (bell) curve. Since S is a measure of the spread of test values, it is inversely proportional to the precision of the results. The greater the S is, the greater the difference between individual results and the less the precision of the analytical method is. The acceptable limits used by most laboratories is plus or minus 2S (95,5%). Usually, minimal acceptable performance of a nonenzymatic method is 5% CV, while electrolytes have a minimal level of acceptable performance of 3% CV, and enzymatic methods 10%. When S and CV increase significantly from the cumulative values, problems due to random error should be reviewed.

The concept of total error refers to all types of errors that produce deviations from the true value of the compound to be determined. There are two main types of errors:

Systematic errors	Random errors
- result in the average value's being different from the true value - might be due to incorrect calibration on the instrument, incorrect assay temperature, or bad reagents - affect the accuracy of the results	- result in a scatter of values about the true value; the values are randomly different from the true value - might be due to personnel errors (pipetting, measuring) - affect the precision of the results

3. Determine the analytical range by analysing a set of standards or a series of dilutions of either control materials or an elevated specimen pool. Plot the observed values versus the concentrations of the samples or versus the relative dilutions for the series of samples and estimate the analytical range.

4. Determine recovery by adding known amounts of the analyte (C) in concentrated form to actual clinical specimens to prepare a new test sample (T). Add the same volume of solvent without analyte to provide a new baseline sample (M).

$$\overline{R} = \frac{T - M}{C} \cdot 100$$

5. Test the interference of common substances such as bilirubin, haemoglobin, lipids, anticoagulants, and common drugs, and of specific substances known to cause problems in the determination of the particular analyte being measured. Interference can be tested by an experiment analogous to the recovery experiment, except that the material added is the suspected interfering substance rather than the analyte to be determined. The results can be expressed in terms of the difference in the concentration between the sample containing the interferent and the baseline sample.

$$\text{Interference} = \overline{y} - \overline{x}$$

$\overline{y}$ - Mean of results with interferent

$\overline{x}$ - Mean of results without interferent

6. Compare the results by the test method with those obtained by a reference analytical method. Specimens from 40-200 patients should be analysed. The comparison with a reference analytical method actually measures the systematic error $|x-\mu|$ of the method regarding the true value. The data obtained can be processed by one of these methods:

a) correlation coefficient
b) linear regression or least squares analysis
c) t-test (student)

a) Correlation coefficient

The correlation coefficient may be helpful in assessing the reliability of the linear regression estimates of slope and y-intercept. It can be calculated from the equation:

Ideally, the value for r should be 1.00. Values less than 1.00 are due to random errors within or between the methods, while systematic errors have no effect on r. For values higher than 0.95, the estimates of slope and y-intercept are not very much affected by variability in the comparative method.

b) Linear regression method

To describe the plot of test values versus reference values from the comparison-of-methods experiment, the best straight line through the data can be determined from the linear regression or least squares analysis.

A straight line is given by an equation of the form (the regression line describing how estimates of the test method values (y) are related to reference method values (x)): $y = a + bx$

y intercept (a)	slope (b)	standard deviation about the regression line ($S_{x/y}$)
$a = \bar{y} - b\bar{x}$	$b = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2}$	$S_{y/x} = \sqrt{\frac{\sum (y_i - Y_i)^2}{n-2}}$

a = the y-intercept; b = the slope; y_i = the value observed for a sample by the test method; Y_i = the value calculated from the regression equation, given the value by the reference method (x_i); n-2 = degrees of freedom.

Ideally:

- slope = 1.00
- y-intercept = 0.0
- $S_{x/y} = 0.0$

Deviations from these ideal values are caused by different kinds of analytical errors:

- proportional errors cause changes in the slope
- constant errors cause changes in the y-intercept
- random errors cause $S_{x/y}$ to increase

Example:

The straight line calculated from the equation: $y = 1{,}2 + 0{,}982x$

- a constant error of 1.2 mg% (y-intercept)
- a proportional error of 1.8 mg% (1-0.982)
- standard deviation = 4.2 mg%, => is expected that 95% of the data points will fall within 8.4 mg% (±2S) of the line

To judge the acceptability of accuracy or systematic error, it is best to determine the magnitude of the error at critical medical decision levels. If the allowable error were 5.0 mg% at a decision level of 100 mg%, the systematic error should be calculated for a concentration of 100 mg%. Therefore, $Y_{100} = 1{,}2 + 0{,}982 \cdot 100 = 99{,}4$.

The difference between $Y_c - X_c = |99{,}4 - 100| = 0{,}6$ provides an estimate of the systematic error at the critical concentration. Because $0.6<5$ the method is acceptable.

In applying linear regression to data from a comparison-of-methods experiment, it is important to graph the data for visual inspection and to calculate the correlation coefficient r whose value has to be higher than 95.

c) t-test (student)

The null hypothesis being tested is that there is no systematic difference between the two analytical methods.

The result of a determination will be $x = x \pm S_x t_p$ where t_0 is the t (student) factor, a factor which multiplied by the standard deviation will determine a certain measurement to fall (with a probability P) in the interval stated by the equation $x = x \pm S_x t_p$.

The value of t can be calculated from the equation:

$$t = \frac{\left|\bar{x} - \bar{y}\right|\sqrt{n}}{\sqrt{\frac{\sum\left[(x_i - y_i) - (\bar{x} - \bar{y})\right]^2}{n-1}}},$$

Generally, P=95%. If the value of t calculated for n-1 degrees of freedom corresponds or it is lower than the corresponding value of P=95% from tables, then the null hypothesis is verified. If the value of t is higher, then between the two methods there are significant differences, the tested method being inexact in comparison with the reference method.

In conclusion, the precision of the method measures the random errors resulted by repeated determination of the same sample, while the accuracy of the method measures the systematic errors by recovery experiments, linearity and comparison of the tested analytical method.

EXPERIMENTAL WORK

Evaluation of the precision and accuracy of manual determination of glucose using the glucose oxidase method

$$\alpha - D - glucose \xrightarrow{glucomutase} \beta - D - glucose$$

$$\beta - D - glucose + H_2O + O_2 \xrightarrow{glucose\ oxydase} D - gluconolactone + H_2O_2$$

$$2H_2O_2 + phenol + 4 - aminoantipyrine \xrightarrow{peroxydase} quinoneimine\ dye\ (red) + 4H_2O$$

Reagents

- Working reagent: phosphate buffer 0.5 mol/l (pH 7.50), phenol 7.5 mmol/l, glucose oxidase 12000 U/l, peroxidase 660 U/l, 4-aminoantipyrine 0.4 mmol/l. The reagent is stable 3 months at 2-8^{0}C or 3 weeks at 20-25^{0}C.
- Glucose standard, 100 mg%

Procedure

- Pipette into 3 test tubes as follows:

Reagents, µl	**Sample**	**Standard**	**Blank**
Working reagent	1000	1000	1000
Diluted serum	100	-	-
Diluted standard	-	100	-
Distilled water	-	-	100

- Incubate the tubes at room temperature for 30 minutes.
- Read the absorbance of sample and standard at λ=546 nm, zeroing the spectrophotometer against the blank. The colour is stable for 60 minutes. The response is linear up to a glucose concentration of 400 mg%.

Calculation

- Calculate serum glucose concentration using the formula:

$$C_{sample}\ (mg/100mL) = \frac{A_{sample}}{A_{standard}} \times C_{standard} = \frac{A_{sample}}{A_{standard}} \times 100$$

- Determine the precision by calculating the mean, standard deviation and coefficient of variation. According to www.westgard.com/biodatabase1.htm, optimum CV ≤ 2,8% for serum glucose determination
- Determine the accuracy based on the bias (displacement). The bias (D) represents the difference between the average value of the control data string and the real value of the glucose concentration in the serum (the real value in our case is 100 mg/dL). The bias is calculated according to the formula (according to www.westgard.com/biodatabase1.htm, optimum D ≤ 2,34% for serum glucose determination):

$D\% = \frac{x-a}{a} \times 100$, where x – mean of the control data string; a – the actual value

Supplementary experimental work

We determine two series of 10 serum glucose levels (in the same serum) obtaining 10 values. These values will be used for the determination of the precision ($\bar{x}$, S and CV), considering a value of CV<5% as acceptable. The accuracy of the method will be calculated using the t test.

Det. No.	I			II		
Glycemia	X_I	$X_I - \bar{x}_I$	$(X_I - \bar{x}_I)^2$	X_{II}	$X_{II} - \bar{x}_{II}$	$(X_{II} - \bar{x}_{II})^2$
1	96	-2	4	98	-1	1
2	102	4	16	101	2	4
3	95	-3	9	99	0	0
4	104	6	36	102	3	9
5	99	1	1	98	-1	1
6	95	-3	9	99	0	0
7	99	1	1	97	-2	4
8	98	0	0	101	2	4
9	96	-2	4	98	-1	1
10	96	-2	4	97	-2	4
Total	980		84	990		28

1. Precision calculation.

Statistical parameter	Formula	Method I	Method II
Mean	$\bar{x}_i = \frac{\sum_{i=1}^{n} x_i}{n}$	$\bar{x}_I = \frac{980}{10} = 98$	$\bar{x}_{II} = \frac{990}{10} = 99$
Standard deviation	$S = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$	$S_I = \sqrt{\frac{84}{9}} = 3,05$	$S_{II} = \sqrt{\frac{28}{9}} = 1,76$
Coefficient of variation	$CV = \frac{S}{\bar{x}_i} \cdot 100$	$CV_I = \frac{3,05 \cdot 100}{98} = 3,11$	$CV_{II} = \frac{1,76 \cdot 100}{99} = 1,78$

Discussion:

- both series of determination have a CV < 5 % => good precision
- the distribution of the values x ± 2S is:
  - **I.** 98 ± 2 · 3,05 = 98 ± 6,1;
  - **II.** 99 ± 2 · 1,76 = 99 ± 3,52.

2. Accuracy calculation.

We compare the accuracy of the method I in comparison with the reference method II. Assume as the null hypothesis that the means of the two data sets are the same. Calculate t and compare it to a tabulated t-value at the desired confidence level and $n_1 + n_2$ -2 degrees of freedom. If the calculated t is larger than the table t value, reject the null hypothesis.

$n_1 = n_2 = 10$ $\quad \overline{x_I} = 98$ $\quad \overline{x_{II}} = 99$ $\quad S_1 = 3,05 \quad S_2 = 1,76$

$$t = \frac{|\overline{x_1} - \overline{x_2}|}{S}\sqrt{\frac{n_1 n_2}{n_1 + n_2}} \qquad S = \sqrt{\frac{S_1^2(n_1 - 1) + S_2^2(n_2 - 1)}{n_1 + n_2 - 2}}$$

$$S = \sqrt{\frac{3,05^2 \cdot 9 + 1,76^2 \cdot 9}{10 + 10 - 2}} = 2,49 \qquad t = \frac{|98 - 99|}{2,49}\sqrt{\frac{100}{10 + 10}} = 0,89$$

We look in the table for P=95% and degrees of freedom $n_1 + n_2$ - 2 = 10 + 10 -2 = 18, obtaining a value for t=1.73. The calculated value of t being 0.89 and because 0.89<1.73 we can conclude that there are no significant differences between the two methods, and the tested method is comparable with the reference method.

To evaluate the qualitative performances of the analytical systems using the linear regression method and the coefficient of variation, we consider that between two variables there is a correlation stated by a regression equation. The correlation intensity between the variables is measured by the correlation coefficient.

Sample Nr.	-	1	2	3	4	5	6	7	8	9
Glycemia value Tested method	y	12	17	31	42	48	63	69	77	92
Glycemia value Reference method	x	10	20	30	40	50	60	70	80	90

3. Correlation coefficient calculation.

$$\mathbf{r = \frac{n\sum xy - \sum x \sum y}{\sqrt{\left[n\sum x^2 - (\sum x)^2\right] \cdot \left[n\sum y^2 - (\sum y)^2\right]}}}$$

$$\mathbf{r = \frac{9 \cdot 28520 - 451 \cdot 450}{\sqrt{(9 \cdot 28500 - 202500)(9 \cdot 28500 - 203401)}} = \frac{53730}{53931,956} = 0,996}$$

$r > 0,95 \Rightarrow$ very good value

4. Regression line calculation.

$$y = a + bx \qquad a = \overline{y} - b\overline{x} \qquad b = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2}$$

$y = 0,36 + 0,995x.$

For error calculation at critical diagnosis value:

- x = 100 mg %
- y = 99,86 mg %
- the error x – y = 100 - 99,86 = 0,14 mg %.

Lab Notes

Use this space for handwritten notes during the practical session.

L2. PCR. ELECTROPHORESIS. SARS-CoV-2 TESTING

LEARNING OBJECTIVES

Describe the structural components and procedural steps of the polymerase chain reaction (PCR).

Distinguish between endpoint PCR, quantitative real-time PCR (qPCR), RT-PCR, and RT-qPCR.

Identify the direct and indirect laboratory methods used for SARS-CoV-2 detection.

Explain the principle of agarose gel electrophoresis and interpret electrophoretic band patterns.

Prepare and run an agarose gel for the visualisation of DNA fragments.

KEY POINTS

PCR amplifies specific DNA sequences through repeated cycles of denaturation (95°C), primer annealing (50–65°C), and extension (72°C).

qPCR allows real-time monitoring of amplification using fluorescent dyes (SYBR Green) or sequence-specific probes (TaqMan), enabling quantification without gel electrophoresis.

RT-PCR and RT-qPCR first convert RNA into cDNA, enabling amplification and quantification of RNA targets, including viral genomes such as SARS-CoV-2.

SARS-CoV-2 is diagnosed by RT-qPCR (gold standard for active infection); rapid antigen tests detect active infection; antibody tests indicate past infection.

Agarose gel electrophoresis separates DNA fragments by size; smaller fragments migrate faster toward the positive electrode.

GENERAL ASPECTS

- SARS-CoV-2 is a single-stranded positive-sense RNA virus belonging to the Family of *Coronaviridae*
- SARS-CoV-2 is the causative agent of the respiratory illness COVID-19 (Coronavirus Disease 2019)
- The main structural proteins of the virus are spike (S), membrane (M), envelope (E), nucleocapsid (N), hemagglutinin-esterase (HE) proteins

SARS-CoV-2 TESTING

The following specimens are relevant for biological testing of SARS-CoV-2:

- Upper respiratory specimens: nasopharyngeal swab, oropharyngeal (throat) swab
- Lower respiratory specimens: sputum, tracheal aspirate/bronchoalveolar lavage
- Whole blood, serum and plasma

The methods used for SARS-CoV-2 testing are:

- Direct methods:
  - detect the presence of SARS-CoV-2 structures (nucleic acid/proteins) in the biological sample (generally respiratory specimens)
  - the gold standard for detecting the presence of SARS-CoV-2 RNA is the PCR technique (molecular tests)
  - the presence of SARS-CoV-2 antigens (proteins) can be detected with lateral flow devices, either by healthcare providers or as point-of-care tests (rapid tests)
  - these methods check for active infections
- Indirect methods:
  - include the detection of antibodies (IgM, IgG) anti-SARS-CoV-2, representing the patient's humoral immune response to the virus
  - are generally performed on blood samples obtained by fingerstick (serological tests)
  - can be either *point-of-care (POC)* tests with lateral flow devices or *laboratory tests such as ELISA* (enzyme-linked immunosorbent assay) or *CIA* (chemiluminescent immunoassay)
  - these methods check for past infections

POLYMERASE CHAIN REACTION

In 1984, an American biochemist, Kary Mullis discovered a method called the polymerase chain reaction (PCR) for amplifying specific DNA sequences. This technique has revolutionized the world of molecular biology. The concept was straightforward: reproducing in a laboratory test tube the DNA replication process that takes place in cells, the outcome being the same: the formation of new complementary DNA (cDNA) strands starting from the existing ones.

PCR Components

- Template DNA (PCR template, DNA to be amplified)
  - The DNA can be genomic DNA (gDNA), cDNA, and plasmid DNA.
  - The template DNA contains the target sequence (enclosed by 2 flanking sequences), representing the sequence to be amplified.

- DNA polymerase
  - All PCR reactions need a DNA polymerase that is active at high temperatures. Taq polymerase is one of the most used enzymes, working optimally at 70 °C - 72 °C. Taq polymerase was isolated from the thermophilic bacterium *Thermus aquaticus* found in hot springs. It is suitable for standard PCR without special requirements.
  - In order to improve reaction performance, new generations of polymerases are being engineered. For example, some enzymes are activated only at high temperatures to reduce non-specific amplification at the beginning of the reaction.
  - The DNA polymerase synthetizes the new strand in the direction 5' to 3'.

- Primers
  - The primers are short single-stranded DNA (approximately 15-30 bases) complementary to the flanking sequences of the target DNA.
  - In order to initiate amplification, DNA polymerases require a short sequence of nucleotides (the primers) to indicate the starting point of the reaction.
  - Primer sequences must be carefully chosen so that it targets only the DNA of interest, avoiding the possibility of binding to another sequence.
  - The primers attach to the 3' end of the parent strand.

- Deoxynucleotide triphosphates (dNTPs)
  - dNTPs act as the building blocks required to synthesize the new DNA strands and include the four basic DNA nucleotides: dATP, dTTP, dCTP, and dGTP. For optimal base incorporation, dNTPs are usually added to the PCR reaction in equimolar amounts.

- PCR buffer
  - the buffer ensures that optimal conditions are kept throughout the PCR reaction. The major components of PCR buffers include magnesium chloride (used as cofactor for the DNA polymerase), tris-HCl and potassium chloride (maintain a stable pH during the reaction).

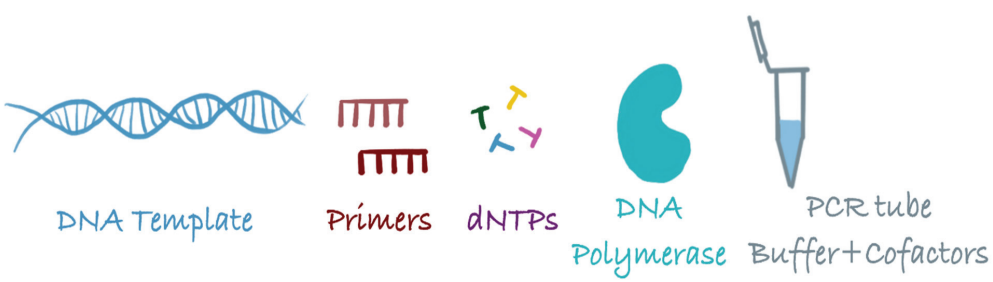


Figure 1. PCR Components

PCR Steps

The PCR amplification cycle consists of three steps: denaturation (strand separation), annealing (hybridization of the primers), and extension (DNA synthesis).

- Denaturation/Strand separation
  - heating the solution up to 95°C for a few seconds (approximatively 15s), separating the two strands of the parent DNA as the hydrogen bonds are broken.

- Annealing/Hybridization of primers
  - the solution is then abruptly cooled to 54°C allowing each primer to hybridize to a DNA strand. Annealing temperatures are usually 50 - 65°C. However, the optimal temperature depends on the primers' sequence and length.
  - one primer hybridizes to the 3' end of the target on one strand, and the other primer hybridizes to the 3' end on the complementary target strand.
  - parent DNA duplexes could rejoin at this temperature, but do not, because the primers are present in large excess.

- Extension/DNA synthesis
  - the solution is then heated to 72°C (74 °C), the ideal working temperature for heat-stable polymerases.
  - The polymerase elongates both primers, synthesizing new strands of DNA, in the direction of the target sequence because DNA synthesis takes place in the 5'-to-3' direction.
  - DNA synthesis occurs on both strands but extends beyond the target sequence.

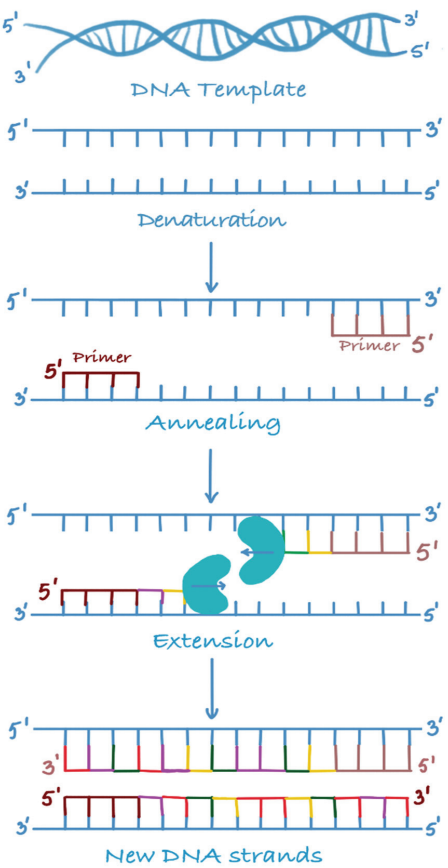


Figure 2. PCR Steps

These three steps - denaturation, annealing, extension - constitute one cycle of the PCR amplification and can be carried out repetitively just by changing the temperature of the reaction mixture. The polymerase's thermostability makes it practical to carry out PCR in a closed container, without adding reagents after the first cycle. Four duplexes containing the target sequence were generated after the completion of the second cycle. Subsequent cycles will amplify the target sequence exponentially. Ideally, after n cycles, the desired sequence is amplified 2^n-fold.

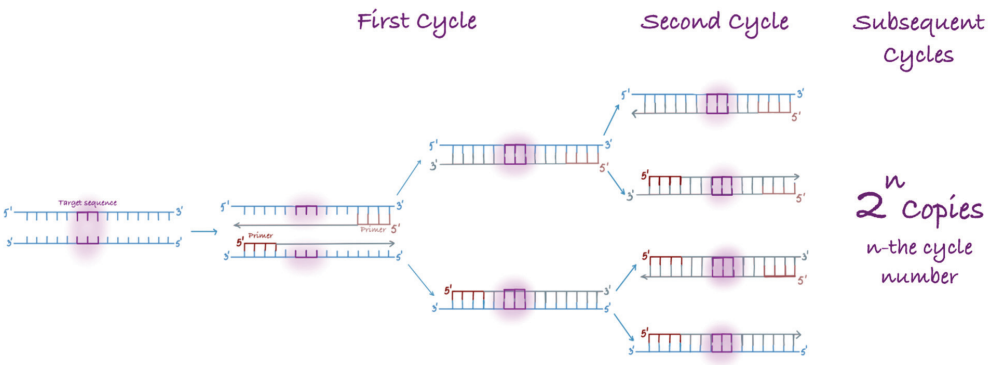


Figure 3. PCR cycles

Characteristics of PCR method:

- the sequence of the target doesn't need to be known; but, it is required the knowledge of the flanking sequences so that complementary primers can be designed.
- the target can be much larger than the primers
- primers do not have to be perfectly matched to flanking sequences to amplify targets.
- the method is highly specific because of the stringency of hybridization at relatively high temperature. Stringency is the required closeness of the match between primer and target.
- PCR is very sensitive; a single DNA molecule can be amplified and detected.

To determine whether amplification has been successful, PCR products may be visualized using gel electrophoresis, which can indicate the presence/absence of the amplicon, its size and approximate abundance. Depending on the purpose, this may be the endpoint (determining whether a gene is present or not), or the PCR product may be the starting point for more complex downstream investigations such as sequencing.

Types of PCR

- Endpoint PCR
  - In general, any method based on Endpoint PCR will require an extra step for physical analysis. Well known second steps of that kind are gel electrophoresis, or DNA sequencing or DNA microarrays.
- Quantitative real-time PCR (qPCR)
  - qPCR is a quantitative technique that allows real-time monitoring of the amplification process and detection of PCR products
  - It can be used to determine the starting concentration of the target DNA, eliminating the need for gel electrophoresis in most of the cases.
  - This is achieved through the inclusion of one of the following:
    - non-specific fluorescent intercalating dyes, such as SYBR Green, that generates fluoresce when bound to double-stranded DNA.
    - sequence-specific fluorescent DNA oligonucleotide probes, such as TaqMan probes, that bind specifically to DNA target sequences within the amplicon. They generate fluorescence by the coupling of a fluorescent molecule on one end and a quencher at the other end; when polymerization occurs, the probe is cleaved and releases the fluorescent molecule (reporter) so that the signal is not absorbed anymore by the quencher.

- For both fluorescent dyes and probes, as the number of copies of the target DNA increases, the fluorescence level increases proportionally, allowing real-time quantification.
- qPCR uses specialized thermal cyclers containing fluorescent detection systems that monitor the fluorescent signal as the amplification takes place.

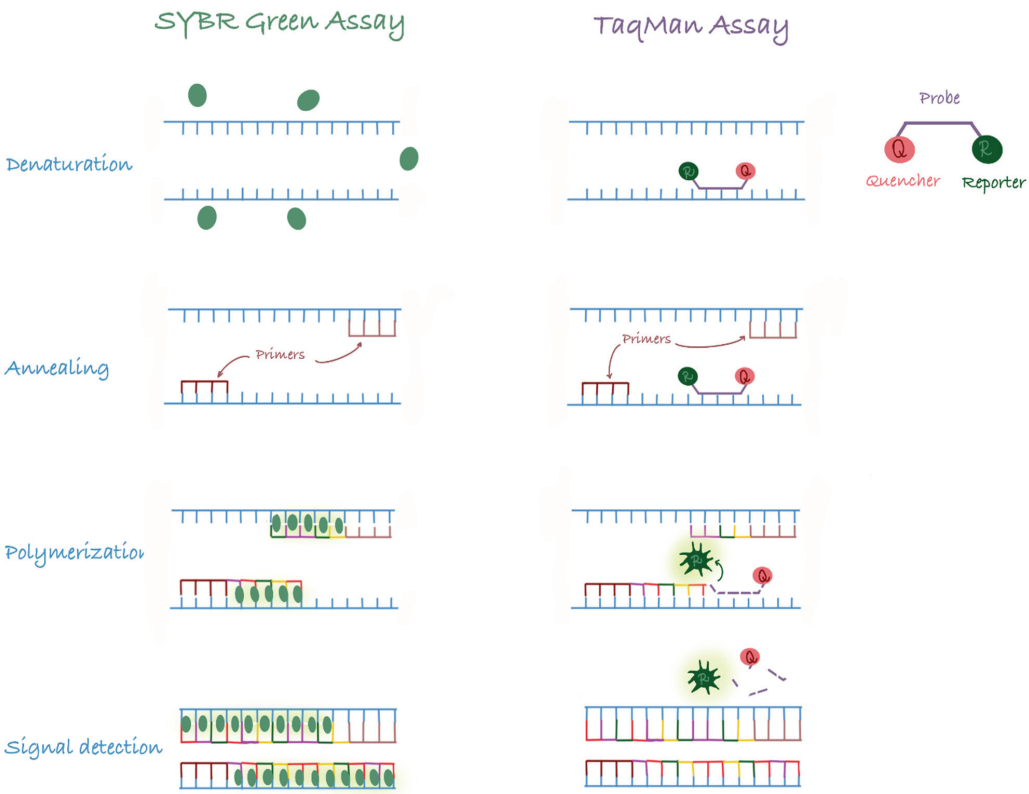


Figure 4. PCR – SYBR Green and TaqMan Fluorescent dyes

- Reverse transcription-PCR (RT-PCR)
  - Reverse transcription is the process of making complementary DNA (cDNA) from single-stranded template RNA
  - The first step of RT-PCR reaction is to synthesize a DNA/RNA hybrid between the RNA template and a DNA oligonucleotide primer.
  - The enzyme that catalyzes the reaction is called reverse transcriptase and has RNase activity that can degrade the RNA portion of the hybrid.
  - Consequently, a single-stranded DNA molecule is synthesized by the DNA polymerase activity of the reverse transcriptase.
  - For successful RT-PCR, high quality and purity starting RNA is essential.

- Reverse transcription-quantitative PCR (RT-qPCR)
  - This method is used for the quantification of RNA in biological samples (either RNA transcripts or derived from viral RNA genomes). The reverse transcription process described above can serve as the first step in qPCR.

- Two approaches can be used when performing RT-qPCR: one-step RT-qPCR and two-step RT-qPCR. In both cases, RNA is first reverse-transcribed into cDNA, which is used as the template for qPCR amplification. In the one step approach, the RT reaction and the PCR reaction occur in the same tube, while in the two-step method, the two reactions are performed separately and sequentially, as two separate experiments.
- Reverse transcription (RT)-PCR and RT-qPCR are two commonly used PCR variants enabling gene transcription analysis and quantification of viral RNA, both in clinical and research settings.

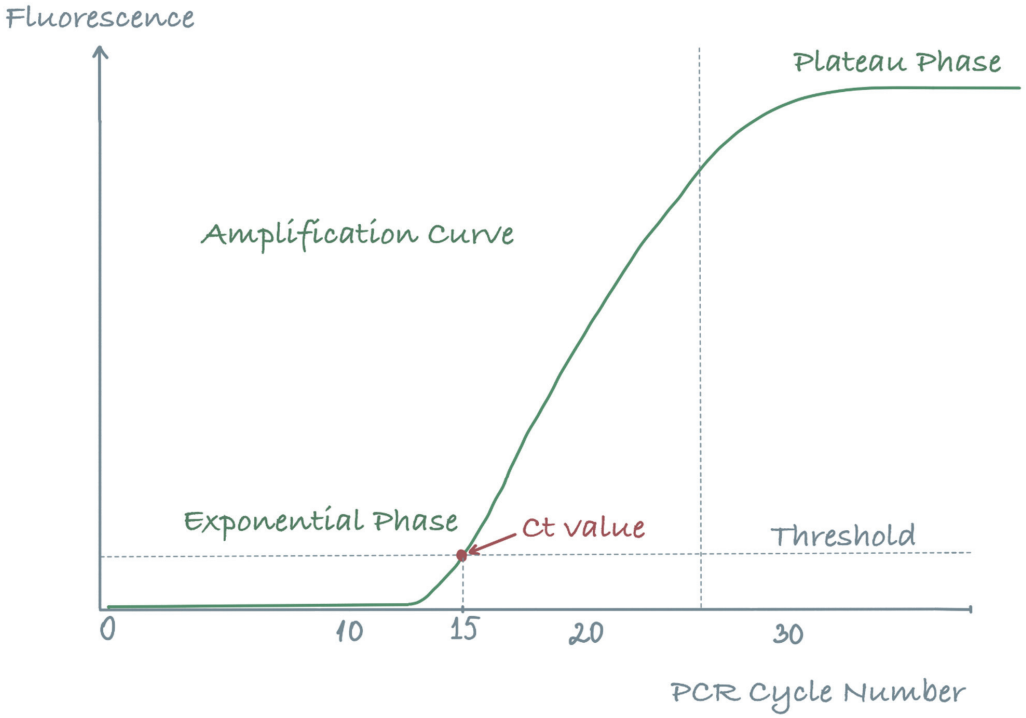


Figure 5. PCR -Amplification Curve

PCR Applications

- Medicine and biomedical research
  - diagnostic testing for disease-associated genetic mutations
  - identification of infectious agents (bacteria, viruses) with the use of specific primers
  - prenatal genetic testing; can identify chromosome abnormalities and genetic mutations in the fetus.
  - a promising method for the early detection of certain cancers; can also be highly informative in monitoring cancer chemotherapy

- Forensics and legal medicine
  - paternity testing
  - forensic investigations to pinpoint samples' sources (Small DNA samples isolated from a crime scene can be compared with a DNA database or with suspects' DNA).

- Environmental microbiology and food safety
  - pathogen detection, not only in patients' samples, but also in food or water, can be vital in diagnosing and preventing infectious disease.

- Genetic research
  - gene transcription analysis, aimed at evaluating the presence or abundance of particular gene transcripts
  - elucidate gene functions
  - In genotyping, PCR can be used to detect sequence variations in alleles in specific cells or organisms. Genotyping of transgenic organisms, such as knock-out and knock-in mice. Genotyping plants in agriculture assists plant breeders in selecting, refining, and improving their breeding stock.

- Sequencing
  - PCR can be used to enrich template DNA for sequencing
  - In Sanger sequencing, PCR-amplified fragments are then purified and run in a sequencing reaction
  - In next-generation sequencing (NGS), PCR is used at the library preparation stage, where DNA samples are enriched by PCR in order to increase the starting quantity of DNA.

ELECTROPHORESIS

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology.

When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Proteins and nucleic acids are separated within a matrix or "gel". Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value.

Electrophoresis gels

The gel itself is composed of either agarose or polyacrylamide, each of which has attributes suitable to particular tasks:

- *Agarose* is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "stiffer" the gel.
  Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques.

- *Polyacrylamide* is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%.
  The preparation of polyacrylamide gels is notably more complex than that of agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders).

⚠ Safety Note: Acrylamide is a potent neurotoxin and should be handled with care! Wear disposable gloves when handling solutions of acrylamide, and a mask when weighing out powder. Polyacrylamide is considered to be non-toxic, but polyacrylamide gels should also be handled with gloves due to the possible presence of free acrylamide.

Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.

Preparing and Running Standard Agarose DNA Gels

The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

- *Electrophoresis chamber* and *power supply*
- *Gel casting trays*, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- *Sample combs*, around which molten agarose is poured to form sample wells in the gel.
- *Electrophoresis buffer*, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- *Loading buffer*, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- ***⚠ Safety Note: Ethidium bromide***, a fluorescent dye used for staining nucleic acids. NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical - wear gloves while handling.
- ***⚠ Safety Note: Transilluminator*** (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration and then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 µg/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

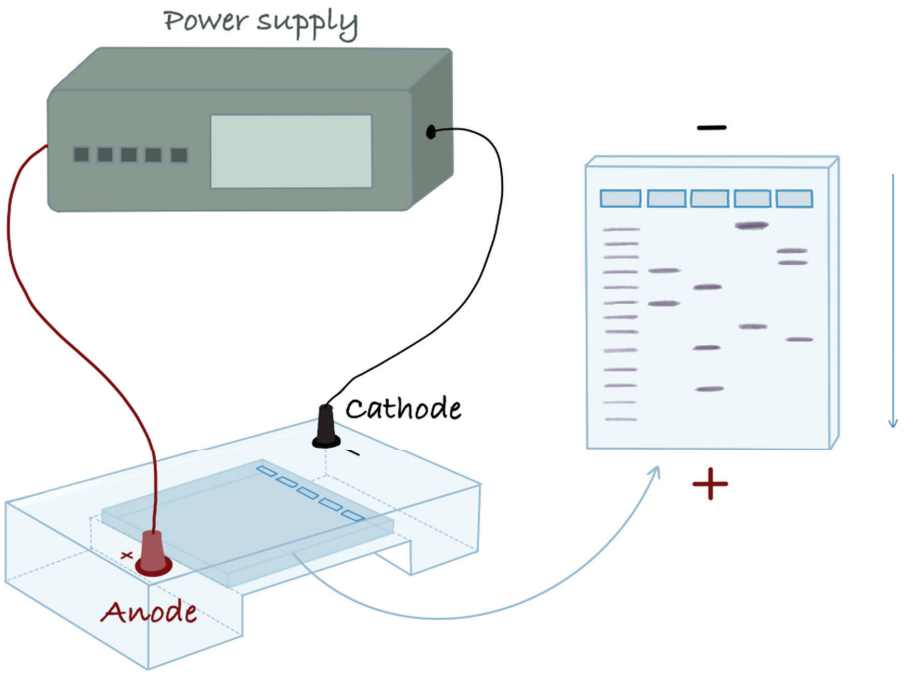


Figure 6. Gel electrophoresis

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weights or number of base pairs, a roughly straight line will appear.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

- *Voltage*: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster that small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).
- *Electrophoresis Buffer*: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

- *Agarose Concentration*: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.
- Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels. As described above, it can be incorporated into agarose gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

Other Considerations

Agarose gels, as discussed above provide the most commonly-used means of isolating and purifying fragments of DNA, which is a prerequisite for building any type of recombinant DNA molecule.

By varying buffer composition and running conditions, the utility of agarose gels can be extended. Examples include:

- Pulsed field electrophoresis is a technique in which the direction of current flow in the electrophoresis chamber is periodically altered. This allows fractionation of pieces of DNA ranging from 50,000 to 5 millon bp, which is much larger than can be resolved on standard gels.
- Alkaline agarose gels are prepared with and electrophoresed in buffers containing sodium hydroxide. Such alkaline conditions are useful for analyzing single-stranded DNA.

EXPERIMENTAL WORK

Agarose gel electrophoresis of DNA fragments

The principle of the method

The DNA fragments migrate differentially in electrical field through an agarose gel matrix, at alkaline pH, according with their size. The results of electrophoresis are different electrophoretic bands which can be visualized in UV light using ethidium bromide as fluorescent marker that is intercalated between the 2 strands of a double stranded DNA.

Steps

- *Agarose gel preparing*
  - Fix the gel tray
  - Weight 0,1 g of agarose in 50 mL beaker
  - Add 20 mL working buffer TAE
  - Warm up the agarose to boiling; to stop evaporation cover the beaker with a glass watch. Boil the mixture till the agarose is complete dissolved
  - When the dissolving is complete, the beaker is introduced in water bath at 60 ºC
  - Add 3 µL of ethidium bromide to the agarose gel and mix.
  - After several minutes, when the solution has reached 60 ºC, it is transferred in the fixed tray very carefully to not form bubbles in the gel
  - Fix the comb in the gel to create the wells for sample
  - Store the gel at the room temperature 20 min to polymerize
  - After 20 min remove the comb
  - Remove the gel tray from the support

- *Sample preparing*
  - 5 µL from DNA sample are mixed with l µL 6x loading solution in 1.5 mL Eppendorf tubes
  - To compare the size of the fragments, a DNA ladder is used. For ladder sample preparation mix 5 µL from DNA Marker are mixed with l µL 6x loading solution in 0.7 mls Eppendorf tubes

- *Sample Loading and Electrophoresis Running*
  - In electrophoresis chambers add 300 ml of 1x TAE buffer solution
  - The gel tray is fixed in the electrophoresis chamber with the wells orientated to the negative electrode (red electrode)
  - Load 10 µL of each sample to each well using a micropipette changing the tip every time after each sample
  - Close the lid of electrophoresis chamber
  - Connect the electrophoresis chamber to the electrical supply
  - The electrical supply is connected to the electrical network and then is fixed at 75V
  - Turn on the electrical supply
  - The migration process takes 20-30 min.
  - When the migration is finished (the brom-phenol blue is nearly at the end of the gel), turn off the power supply

- *UV visualization*
  - Remove the tray gel from the electrophoresis chamber and wipe it out with filter paper
  - Insert the gel tray under the protector screen of the UV transilluminator
  - Turn on the transilluminator
  - Compare the fragment size of PCR product with DNA Marker to confirm the size of the product and the success of PCR reaction.

Lab Notes

Use this space for handwritten notes during the practical session.

L3. BIOCHEMICAL BLOOD EXAM

LEARNING OBJECTIVES

Describe the composition and main components of blood (plasma and formed elements).

Differentiate between whole blood, serum, and plasma, and identify appropriate clinical uses for each.

Recognise the common preanalytical error sources (haemolysis, lipemia, icterus) and understand their effects on test results.

Explain the role of anticoagulants and the appropriate use of different blood collection tubes.

Perform and interpret a rapid whole-blood glucose determination.

KEY POINTS

Blood consists of a liquid phase (plasma, 50–60%) containing proteins, electrolytes, and metabolites, and a solid phase (erythrocytes, leucocytes, thrombocytes).

Serum is plasma without coagulation factors; plasma retains all coagulation factors due to the anticoagulant added during collection.

Haemolysis, lipemia, and icterus are the three principal preanalytical interferences that can falsify biochemical test results through spectral, chemical, or dilutional effects.

Anticoagulant choice is test-dependent: EDTA for haematology, citrate for coagulation studies, fluoride for glucose preservation.

Blood glucose (determined by the glucose oxidase method) is the central marker of carbohydrate metabolism and the most commonly requested biochemical test.

GENERAL ASPECTS

Blood connects all the cells in the body, and changes in its composition can indicate either a pathological or physiological condition. Blood makes up 6-8% of the total body weight, with a healthy adult having around 5 liters.

Blood is the primary biological sample tested in the laboratory. Biochemical blood examination is required for establishing the positive diagnosis, differential diagnosis as well as for the monitorization of treatment and disease evolution. There are several steps which are required for blood analyses.

BLOOD COMPOSITION

It is considered a liquid, heterogeneous tissue, consisting of two main components:

- Liquid phase: Represented by plasma, an aqueous solution of proteins, mineral salts, and buffered at a pH of 7.35-7.45. Plasma accounts for 50-60% of the blood volume. Plasma is a transparent, slightly yellowish liquid composed of:
  - water,
  - inorganic substances (such as sodium, potassium, calcium, magnesium, phosphate, chloride, zinc, iron, and bicarbonate),
  - nitrogenous organic substances (like proteins, urea, creatinine, bilirubin), and
  - non-nitrogenous organic substances (such as glucose, cholesterol, triglycerides, free fatty acids, and phospholipids).
- Solid phase: Represented by the formed elements, which are suspended in the liquid phase. The blood cells make up 40-45% of the blood volume and consist of red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes).

BLOOD COLLECTION

Depending on the collection site (parts of the circulatory system), the blood specimens can be either capillary, venous, or arterial. Each type of sample serves specific purposes in medical diagnostics, as they provide varying information about the body's health.

- *Capillary Blood:* Collected from capillaries of the fingertip (for adults) or heel (for infants). Usually obtained through a fingerstick method, even in point-of-care settings. This type of blood is typically used for rapid testing, like glucose measurements.
- *Arterial Blood:* Collected from an artery (often the radial artery) for tests that measure blood gases (ABG tests), such as oxygen and carbon dioxide levels, and pH.
- *Venous Blood:* Collected from a vein (often the cubital vein, but also from the jugular veins in children and from the fontanelles in infants), this is the most common type used for a wide range of tests, including plasma and serum-based tests.

Currently, the blood used in analyses is obtained in the fasting state. The level of the sodium, potassium, calcium, chloride, urea, creatinine, uric acid, total proteins, cholesterol, amylase, alkaline phosphatase, transaminase and cholinesterase remains unchanged in the blood even in the case of light breakfast, but usage of the blood after 8-10 hours fast is recommended.

The body position during the process of blood drawing could raise the value of total serum proteins from 6,6 g/dl in clinostatic position to 7,6 g/dl in orthostatic position.

BLOOD SPECIMENS

There are several types of blood specimens commonly collected for laboratory testing, depending on the type of test required. The main types include:

- *Whole Blood:*
  - Whole blood is collected without separating the plasma or cells.
  - Whole blood is used for different determinations only in the case when the concentration of those analyzed compounds is equally in erythrocytes and plasma (the glucose is an example).
  - It's typically used for tests like complete blood count (CBC), blood typing, and certain metabolic assays.
- *Serum:*
  - Serum is obtained from blood that has been allowed to clot, from blood drawn without an anticoagulant.
  - To obtain serum the blood sample can be centrifuged. The serum represents the supernatant phase, while the lower phase is the coagulum (clot).
  - The coagulum also forms if the blood stays for 30-60 minutes at room temperature or 15-20 minutes at 37ºC. The coagulum is a fibrin network containing blood figurative elements.
  - The serum does not contain the blood coagulation factors.
  - The serum is commonly used for biochemical, immunological, and serological tests.

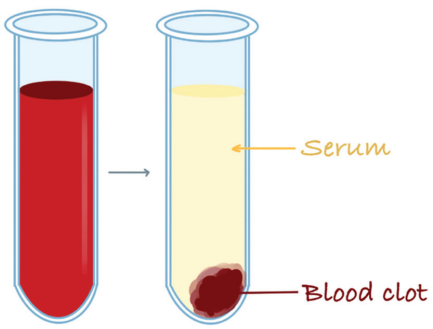


Figure 1. Separation of serum

- *Plasma:*
  - Plasma is obtained from blood drawn on anticoagulant (fluoride, oxalate, citrate, EDTA, heparin, etc.).
  - To obtain plasma the blood sample is centrifuged, with the figurative elements of the blood remaining as pellet on the bottom of the tube and the plasma being recovered as the supernatant.
  - In contrast to serum, plasma contains the coagulation factors.
  - The plasma is used for tests requiring the measurement of clotting factors, electrolytes, hormones, and certain proteins.

There is no difference between the concentrations of serum and plasma components, except some enzymes (acid phosphatase, LDH, aldolase) that are liberated from thrombocytes during the blood coagulation process.

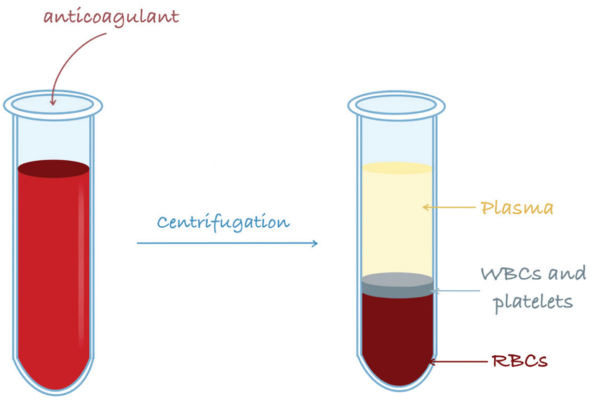


Figure 2. Separation of plasma

Biochemical determinations in plasma as well as for serum should be made in the range of 4 hours from blood drawing in the case of blood storage at room temperature and without separation of the red cells. If the red cells are separated, the serum and plasma could be stored at 4ºC all night. The determinations performed after 24 h require the preservation of the serum and plasma by freezing.

Anticoagulants are substances that added to the collected blood to stop the coagulation process. Anticoagulants work by binding calcium (EDTA, citrate, oxalate - form insoluble calcium salts) or interfering with other steps in the coagulation cascade (heparin - binds thrombin). Examples of coagulants are:

- EDTA, ethylenediaminetetraacetate (sodium or potassium salts) is the preferred anticoagulant for hematology procedures (purple vacutainer) because it does not distort cellular morphology or produce cellular artifacts. It is not recommended for calcium, electrolytes and alkaline phosphatase determinations.
- Oxalate (sodium, potassium or lithium salts) is not indicated in electrolytes determinations (sodium or potassium ions), calcium and enzymes determinations (LDH)
- Citrate (sodium or potassium salts) is used to prevent clotting for coagulation studies (blue vacutainer) or the determination of erythrocyte sedimentation rate (black vacutainer). It has the same disadvantages as oxalate.
- Heparin (sodium or lithium salts) is a polysaccharide that prevents blood clotting by inactivation of the blood clotting chemicals thrombin and thromboplastin (green vacutainer). It is the best anticoagulant for cytogenetic studies, special hematology tests (osmotic fragility and plasma hemoglobin). Since heparin is the physiological anticoagulant, it reserves best the physical and chemical properties of the blood. With ammonium heparin all metabolites, electrolytes and enzymes could be assessed except ammonium and acid phosphatase.
- Fluoride (sodium or potassium salts) is used in glucose determinations because it inhibits the glycolysis process being useful in the case when the glucose determinations are performed after more than 4 hours (grey vacutainer). Also, this anticoagulant is not recommended for electrolytes determination.
- ACD-solution contains citric acid, sodium citrate, and glucose in distilled water (yellow vacutainer). It is used for preservation of donated blood.

Error sources

The normal aspect of serum and plasma is pale-yellow and clear. Serum and plasma analysis should be avoided when the normal aspect is modified. There are three major modifications that can lead to interferences:

- *Hemolyzed serum or plasma (pink to red color)*
  - Hemolysis is usually an in vitro artefact due to poor venipuncture technique, freezing of whole blood samples, delayed separation of serum or plasma from cells, or delayed sample submission.
  - Hemolysis can also occur in vivo especially when the intracellular concentration of the analyzed compounds is higher comparative to their concentration in serum (LDH, ALAT, ASAT, potassium).
  - Interferences of hemolysis with biochemical tests are:
    - Increased absorbance: Released hemoglobin increases absorbance in the hemoglobin spectral range. This can interfere with the determination of bilirubin or cholesterol
    - Inhibition of reactions: Released hemoglobin can directly inhibit chemical reactions.
    - Analyte release: Release of analytes found in high concentrations in red blood cells will falsely elevate the values of these analytes.
    - Enzyme release: Release of enzymes which participate in chemical reactions, e.g. adenylate kinase
    - Water release: Release of red blood cell water dilutes analytes.
- *Icteric serum or plasma (orange-brown color)*
  - Icteric serum or plasma occurs when the blood highly concentrated in bilirubin, in jaundice
  - Interferences of hyperbilirubinemia with biochemical tests are:
    - Modified absorbance: The spectral properties of bilirubin could significantly interfere with the colorimetric determinations at 400-500 nm.
    - Chemical binding: Bilirubin has the ability to react with other reagents.
- *Lactescent serum or plasma (turbid/milky aspect)*
  - Lipemia (latescence) is caused by increased triglycerides (as chylomicrons or very low-density lipoproteins) concentration in the blood
  - Interferences of hemolysis with biochemical tests are:
    - Light scattering: Results in falsely increased absorbance readings of some analytes such as total bilirubin.
    - Volume displacement: This falsely decreases values of some analytes such as electrolytes.
    - Hemolysis: Lysis of erythrocytes is enhanced in the presence of lipemia.

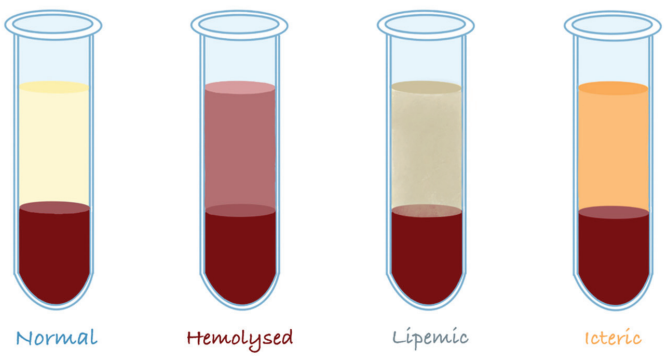


Figure 3. Serum/plasma collection errors

BIOCHEMICAL BLOOD EXAM

The biochemical examination of the blood aims to investigate the glucose, lipid and protein metabolisms as well as some systems and apparatus of the human body.

- *The biochemical examination of glucose metabolism:*
  - Glucose determination (the most frequently used)
  - Glucose tolerance test to find the cases with decreased glucose tolerance that appear prior diabetes mellitus installation.
  - Glycated hemoglobin (Hemoglobin A1c test) that gives information about the level of glucose from 6-8 weeks prior analysis.
- *The biochemical examination of lipid metabolism:*
  - Total cholesterol level: Desirable - Less than 200 mg/dL, Borderline high risk (200–239 mg/dL), High risk (240 mg/dL and over)
  - HDL and LDL-cholesterol. This determination is used in diagnosis of primary and secondary hyperlipoproteinemia.
  - Serum triglyceride determination. Unlike cholesterol levels which are constant, the triglyceride levels fluctuate considerably daily and are highest 1-4 hours after meals.
- *The biochemical examination of protein metabolism:*
  - Total protein determinations from serum (that permit investigation of organ functionality - liver, kidney)
  - Protein electrophoresis. Allows for analysis of ratio between the protein fractions (albumins, globulins, globulin fractions).
- *The biochemical examination of mineral metabolism (serum ions determinations):*
  - Serum cations: sodium, potassium, calcium, magnesium, iron
  - Serum anions: phosphate, chloride
- *The biochemical examination of acid-base equilibrium:*
  - Alkaline reserve
  - Blood pH
- *The biochemical examination of the digestive system:*
  - ALT = alanine transaminase (GPT = glutamate-pyruvate transaminase)
  - AST = aspartate transaminase (GOT = glutamate-oxaloacetate transaminase)
  - Gamma glutamyl transferase/transpeptidase (GGT)
  - Alkaline phosphatase (ALP)
  - Cholinesterase
  - Amylase
  - Lipase
- *The biochemical examination of the cardiovascular system:*
  - Creatine kinase or creatine phosphokinase (CPK, CPK-MB)
  - Lactate dehydrogenase (LDH)
  - Aspartate transaminase (AST)
- *The biochemical examination of the reno-urinary system:*
  - Urea
  - Creatinine
  - Endogenous creatinine clearance
  - Cystatin C
  - Uric acid *

EXPERIMENTAL WORK

Rapid test for determination of blood glucose level

Principle of the method

β-D-glucose is enzymatically oxidized by glucose oxidase in the presence of water and oxygen to guconolactone and hydrogen peroxide. The H_2O_2 resulted from that reaction is decomposed producing water and atomic oxygen that oxidize the o-tolidine to a blue-colored compound.

$$\alpha - D - glucose \xrightarrow{glucomutase} \beta - D - glucose$$

$$\beta - D - glucose + O_2 + H_2O \xrightarrow{glucoseoxidase} D - gluconolactone + H_2O_2$$

$$2H_2O_2 + o - toluidine \xrightarrow{peroxidase} blue\ compound + H_2O$$

Glycemia determinations

Glucose serves as a primary energy source for most cells in the body, particularly for the brain and red blood cells, which rely almost entirely on blood glucose for energy. The brain requires blood glucose levels to remain within a specific range for normal function; concentrations below 30 mg/dL or above 300 mg/dL can result in confusion or unconsciousness. Plasma glucose levels are typically 10–15% higher than whole-blood glucose levels, with an even greater difference post-meal.

Home glucose meters (rapid tests) measure whole-blood glucose, while laboratory tests generally assess plasma glucose. The rapid method for blood glucose determination is particularly useful for evaluating the effectiveness of diabetes treatments at home or in resource-limited medical settings where quick assessments are necessary. Monitoring one's blood glucose levels using a glucose meter is commonly known as self-monitoring of blood glucose, or **SMBG**.

Rapid tests utilize plastic strips with reagents immobilized on one end, enabling the required reactions. By pricking the finger with a sterile lancet and placing a drop of blood in the reagent zone on the strip, the reaction occurs. A portable photometer measures the absorbance of the resulting colored product, displaying the result in mg/dL. Plasma-calibrated meters convert whole-blood readings to plasma-equivalent values, aligning with the standard used by healthcare professionals.

Some glucose meters allow testing from alternative sites, such as the upper arm, forearm, thumb base, or thigh. While convenient, these alternative sites may produce results that differ from fingertip readings due to slower glucose level changes in those areas. This discrepancy is due to physiological differences rather than the meter's accuracy.

Oral Glucose Tolerance Tests

In Oral Glucose Tolerance Test (OGTT) blood glucose is measured two hours after the patient consumes 75 grams of glucose. A reading of 200 mg/dL or greater indicates diabetes.

Glycated Hemoglobin (HbA1c Test)

With this test, a 1% change in HbA1c corresponds to approximately a 30 mg/dL (1.67 mmol/L) shift in average blood glucose levels. For example, an HbA1c of 6% reflects an average glucose level of 135 mg/dL (7.5 mmol/L), while an HbA1c of 9% corresponds to an average glucose level of 240 mg/dL (13.5 mmol/L). Maintaining HbA1c close to 6% indicates good diabetes control and minimizes the risk of complications. Higher HbA1c levels are associated with increased complications.

CASE STUDY

1. Patient: **BC, sex: F, 70 years old**

The patient presents with the following symptoms: general fatigue, obesity, poor eyesight (poor vision), eczema, dysuria, pollakiuria. The doctor recommends performing the following biochemical analysis:

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	240 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.4 mg/dl	<0.2 mg/dl
HbA1c	9 %	4-6 %	**Total Bilirubin**	1.5 mg/dl	<1 mg/dl
ALAT	20 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	5.5 g/dl	6.2-8 g/dl
ASAT	19 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	13.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	55 mg/dl	15-45 mg/dl	**hsCRP**	6 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3 mg/l high CV risk
Creatinine	1.7 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.09 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	90 ml/min	95-150 ml/min	**Cystatin C**	1.30 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	22 mEq/l	22-27 mEq/l
Amylase	77 U/l	50-100 U/l	**Na+**	135 mEq/l	135-145 mEq/l
Total Cholesterol	255 mg/dl	140- 200 mg/dl	**K+**	5.4 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	10 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	300 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	209 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	5 U/l	2-10 U/l	**GGT**	22 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	180 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.030	1.015- 1.025
pH	9.0	6.5
Proteins	+(30mg/dl)	negative
Glucose	+(100mg/dl)	negative
Ketone bodies	+(15mg/dl)	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	+	negative
Leucocytes	+(25/µl)	negative
Red blood cells	negative	negative

Compare these values with the normal values and establish a possible diagnosis.

2. Patient: **CD, sex: M, 18 years old**

The patient presents with the following symptoms: fatigue, anorexia, nausea, vomiting, acetone breath, dehydration, weight loss, polyuria. The doctor recommends performing the analysis below. Compare these values with the normal values, and establish a possible diagnosis.

Biochemical examination of blood

Denomination	**Result**	**Reference range**	**Denomination**	**Result**	**Reference range**
Glycemia a jeun (fasting blood glucose)	400 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	10 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	25 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	7.0 g/dl	6.2-8 g/dl
ASAT	20 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	14.6 g/100ml	F: 12-16 g/dl M: 14-18 g/dl
Urea	50 mg/dl	15-45 mg/dl	**hsCRP**	5 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3 mg/l high CV risk
Creatinine	1.1 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.1 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	100 ml/min	95-150 ml/min	**Cystatin C**	0.89 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	18 mEq/l	22-27 mEq/l
Amylase	73 U/l	50-100 U/l	**Na+**	125 mEq/l	135-145 mEq/l
Total Cholesterol	150 mg/dl	140- 200 mg/dl	**K+**	7.9 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	40 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	100 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	222 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	8 U/l	2-10 U/l	**GGT**	19 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	188 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	**Result**	**Reference range**
Density	1.030	1.015- 1.025
pH	5.0	6.5
Proteins	negative	negative
Glucose	+++(1000mg/dl)	negative
Ketone bodies	++(40mg/dl)	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

Lab Notes

Use this space for handwritten notes during the practical session.

L4. BIOCHEMICAL URINE EXAM

LEARNING OBJECTIVES

Describe the composition and normal physical and chemical properties of urine.

Select the appropriate urine collection method for a given diagnostic purpose.

Perform and interpret macroscopic urinalysis, including assessment of colour, clarity, and specific gravity.

Identify and interpret the findings of a biochemical urine strip test (dipstick analysis).

Examine and interpret the microscopic appearance of urinary sediment elements.

KEY POINTS

Normal urine consists of approximately 95% water and hydro-soluble compounds: urea, uric acid, creatinine, and electrolytes. Composition varies with diet, hydration, and renal function.

Urine collection method (random, first-morning void, 24-hour, catheter) must be matched to the specific analytical requirement and properly documented.

Macroscopic examination assesses colour, transparency, odour, and specific gravity (normal range: 1.005–1.030).

Biochemical dipstick testing provides rapid, semi-quantitative results for proteins, glucose, ketones, blood, bilirubin, urobilinogen, leucocyte esterase, and nitrites.

Microscopic sediment examination identifies cellular elements (erythrocytes, leucocytes, epithelial cells), casts, and crystals, and guides clinical interpretation.

GENERAL ASPECTS:

Urine is a biological fluid, a liquid by-product of metabolism

The production of urine involves a process consisting of 3 steps (Figure 1):

- glomerular filtration
- reabsorption
- secretion

After production, urine is excreted from the body in a process known as urination (micturition), where urine travels in the following direction: kidneys → ureters → urinary bladder → urethra

Through urination, some rich in nitrogen substances are cleared from the bloodstream (amino acids catabolism generates ammonium ion NH_4+, a neurotoxic product, which needs to be converted into urea before excretion)

Urine plays a role in the earth's nitrogen cycle (soil fertilizer and growth-promoter in plants)

URINE COMPOSITION

In physiological state urine has a specific composition, and physical and chemical properties

The composition depends on several factors such as: alimentation, intermediary metabolism, functionality of different organs (kidney, liver) and medication

Urine consists of water (around 95%) and hydro-soluble compounds:

- Organic compounds: urea, uric acid, creatinine, urobilinogen, amino acids, and other compounds conjugated with sulfuric acid, glucuronic acid, and glycine
- Inorganic compounds: cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+), anions (Cl^-, SO_4^{2-}, HPO_4^{2})

URINE COLLECTION

In most cases, urine is collected by the patient upon micturition. Occasionally, urine can be obtained by the doctor through catheterization or suprapubic aspiration. The conditions of urine collection are different, depending on the analyte and the required test (Table 1).

Important considerations:

- 50 mL of urine is sufficient for all the analyses
- Urine specimens must be collected in clean and covered containers
- For bacteriological exams the containers should be sterile (the specimens are to be cultured)
- The containers should be brown or wrapped in aluminum foil if the desired analytes are susceptible to deteriorate from exposure to light (bilirubin, porphyrins)
- The urine specimens must bear the proper patient identification and the day and time(s) of collection to ensure that a proper result will be issued

Table 1. Urine collection methods

Urine type	*Collection time*	*Tests required*	*Non-recommended tests*
"Clean catch" (midstream) urine sample	1st urine collected in the morning	- bacteriological exam - short tests using reagent strips - test to evidence the nitrites existence in the urine collected in sterile recipients - macroscopic exam of urine sediment - semiquantitative urinary proteins - others biochemical qualitative determinations	- quantitative determination of all biochemical compounds (that is not indicated for all situations when the spontaneous urine is used)
	2nd urine collected in the morning	- urine exams using reagent strips - semiquantitative urinary proteins and glucose determination	- test to evidence the nitrites existence in the urine
	Random urine	- semiquantitative urinary glucose postprandial determination	- bacteriological exam - microscopic exam of urine sediment
Suprapubic aspiration	-	- bacteriological exam	- quantitative analysis of all biochemical components
Urine collected during a specific period of time	Usually for 24 h (or 12 h for some determinations)	- quantitative determination of all biochemical components - Addis-Hamburger test (Addi's no.)*	- bacteriological exam - microscopic exam of urine sediment

*Quantitative estimation of urinary cellular excretion. Method for counting the sediment (casts and cells) in a 12-hour (15: 24 hours) urine sample

URINE SPECIMENS

- *The first urine in the morning specimen*:

Is used for qualitative analyses (proteins, glucose, bilirubin, urobilinogen, ketone bodies)
Is the least influenced by alimentation and physical effort
Is more concentrated than the urine collected during the day, which means that some trace components can be better detected
Is not recommended for patients receiving intensive diuretics treatment (massive dilution)
Instructions to be followed:

- To collect uncontaminated specimens, hand-washing and local hygiene are crucial
- Collect the specimen immediately on rising from a night's sleep (other 8-hour periods may also be used to accommodate a patient's life-style)
- Collect from the midstream, as the first part of the urinary jet might be contaminated with cells or bacteria from the urethra or genitals (however, first jet might be required for diagnosing some urethral disorders)

- *24 hours urine specimen*:

Is used for quantitative analyses (total urinary proteins, carbohydrates, electrolytes)
Instructions to be followed:

- The procedure starts at 8 o'clock in the morning, but the first urine collected in the morning is not kept
- Urinary fractions collected during the day and night are stored in the same container
- Urine should be kept it in the refrigerator or a cool place during the collection period
- Only 50 mL should be sent to the laboratory (but it is necessary to label the container with the total volume collected during the procedure)
- The urine collected at 8 o'clock next day in the morning is stored in another container (its volume is used for diuresis determination)

- *The random (spot) specimen*:

May be collected at any time during the day or the night

- *The timed specimen:*

Is collected at a specified time in the 24-hour period (2-4 PM: urobilinogen) or at another specified time in relation to another activity (2 hours after eating to test glycaemia)

Table 2. Urine specimens

Specimen	*Reason*	*Use*
First morning or 8-hr	Most concentrated urine excretion	Protein, nitrite, microscopic analysis
Midstream/clean catch	Avoids contamination of specimen	Culture of bacteria and microscope analysis
Random (spot)	Convenience	Routine urinalysis
Fasting	Metabolism status	Glucose for diabetes testing
Timed	Excretion rate is determined 24 h	Quantitative chemical analysis

URINE PRESERVATION

Ideally, urine specimens are examined immediately after collection (maximum 2-3 hours after collection), to maintain the characteristics of urine. However, this is not always possible, and appropriate steps on preservation are required (Table 3):

- storage of the sample in the fridge (4-8°C) for 24-36 hours
- storage of the sample in the refrigerator (-20°C) for prolonged time
- adding preservatives
  - preservatives are specific for the parameters analyzed (Appendix 1)
  - some general preservatives are:
  - sodium azide – for the determination of proteins, glucose, amylase, creatinine, urea and uric acid
  - HCl 6 mM or thymol 1% – for the determination of inorganic phosphate
  - toluene, xylene, chloroform (5-10 ml)
  - preservatives might interfere with certain tests (chloroform should not be used if glucose or ketone bodies are to be determined)
  - the bottle should bear a label indicating the presence of any preservative that is used

Table 3. Urine preservation methods

Method	*Use*	*Disadvantage*
Refrigeration	Preservation of all urines for 24 hours	Unsatisfactory for longer periods of time
Freezing	For prolonged storage	Destroys cells
Formalin	Good for preserving cells and casts	Interferes with urobilinogen and leukocyte esterase on reagent strip tests and bacterial culture
Chlorhexidine	Preservative for glucose	Interferes with protein test on reagent strip
Preservative tablets	Routine urinalysis and cells	Interferes with specific gravity, sodium, potassium, and hormone studies

URINALYSIS

Urinalysis is the process of examining the urine by making use of a set of chemical and microscopic test. It offers useful information about the intermediary metabolism or organs functionality. Urinalysis can be used to screen for:

- urinary tract infections
- renal (kidney) diseases
- diseases of other organs that result in the appearance of abnormal metabolites (break-down products) in the urine

Summary urine examination includes:

1. Macroscopic exam (Physical examination)
2. Biochemical exam
3. Microscopic exam

MACROSCOPIC EXAMINATION

a) Volume

- *Normal values:* 1500 mL in men and 1200 mL in women
  - Diuresis = the normal quantity of urine eliminated in 24 hours
  - has greater values during the day (3/4) than during the night (1/4)
  - has greater values in standing position (clinostatism) than resting position (orthostatism)
  - is influenced by liquid ingestion, alimentation, temperature, body mass and emotional factors
- *Clinical correlations:*
  - the volume can be determined only on 24 hours urine specimens
  - the volume determination is not included in the summary urine examination
  - variations in the volume of urine excreted in 24 hours: polyuria, oliguria, anuria
  - variations in the rhythm of urine excretion in 24 hours: nocturia, opsiuria
    - Polyuria
      - definition: excretion of more than 2000 mL of urine / 24 h
      - *physiological conditions* (nocturia is absent): cold weather, emotions, ingestion of high quantities of fluids or vegetables rich in water, excessive coffee intake, certain drugs (diuretics)
      - *pathological conditions* (nocturia is absent): diabetes mellitus, diabetes insipidus, glomerulonephritis, hyperthyroidism, after pain crisis (angor pectoris = chest pain, colic, intensive cramps), after epileptic crisis
    - Oliguria
      - definition: excretion of less that 1000 mL of urine / 24h
      - *physiological conditions*: ingestion of small quantities of fluids during the day, excessive water loss through sweat, dehydration
      - *pathological conditions*: kidney stone, heart failure, dehydration through vomiting, diarrhea, hemorrhages, hypothyroidism
    - Anuria
      - definition: excretion of < 150 mL of urine /24h (kidneys lose the ability to produce urine)
      - *pathological conditions*: all the disease that produce oliguria, if the involved factors are more aggressive or of a longer duration

- Nocturia
  - definition: urine excretion during the night
  - physiological conditions: aging
  - *pathological conditions*: benign prostate hypertrophy, prostate tumors, urinary bladder infections, diabetes mellitus, urinary bladder prolapse, uterine prolapse
- Opsiuria
  - definition: delay in urine excretion after water ingestion
  - *pathological conditions*: Addison's disease

b) **Colour**

- *Normal values:* pale yellow
- *Physiological modifications:*
  - more intense colour: acidic urine and urine excreted during the night
  - weak colour: diuretics treatment
  - blue-green: methylene blue ingestion
  - red: aspirin, beetroot
- *Pathological modifications:*
  - weak colour: renal diabetes, renal sclerosis, renal failure associated with polyuria
  - yellow-orange: acute fever, abundant sweat
  - red: haematuria, haemoglobinuria, porphyria
  - grey: methemoglobinemia, sarcoma, phenol intoxication
  - brown: icteric syndromes
  - green-red: acute nephrites
  - black: alkaptonuria

c) Aspect

- *Normal values*: clear, transparent
- *Physiological modifications:*
  - after a few hours a deposit can appear and it is formed by the desquamation of urinary tract cells (also vaginal cells in women)
  - alkaline/neutral urine may be unclear even after elimination, due to alkaline phosphates
  - to make a differentiation between normal and pathological urine some methods can be used
- *Pathological modifications:*
  - renal diseases in which erythrocytes, leukocytes, mucus or casts can be seen
  - turbid urine must be analyzed only after it has been cleared through sedimentation
  - in diseases that associate polyuria, the urine is very clear and shows no sediment

d) Smell

- *Normal values:* vague smell, of bitter almonds, which is given by the volatile acids
- *Physiological modifications:*
  - more intense smell: when concentrated
  - unpleasant smell: ingestion of garlic, asparagus, horseradish
- *Pathological modifications:*
  - Ammonia smell in infectious diseases or tumors at the renal level
  - Putrescide (rotten) smell in anaerobic bacterial infections
  - Sour-apples smell in diabetes mellitus

BIOCHEMICAL EXAMINATION

The biochemical exam of urine uses urinary strips. These urinalysis test strips are simple, easy to use reagent strips for the detection of key diagnostic chemical markers in human urine. The tests using strips are important in general medicine as a screening method to identify the diseases at an incipient stage; also, the strips are used for the treatment monitoring and therapeutic strategies orientation.

They facilitate semiquantitative analyses of several compounds such as: urobilinogen, bilirubin, ketones bodies, ascorbic acid, glucose, nitrites, leucocytes and also analyses the urinary pH or density (gravity). The strip contains several distinct zones, each of them corresponding to a parameter to be tested.

When the strip is introduced in urine each zone will color specifically. The colours obtained will be compared with an etalon scale provided by the test; it makes the connections between the concentration of the analysed parameter and the color modification. A strip comprises 4 layers: a nylon protector layer, a paper to which chemically specific reagent pads are affixed, an absorbent paper layer and a plastic support.

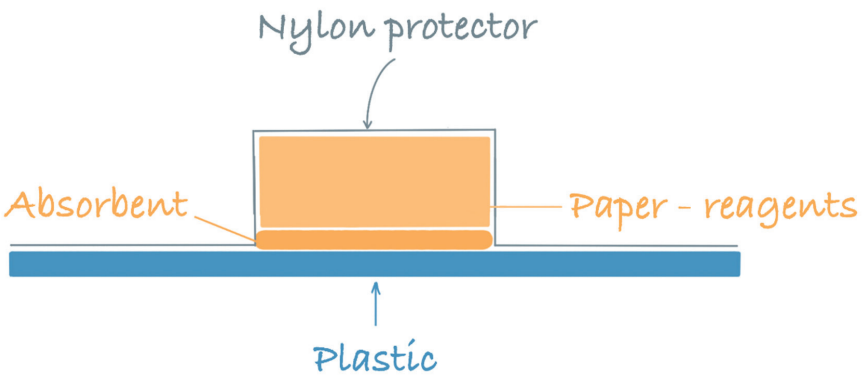


Figure 1. Urinary strip components

The reagent pads react with the sample urine to provide a standardized visible color reaction within 30 seconds to one minute depending on the specific panel screen. The color is then visually compared to the included color chart to determine the level of each chemical factor. Test results may provide useful information regarding carbohydrate (sugar) metabolism (diabetes), kidney function, acid-base balance, bacteriuria, occult blood, high leukocytes (infection) and other conditions of overall health.

The usage of urinary strips is recommended:

- in diseases of renal apparatus and urogenital tract when these diseases are asymptomatic for a long period of time (for example renal insufficiency is the terminal stage of different nephropathies)
- in metabolic diseases (especially diabetes mellitus type 2) glucose and ketone bodies are analyzed
- in hepatic diseases and anemia when urobilinogen and bilirubin are analyzed.

Procedure:

- Collect a fresh urine sample in a clean, dry container preferably glass (if the container is not sterile the nitrites are not analyzed). First morning samples contain the highest concentration of target markers.
- Remove one reagent strip from the bottle and immediately replace the container cap, minimizing the exposure of the remaining test strips to light and air.
- Completely immerse the reagent pads of the strip in the urine sample (2 sec.) and then remove immediately to avoid dissolving out the reagent pads.
- While removing the reagent strip, run the edge of the strip against the rim of the specimen container to remove excess urine. Hold the strip in a horizontal position to prevent possible cross contamination of chemicals located in adjacent reagent pads.
- Compare the colour change of reagent pads to the corresponding colour chart on the bottle label. Read results according to the chart's time frame for each panel tested [No more than 2 min. should be taken to read the strip (the leucocytes are read after 60 sec; in the range of 60 sec-120 sec)].

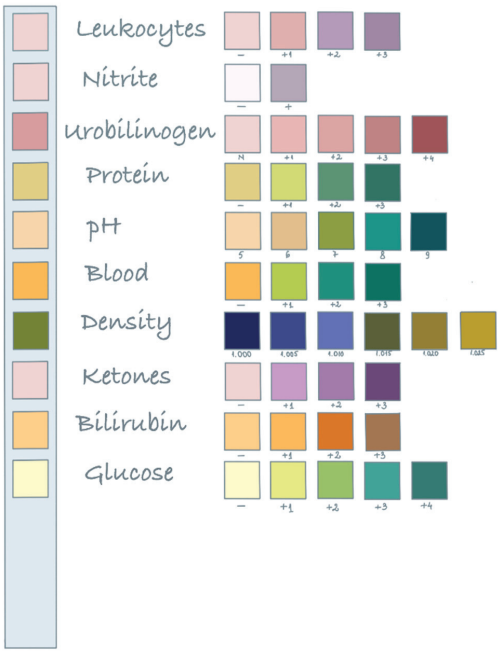


Figure 2. Urinary strip testing

a) pH

- *Normal values and:* 5-7
- *Clinical correlations:*
  - normally, urine is an acidic biological fluid, whose pH is mainly determined by the ratio between alkaline and acidic phosphates from urine
  - in plasma NaH_2PO_4:Na_2HPO_4=1:5 (pH=7.4), whereas in urine NaH_2PO_4:Na_2HPO_4=5:1
  - compared to blood, urine has a wider pH range
- *Physiological modifications:*
  - urine pH is influenced by nutrition:
    - a nutrition rich in meat will correspond to an acidic urine (5.2-5.3)
    - a nutrition based on vegetables will correspond to an alkaline urine (7.0-7.5)
    - a mixed nutrition will lead to a weak acidic urine
    - bicarbonates and mineral water will lead to an alkaline urine
  - urine pH is influenced by the nictemeral (circadian) rhythm
    - the most acidic urine is eliminated at midnight
    - the most alkaline one in the morning
- *Pathological modifications:* (nutrition-independent)
  - low pH: inanition, intoxications, renal failure, diabetic ketoacidosis
  - high pH: profuse vomiting, urinary tract infections (urethritis, cystitis, pyelitis, pyelonephritis), severe hypokalemia, intoxication, fever

b) Density

- *Normal values*: 1.015 - 1.025 g/cm^3
- *Clinical correlations:*
  - normosthenuria = normal urine density
  - the density varies directly proportional to the amount of substances dissolved in the urine and to the intensity of the color and inversely proportional to the amount of urine emitted
  - at a density lower than 1.010 g/cm^3, leucocytes and erythrocyte are being lysed which might give false negative results when analyzing the urinary sediment
- *Physiological modifications:*
  - low density: ingestion of high amount of fluids
  - high density: dehydration, excessive sweating
- *Pathological modifications:*
  - Hypersthenuria (density > 1.025): diabetes mellitus, proteinuria, dehydration, fever
  - Hyposthenuria (density < 1.015): diabetes insipidus, hyperhydration
  - Isosthenuria (density = 1.002): severe renal failure, showing the incapacity of the kidney to concentrate or dilute urine

c) Leucocytes

- *Normal values:* Absent
- *Clinical correlations:*
  - the leucocytes in urine represent an important indicator of renal and urinary tract inflammatory processes: bacterial infections, non-bacterial infections caused by viruses, parasitical infections, intoxications
  - in most cases, the positive test for leucocytes indicates bacterial infection of urinary tract
  - however, the presence of leucocytes in urine could be caused by a contamination with secretions of genital apparatus
  - sometimes, in chronic inflammations, a strong reaction of leucocytes could be detected in the absence of bacteria. This situation is a unique symptom of chronic pyelonephritis, tuberculosis or different tumors.
- *Pathological modifications:*
  - leukocyturia: acute inflammatory processes of the urinary tract (bacterial infections and non-bacterial infections caused by viruses or parasites - urethritis, cystitis, pyelitis, pyelonephritis), intoxications, chronic inflammations = abacterial leukocyturia (chronic pyelonephritis, tuberculosis or different tumors)

d) Nitrites

- *Normal values:* Absent
- any nitrate from urine may be reduced to a nitrite by bacteria-mediated process:

$$Nitrate \rightarrow bacterial\ reduction \rightarrow Nitrite$$

- *Clinical correlations:*
  - under normal conditions, the urine does NOT contain nitrites. Bacteria-free urine does not contain any nitrite.
  - the presence of nitrite in the urine is a specific indicator of bacteriuria, and when positive, should be taken seriously
  - a negative result gives no assurance that significant infection is not present. Urine may not have been retained in the bladder long enough for bacteria to have reduced nitrate, and this will invariably be the case when a urinary catheter is in place. Moreover, certain pathogens, such as *Streptococcus faecalis*, do not reduce nitrate at all.

- the administration of nitrites containing therapy does not lead to nitrites excretion in urine
- following antibiotics or chemotherapy, enzyme metabolism and microbial population are inhibited, thus the test's result will be negative. This is the reason for any antimicrobial therapy should be withdrawn at least 3 days before testing.

- *Pathological modifications:*
  - nitrituria: bacterial urinary tract infections

e) Erythrocytes (RBCs)

- *Normal values:* Absent
- *Clinical correlations:*
  - Even small increases in the number of RBCs in urine are significant. Numerous diseases of the kidney and urinary tract, as well as trauma, medications, smoking, or strenuous exercise, can cause hematuria (RBCs in urine) or hemoglobinuria (hemoglobin in urine).
  - This test cannot determine the severity of disease (in other words, a high amount of blood in urine does not mean the disease is more advanced, nor does a low amount of blood in urine mean that disease is less advanced).
  - This test also cannot identify where the blood is coming from. For instance, contamination of urine with blood from hemorrhoids or vaginal bleeding cannot be distinguished from a bleed in the urinary tract.
- *Physiological modifications:*
  - pseudo hematuria: pink or reddish urine can be produced by excessive consumption of beets, berries, or rhubarb; food coloring; and certain laxatives and pain medications
- *Pathological modifications:*
  - depending on severity of bleeding:
    - *gross* (*macroscopic*) *hematuria*: there is sufficient blood present to color the urine red or brown
    - *microscopic hematuria*: the urine is visually normal in color but is found to contain blood on chemical analysis or microscopic evaluation
  - depending on the site of bleeding:
    - *glomerular hematuria*: urinary RBCs have an irregular size distribution and tend to be smaller than peripheral RBCs. Erythrocytes of glomerular origin tend to be distorted with variation in size and shape. This is thought to result from the red cells passage through the nephron.
    - *nonglomerular hematuria*: urinary RBCs are more uniform in size distribution and have a larger mean volume with the peak volume being greater than that of peripheral RBCs
  - depending on when the bleeding occurs:
    - Onset of urination (initial hematuria) - urethra or prostate (men)
    - Throughout urination (total hematuria) - bladder, ureter, or kidneys
    - End of urination (terminal hematuria) - bladder or prostate (men)
  - causes of hematuria:
    - primary parenchymal disease such as glomerulonephritis (associated with proteinuria and hypertension), polycystic kidney disease, renal calculi, upper or lower tract infections (cystitis or prostatitis), tumors of the urinary tract
    - drug reactions (e.g., penicillin, aminoglycosides, NSAIDs)
    - trauma (Jogger's hematuria results from repeated jarring of the bladder during jogging or long-distance running)
  - Symptoms may indicate the site and/or cause of bleeding:
    - Abdominal pain - Inflammation of the kidney or ureter caused by trauma, infection, or tumor

- Decreased urinary force, hesitance, or incomplete voiding - lower urinary tract, benign prostate hyperplasia, tumor
- Fever - infection, typically kidney infection or ureter infection
- Pain in the flank - kidney trauma or tumor
- Urinary urgency, pain, or frequency - bladder cancer

f) Proteins

- *Normal values:* Absent
- *Clinical correlations:*
  - Small quantities of albumin are normally filtered at the glomerulus and reabsorbed by the proximal tubule. The protein that escapes reabsorption does not exceed 150 mg/24 hr so that proteinuria in excess of this amount is regarded as pathologic.
  - *"Functional" proteinuria* is a term applied to the transient proteinuria that accompanies fever, exercise, or congestive heart failure and is probably hemodynamically mediated within the kidney. Proteinuria rarely exceeds 500 mg/24 hr and resolves when the initiating event is reversed.
  - If persistent proteinuria is present, a complete urinalysis including a careful inspection of the urinary sediment is mandatory. Red cell casts and oval fat bodies must be searched for carefully; presence of red cells or red cell casts indicates glomerular disease; lipid-laden tubular cells (oval fat bodies) are found in nephrotic syndrome of a variety of causes. The diagnostic criteria of nephrotic syndrome include heavy or nephrotic-range proteinuria, hypoalbuminemia, oedema, hyperlipidemia and lipiduria. Renal function studies including blood urea nitrogen, serum creatinine concentration, and creatinine clearance should be performed.

- *Physiological modifications:*

- *benign proteinuria*: is due to prolonged orthostatism, lordosis, dehydration, emotional and physical stress, fever, heat injury, inflammatory process. Benign proteinuria has a benign prognosis and is associated with only a minor degree of proteinuria. This proteinuria can appear at healthy persons under 30 years, with no renal disease. The test for orthostatic proteinuria involves a urine collection in the evening followed by confinement to bed until the next morning, when a recumbent sample is obtained. The patient is then allowed to ambulate for a few hours and a third sample is obtained. Presence of protein in the first and third sample with absence in the second confirms the diagnosis of orthostatic proteinuria.

- *Pathological modifications:*
  - *depending on etiology*
    1. *Prerenal proteinuria:* occurs when increased concentrations of low molecular weight proteins present in the plasma overwhelm tubular resorptive capacity. Can be found in colic, epileptic seizures, heart attack, stroke, head trauma, postoperative. In this case, proteinuria disappears after the underlying condition is solved.
    2. *Renal proteinuria*: renal failure, glomerulonephritis, nephrotic syndrome, nephritis, polycystic kidney, and certain inflammatory, infectious, and metabolic diseases.
    3. *Postrenal proteinuria:* occurs due to inflammatory/infectious processes in the urinary bladder or prostate, or due to hemorrhage.

- *depending on the mechanism*
    i. *Glomerular proteinuria*: Glomerular disease is the most common cause of pathologic proteinuria. Several glomerular abnormalities alter the permeability of the glomerular basement membrane, resulting in urinary loss of albumin and immunoglobulins. Glomerular malfunction can cause large protein losses; urinary excretion of more than 2 g per 24 hours is usually a result of glomerular disease.
    ii. *Tubular proteinuria*: occurs when tubulointerstitial disease prevents the proximal tubule from reabsorbing low-molecular-weight proteins (part of the normal glomerular ultrafiltrate). When a patient has tubular disease, usually less than 2 g of protein is excreted in 24 hours. Tubular diseases include hypertensive nephrosclerosis and tubulointerstitial nephropathy caused by nonsteroidal anti-inflammatory drugs.
    iii. *Overflow proteinuria*: low-molecular-weight proteins overwhelm the ability of the proximal tubules to reabsorb filtered proteins. Most often, this is a result of the immunoglobulin overproduction that occurs in multiple myeloma. The resultant light-chain immunoglobulin fragments (Bence Jones proteins) produce a monoclonal spike in the urine electrophoretic pattern.

Table 4. Types of proteinuria based on the pathogenic mechanism

Type	Cause	Pathophysiologic features
Glomerular	Primary or secondary glomerulopathy	Increased glomerular capillary permeability to proteins
Tubular	Tubular or interstitial disease	Decreased reabsorption of proteins in the glomerular filtrate
Overflow	Monoclonal gammopathy, leukemia	Increased production of low-molecular-weight proteins

g) Glucose

- *Normal values:* Absent
- *Clinical correlations:*
    - Less than 0.1% of glucose normally filtered by the glomeruli appears in the urine, and less than 130 mg should appear in the urine over a 24-hour period. Glucose is present in glomerular filtrate but is reabsorbed by the kidney's proximal tubule. If the blood glucose level exceeds the capacity of the tubules to reabsorb all the glucose present in the glomerular filtrate, the renal threshold is reached and glucose spills into the urine.
    - A finding of glycosuria indicates that the person is hyperglycemic or has a lowered renal threshold for glucose. The renal threshold for glucose is approximately 160 to 190 mg/dl of blood; glucose does not appear in the urine until the blood glucose rises above this level.
- *Physiological modifications:*
    - glycosuria: after eating a heavy meal or during times of emotional stress
    - in pregnancy, the renal threshold for glucose may be lowered so that small amounts of glycosuria may be present
- *Pathological modifications:*
    - *glycosuria in diabetes mellitus:* Urine glucose tests are used to screen for diabetes, to confirm a diagnosis of diabetes, or to monitor diabetic control. Postprandial testing for glycosuria is more efficient in identifying diabetes mellitus than the test performed using first morning urine.

- *renal glycosuria*: is a rare condition in which the simple sugar glucose is eliminated (excreted) in the urine despite normal or low blood glucose levels. With normal kidney (renal) function, glucose is excreted in the urine only when there are abnormally elevated levels of glucose in the blood. However, in those with renal glycosuria, glucose is abnormally eliminated in the urine due to improper functioning of the renal tubules, which are primary components of the filtering units of the kidneys (nephrons). In most affected individuals, the condition causes no apparent symptoms (asymptomatic) or serious effects. When renal glycosuria occurs as an isolated finding with otherwise normal kidney function, the condition is thought to be inherited as an autosomal recessive trait.

h) Ketone bodies

- *Normal values:* Absent
- *Clinical correlations:*
  - Ketones are metabolic end products of fatty acid metabolism. In healthy individuals, ketones are formed in the liver and are completely metabolized so that only negligible amounts appear in the urine. However, when carbohydrates are unavailable or unable to be used as an energy source, fat becomes the predominant body fuel instead of carbohydrates and excessive amounts of ketones are formed as a metabolic by-product. A finding of ketones in the urine indicates that the body is using fat as the major source of energy.
  - Three ketone bodies that appear in the urine when fats are burned for energy (acetone, acetoacetic acid and beta-hydroxybutyric acid). Normally, the urine should not contain enough ketones to give a positive reading. As with tests for glucose, acetone can be tested by a dipstick or by a tablet. The results are reported as small, moderate, or large amounts of acetone. A small amount of acetone is a value under 20mg/dl; a moderate amount is a value of 30-40mg/dl, and a finding of 80mg/dl or greater is reported as a large amount.
  - Screening for ketonuria is done frequently for acutely ill patients, presurgical patients, and pregnant women. Any diabetic patient who has elevated levels of blood and urine glucose should be tested for urinary ketones. In addition, when diabetic treatment is being switched from insulin to oral hypoglycemic agents, the patient's urine should be monitored for ketonuria. The development of ketonuria within 24 hours after insulin withdrawal usually indicates a poor response to the oral hypoglycemic agents. Diabetic patients who use oral hypoglycemic agents should have their urine tested regularly for glucose and ketones because oral hypoglycemic agents, unlike insulin, do not control diabetes when an acute infection or other illness develops.
  - In conditions associated with acidosis, urinary ketones are tested to assess the severity of acidosis and to monitor treatment response. Urine ketones appear before there is any significant increase in blood ketones; therefore, urine ketone measurement is especially helpful in emergency situations. During pregnancy, early detection of ketonuria is essential because ketoacidosis is a factor associated with intrauterine death.
  - In nondiabetic persons, ketonuria may occur during acute illness or severe stress. Approximately 15% of hospitalized patients may have ketonuria, even though they do not have diabetes. In a diabetic patient, ketone bodies in the urine suggest that the patient is not adequately controlled and that adjustments of medication, diet, or both should be made promptly. In the nondiabetic patient, ketonuria reflects a reduced carbohydrate metabolism and excessive fat metabolism.
- *Physiological modifications:*
  - ketonuria: starvation, fasting, high protein, or low carbohydrate diets, prolonged vomiting, and anorexia, pregnancy, lactation
- *Pathological modifications:*
  - Metabolic abnormalities such as diabetes, renal glycosuria, glycogen storage disease, hyperthyroidism, fever

i) Bilirubin

- *Normal values:* Absent
- *Clinical correlations:*
  - albumin-bound bilirubin (indirect bilirubin) is not water soluble and will never appear in the urine
  - conjugated bilirubin (with glucuronic or sulfuric acid) is water soluble and may appear in urine (in pathological situations)
  - if the bile ducts are obstructed, direct bilirubin will build up to a high enough level that some of it will escape from the liver into the blood
  - the presence of conjugated bilirubin in detectable amounts (greater than 0.2 mg/dl) does not enable one to confidently distinguish between hepatocellular and obstructive jaundice, but does not commonly occur when hyperbilirubinemia is consequent to hemolysis
  - urobilinogen and bilirubin tests, taken together, help to distinguish between hemolysis, hepatocellular disease, and biliary obstruction (Table 5)
- *Pathological modifications:*
  - hyper bilirubinuria: biliary strictures, cirrhosis, gallstones in the biliary tract, hepatitis with associated biliary obstruction, surgical trauma affecting the biliary tract, tumors of the liver or gall bladder

Table 5. Level of urobilinogen and bilirubin in urine

	Health	Hemolysis	Hepatic disease	Biliary obstruction
Urine urobilinogen	Normal	Increased	Increased	Low/Absent
Urine bilirubin	Negative	Negative	Positive/Negative	Positive

j) Urobilinogen

- *Normal values:* Present in concentrations of 0.1 to 1.0 mg/dL or about 0.5 to 2.5 mg/24 hour
- *Clinical correlations:*
  - urobilinogen is a by-product of the degradation (reduction) of conjugated bilirubin
  - when conjugated bilirubin reaches the bowel, bacterial action produces urobilinogen (and further mesobilinogen and stercobilinogen), which is partly reabsorbed into the portal circulation (~20 percent is absorbed into the circulation and excreted by the kidneys)
  - the three compounds in this group, mesobilinogen, stercobilinogen, and urobilinogen, are colorless. When they are oxidized to their corresponding bilins, they contribute to the color of the stool and urine
- *Physiological modifications:*
  - urobilinogen is not present in neonates because the bacteria that reduce bilirubin to urobilinogen do not exist in newborns as they do in adults
  - antibiotic use in children and adults can result in decreased urobilinogen levels
- *Pathological modifications:*
  - increased urobilinogen in urine: hemolysis (increased production of bilirubin), hepatocellular disfunction (decreased hepatic clearance of urobilinogen from the portal circulation)
  - decreased urobilinogen in urine: biliary obstruction (decreased delivery of conjugated bilirubin to the bowel).

MICROSCOPIC EXAMINATION OF THE URINARY SEDIMENT

As part of a urinalysis, the urine sediment is centrifuged and examined microscopically for crystals, casts, red blood cells, white bloods cells, and bacteria or yeast. Because examination of urinary sediment provides a direct sampling of urinary tract morphology, it provides important information useful for both diagnosis and prognosis. Microscopic examination of urine sediment is usually performed in addition to routine procedures. Although commercial stains are available to highlight cellular elements, examination of unstained urine is usually adequate.

This examination requires a degree of skill acquired through practice under the immediate supervision of an experienced technician. The specimen used for microscopic examination should be as fresh as possible. Red cells and many formed solids tend to disintegrate upon standing, particularly if the specimen is warm or alkaline.

Correct interpretation of the microscopic urinalysis depends on proper specimen collection and preparation. The patient should be instructed on the proper technique for clean catch urine collection. Do not assume that the patient knows what to do from previous experience. Review how to cleanse the genitalia and instruct the patient to collect a midstream specimen.

1. Pour 5 to 10 millilitres of urine into a test tube. Conical bottom test tubes are preferred because they allow for better pellet formation.
2. Place the tube in the centrifuge and balance with a second tube filled with water (or another urine specimen) of equal volume. Spin the urine for about 10 minutes at 2000 rpm (rotations per minute).
3. Remove the tube and decant the supernatant into the sink.
4. Resuspend the sediment in the residual urine that clings to the bottom of the tube by tapping the tube against a hard surface several times.
5. Place a drop of the resuspended sediment on a 76 x 26 mm glass microscope slide using a pipette or by holding the tube upside down and carefully tapping it the slide until one drop falls onto the slide.
6. Place a 20 x 20 mm coverslip over the drop and place under the microscope.
7. Place the slide under the scope and begin the examination under low power. Be sure to use a low light source (adjust he iris and condenser). Too much light makes the cellular and crystalline elements harder to see. Scan the slide under low power (20X) to locate areas of interest.
8. Look for casts just inside the perimeter of the cover slip.
9. Then, switch to high dry magnification (40X) and examine ten random fields in the central part of the coverslip. Count the numbers of red cells and white cells in each and report the range of findings. If the field is covered with cells, report as "TNTC" (too numerous to count) or "packed."
10. Add to the report an estimate of bacteria density, any casts and other structures noted.

Example of microscopic urinalysis report: 5-10 RBC/HPF, 15-25 WBC/HPF, few bacteria, occasional hyaline cast, no epithelial cells.

Components of urinary sediment

- Organized urinary sediment

Urinary sediment cells

  - leukocytes
  - erythrocytes
  - epithelial cells: Renal tubular epithelial cells, Transitional epithelial cells (urothelial cells), Squamous epithelial cells

- casts

- Unorganized urinary sediment

Crystals

  - calcium oxalate
  - uric acid
  - triple phosphates (magnesium ammonium phosphate)
  - cystine crystals

Bacteria

a) Urinary sediment cells

Leukocytes (white blood cells)

Leukocytes designate all the haemoglobin free blood cells. These cells belong to the reticuloendothelial system. White blood cells in urine have lobed nuclei and refractile cytoplasmic granules. Based on their nuclear aspect, white blood cells can be divided into two categories: mononuclear cells and polynuclear cells. Lymphocytes and monocytes are the main mononuclear cells and polynuclear are subdivided into neutrophilic, eosinophilic and basophilic cells.

In the urinary sediment, the term leukocyte is usually interpreted as polynuclear, mostly neutrophils. The reason for this situation is that the neutrophils are, by far, the most abundant leukocytes in urine. In a normal specimen, up to 5 neutrophils / high-power field (40X) can be observed. High neutrophil counts are usually related to an inflammation process.

Mononuclear leukocytes are occasionally seen. High mononuclear counts are usually related to a high blood count pathology, infiltrating the urinary space. Under bright field microscopy and without staining, identification of the different types of white blood cells is almost impossible. The term leukocytes is indicated for the routine microscopy. The subclasses of leukocytes can be identified by staining with the Wright or PAP stain. Efficiency of these staining procedures is highly dependent of the conservation state of the cells. Staining with these represents a workload, not justified for the routine specimen.

Erythrocytes (red blood cells)

Haematuria is defined as a high urine red blood cell count sustained over three specimens taken on different days due to: glomerular damage, tumours which erode the urinary tract anywhere along its length, kidney trauma, urinary tract stones, renal infarcts, acute tubular necrosis, upper and lower urinary tract infections, nephrotoxins, and physical stress.

Red cells may also contaminate the urine from the vagina in menstruating women or from trauma produced by bladder catheterization. Some cases of idiopathic haematuria have been reported in the literature. Red blood cells originating from an external source, like vaginal bleeding, are not a

true haematuria. In a normal urine, less than 1,5 million erythrocytes are found in a 24-hour specimen. This normal value represents a count of less than 5 RBC/hpf (high power field). Two types of haematuria can be seen in urine:

- *Lower urinary tract hematuria*
  - this hematuria is usually associated with a lower urinary tract disease
  - RBCs have their typical shape and colour (refractile disks)
  - with hypertonicity of the urine, the RBCs begin to have a crenated appearance
- *Dysmorphic hematuria*
  - this hematuria is usually related with a glomerular bleeding
  - RBCs have a great variation in the size of the cells (anisocytosis), many ghost cells, and by a high percentage of dysmorphocytosis (>20%)
  - RBCs have bizarre shapes and projections of the cell membrane called blebs

Epithelial cells

- *Renal tubular epithelial cells*

Are usually larger than granulocytes, contain a large round or oval nucleus and normally slough into the urine in small numbers. However, with nephrotic syndrome and in conditions leading to tubular degeneration, the number sloughed is increased.

When lipiduria occurs, these cells contain endogenous fats. When filled with numerous fat droplets, such cells are called oval fat bodies. Oval fat bodies exhibit a "Maltese cross" configuration by polarized light microscopy. These cells are usually seen in a context of heavy proteinuria.

- *Transitional epithelial cells (urothelial cells)*

Come from the renal pelvis, ureter, or bladder have more regular cell borders, larger nuclei, and smaller overall size than squamous epithelium. Transitional cells are normal elements of the urinary sediment. The cell's shape changes slightly according to the section of the lower urinary tract. The presence of transitional cells is more frequent in the elderly population.

Contrarily to the isolated cells, the presence of transitional epithelial fragments is almost always associated to an abnormal situation. In the majority of cases, the cells have a normal aspect and form a thin sheet where it is easy to delimit each cell. These sheets are said to have a brick-wall-like aspect. The presence of this type of fragments can be the result of a urinary catheter or of another condition that provokes an erosion of the surface of the bladder's epithelium. In some sustained irritating conditions, transitional cells can become reactive. In these conditions, the cells and the nucleus increase in size. The size of cells can be variable, but the ratio nucleus/cytoplasm is well preserved. Binucleated cells can be seen.

- *Squamous epithelial cells*

Originate from the skin surface or from the outer urethra. Their significance is that they represent possible contamination of the specimen with skin flora.

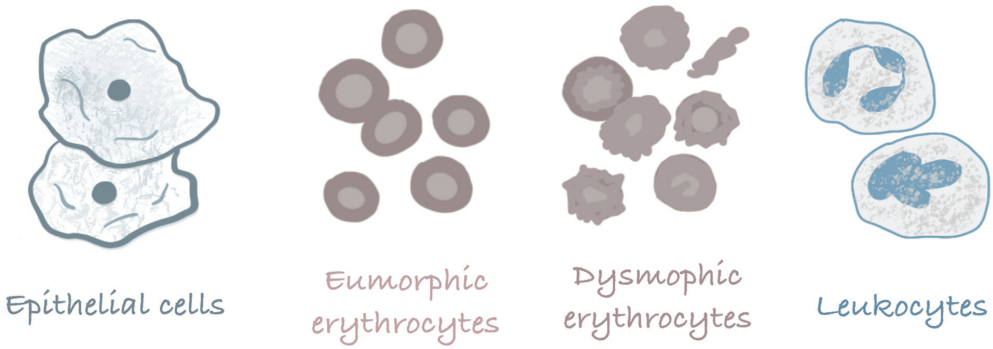


Figure 3. Urinary sediment cells

b) Casts

Casts are urinary sediments which are formed by coagulation of albuminous material in the kidney tubules. The Tamm-Horsfall protein is the cast fundamental substance.

It is believed that all the elements needed for cast formation are in place. Some factors (found in abnormal urines) seem to promote casts formation while others (found in normal urines) have an inhibiting effect. Casts are formed when the equilibrium between promoters and inhibitors is broken. Factors known to be promoters of cast formation are: albumin, urinary stasis, cellular debris, low glomerular filtration rate, acid pH, presence of certain proteins (Bence-Jones, myoglobin, haemoglobin), osmolality between 200 and 400 mOsm/Kg.

Casts are cylindrical and vary in diameter. The sides are parallel, and the ends are usually rounded. Casts in the urine always indicate some form of kidney disorder and should always be reported. If casts are present in large numbers, the urine is almost sure to be positive for albumin.

There are several types of casts: hyaline casts, red cell casts, white blood cell casts, granular casts, epithelial casts, waxy casts, fatty casts.

- Hyaline casts are the most frequently occurring casts in urine. Hyaline casts can be seen in even the mildest renal disease. They are colourless, homogeneous, transparent, and usually have rounded ends.
- Red cell casts indicate renal hematuria. Red cell casts may appear brown to almost colourless and are usually diagnostic of glomerular disease.
- White cell casts are present in renal infection and in non-infectious inflammation. The majority of white cells that appear in casts are hyper-segmented neutrophils.
- Granular casts almost always indicate significant renal disease. However, granular casts may be present in the urine for a short time following strenuous exercise. Granular casts that contain fine granules may appear grey or pale yellow in colour. Granular casts that contain larger coarse granules are darker. These casts often appear black because of the density of granules.
- Epithelial casts are rarely seen in urine because renal disease that primarily affects the tubules is infrequent. Epithelial casts may be arranged in parallel rows or haphazardly.
- Waxy casts result from the degeneration of granular casts. Waxy casts have been found in patients with severe chronic renal failure, malignant hypertension, and diabetic disease of the kidney. Waxy casts appear yellow, grey, or colourless. They frequently occur as short, broad casts, with blunt or broken ends, and often have cracked or serrated edges.
- Fatty casts are seen when there is fatty degeneration of the tubular epithelium, as in degenerative tubular disease. Fatty casts also result from lupus and toxic renal poisoning. A typical fatty cast contains both large and small fat droplets. The small fat droplets are yellowish-brown in colour.

Figure 4. Different types of casts

c) Crystals

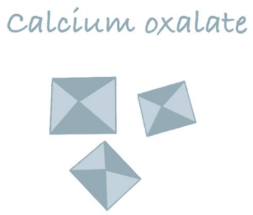
Except for the cystine crystals and a few others, the majority of crystals found in the urinary sediment are of limited clinical value. It is tempting to associate crystals with a risk of urolithiasis, but the majority of patients with a crystalluria does not have and will not develop kidney stones. Many benign situations can provoke crystal formation. In the majority of cases, the crystals found in urine are not present in the freshly voided specimen. Alkalization and refrigeration are promoters of crystals formation.

Table 6. Level of urobilinogen and bilirubin in urine

Type		*Apparition (%)*
Calcium oxalate	Calcium oxalate mono-hydrate (Whewellite)	70
	Calcium oxalate di-hydrate (Weddellite)	
Calcium phosphate	Hydroxyl-apatite	10
	Carbonate-apatite	
	Calcium hydrogen phosphate (Brushite)	
	Calcium hydrogen phosphate (Whitlockite)	
Triple phosphate = Magnesium ammonium phosphate (Struvite)		5-10
Uric acid		<5
Cystine		1

It is impossible to dissolve the quantity of calcium, phosphate, and oxalate eliminated in a 24-hours urine specimen into 1 to 2 litters of water. It is therefore necessary to conclude that substances inhibiting the crystallization are present. Known inhibitors of urinary crystallization are pyrophosphate, citrate, magnesium, and certain macromolecules. The Tamm-Horsfall protein is believed to be an important calcium oxalate inhibitor. This role is thought to be due to the sialic acid residue of the protein. While the fully sialated protein is an inhibitor, the sialic residues lacking protein is a crystallization promoter.

Calcium oxalate crystals



In the urinary sediment, one can find two forms of calcium oxalate crystals. The most frequent form is the di-hydrated calcium oxalate. The mineralogical name of the calcium oxalate 2(H2O) is Weddellite. The classic crystal shape is the eight-face bi-pyramid. In bright field microscopy, the weddelite crystals are recognized easily by their shape that reminds a mail envelope. The second form is the mono-hydrated calcium oxalate whose mineralogical name is Whewellite. The two forms have different crystallographic characteristics. It seems that the calcium/magnesium ratio plays an important role in the formation of the calcium oxalate crystals. Crystals of calcium oxalate are found mainly in acidic urine, but these can also be seen in slightly alkaline specimens.

In some cases, the crystallization of the calcium oxalate is massive and catastrophic. A typical example of the oxalate clinical catastrophe is the cases of ethylene glycol poisoning. In this situation, one can find oxalate crystals in the patient's tissues. The toxicity syndrome affects organs like the liver, the kidney, and the brain and is accompanied by a metabolic acidosis. Naturally, the oxalate crystalluria is massive, and is predominated by the ovoid crystals (Whewellite) forming microlithes. Calcium oxalate casts are highly significant. These imply that the oxalate crystals were already formed when the urine was at its maximum dilution.

Uric acid crystals

Uric acid crystals can appear under several shapes. The classic crystals are thin rhombus shaped plates with more or less eroded tops. The other forms are the hexagonal plate, the needle and the rosette. Uric acid crystals usually have a characteristic yellow color. The intensity of the color depends on the thickness of the crystal, thus very thin plates seem colorless, while the massive crystals have a color that tends to be brown. Under polarized light, uric acid shows a polarization color, and with thicker crystals, a series of concentric black lines. The color variation seen under polarized light is quite typical of uric acid.

Approximately 66 to 75% of the uric acid is eliminated by the urine. The quantity to eliminate depends mostly on the diet (meat). In increased uricosuria, values > 4,5 mmol/d are observed. Uric acid crystals are formed when the urinary pH is <5,5 since the pK of uric acid is 5,5. Uric acid crystalluria is mainly due to a poor dilution volume at an acid pH, or due to an overproduction. In most cases, this finding is of little clinical value and represents a pinpoint situation.

Triple phosphates crystals

Triple phosphates (magnesium ammonium phosphate, Struvite) are found in urines whose pH is superior to 6,5. The primary factor to the triple phosphate crystals formation is the ammonia concentration. Alkalinisation of a urine specimen with ammonia generates triple phosphates while alkalinisation with sodium hydroxide does not. A normal freshly voided specimen contains little free ammonia; this substance is mainly generated by urea splitting bacteria (ex: proteus species). Triple phosphates are usually associated with bacterial growth. With a first-morning fresh specimen, triple phosphates can indicate urinary tract infection. Otherwise, triple phosphates are of little clinical value.

Cystine crystals

Cystine crystals is a clinically significant finding. The cystine is seen as colorless hexagonal plates. The solubility of the cystine is much larger in alkaline urine, with the result that the former is rarely found in alkaline urine. These colorless crystals can be difficult to distinguish from the hexagonal plate form of uric acid crystals. But, in this case, the examination of the crystals under polarized light will probably show birefringent crystals with a polarization color interference. Cystine crystals are quite rare.

Cystine crystals are found in urine of almost exclusively patients with a genetic disease giving an impairment with the tubular reabsorption of the basic aminoacid: lysine, arginine, ornithine and cystine. This disease is called cystinuria. For a few patients with cystinuria, stones will develop. The urolithiasis is highly dependent of the urinary pH and water intake. Cystine is less soluble at a pH lower than 5,0 (saturation 300 mg/l); saturation is of 500 mg/l at a pH of 7,4.

d) Bacteria

Bacteria are common in urine specimens because of the abundant normal microbial flora of the vagina or external urethral meatus and because of their ability to rapidly multiply in urine standing at room temperature. Therefore, microbial organisms found in all but the most scrupulously collected urines should be interpreted in view of clinical symptoms.

Diagnosis of bacteriuria in a case of suspected urinary tract infection requires culture. A colony count may also be done to see if significant numbers of bacteria are present. Generally, more than 100,000/ml of one organism reflects significant bacteriuria. Multiple organisms reflect contamination. However, the presence of any organism in catheterized or suprapubic tap specimens should be considered significant.

Lab Notes

Use this space for handwritten notes during the practical session.

L5. BIOCHEMICAL INVESTIGATIONS OF THE CARDIOVASCULAR SYSTEM

LEARNING OBJECTIVES

Identify the major biochemical markers used in the assessment of cardiovascular disease.

Explain the time course and diagnostic significance of key cardiac biomarkers after myocardial injury.

Describe the role of lipid profile parameters in cardiovascular risk assessment.

Interpret laboratory results in the context of common cardiovascular pathologies.

KEY POINTS

Troponins I and T are the most sensitive and specific markers for myocardial injury; they are the current gold standard for acute myocardial infarction (AMI) diagnosis.

CK-MB rises 4-6 hours after AMI, peaks at 12-24 hours, and normalises within 48-72 hours; troponins remain elevated for 7-14 days.

Lipid profiling (total cholesterol, LDL-C, HDL-C) is essential for assessing long-term cardiovascular risk and monitoring treatment.

BNP and NT-proBNP are markers of cardiac volume overload used in the diagnosis and monitoring of heart failure.

D-dimers are increased after a blood clot is degraded by fibrinolysis.

GENERAL ASPECTS

The most common pathological change that affects cardiac function is **ischemia** (lack of adequate blood flow to ensure the oxygen necessary for the functioning of the myocardium). Although the heart pumps several liters of blood every minute, vascularization and arterial blood transport to the heart is carried out through the coronary arteries which can be affected by **atherosclerosis** (chronic process of damage to the vascular endothelium through which atherosclerotic plaques are produced, that can partially or totally block the vascular lumen, consequently reducing blood flow; also atherosclerotic plaques favor the appearance of blood clots that can totally or partially block the branches of the coronary arteries, causing **acute coronary syndrome-ACS**).

The group of diseases that develop as a result of affecting the coronary blood flow constitutes coronary disease, its most serious manifestation being the **acute myocardial infarction (AMI)**, when the coronary arteries or their branches are totally blocked, and necrosis of the myocardial cells occurs.

BIOMARKERS OF ISCHEMIC HEART DAMAGE

Depending on the size of the necrotic territory, cell death will cause the release of numerous biochemical compounds, more or less specific to the myocardium, the determination of which can establish a rapid positive diagnosis, essential for saving the patient's life.

Among the biomarkers associated with ischemia and necrosis of cardiac cells, currently, the most specific and sensitive biomarker is considered to be cardiac troponin. Other cardiac biomarkers useful in diagnosis are: creatine kinase (CK: total CK and the CK-MB isoenzyme), AST (GOT, ASAT), LDH, myoglobin, but their specificity is lower as they are also found in other tissue territories.

The evolution of the concentration of cardiac biomarkers for myocardial cell necrosis, as well as biomarkers for differentiating it from other pathologies, is different in the time interval elapsed since the ischemic event (Table 1 and Figure 1).

Table 1. The main characteristics of cardiovascular biomarkers

Cardiac marker	Molecular weight (GM, Da)	Debut (hours)	Maximum (hours)	Disappear (days)
CK	80000	4-8	18-30	3-4
CK-MB	80000	3-8	12-24	2-3
LDH	135000	8-12	48-72	8-14
Myoglobin	18000	1-3	6-9	1
Troponin I	24000	3-8	24-48	4-10
Troponin T	37000	3-8	24-48	4-10
ASAT (GOT)		72-96	5-6 days	12-14
D-Dimer	200000	4-8	12-24	2-4
B-type natriuretic peptide (BNP)		8-12	12-24	2-3

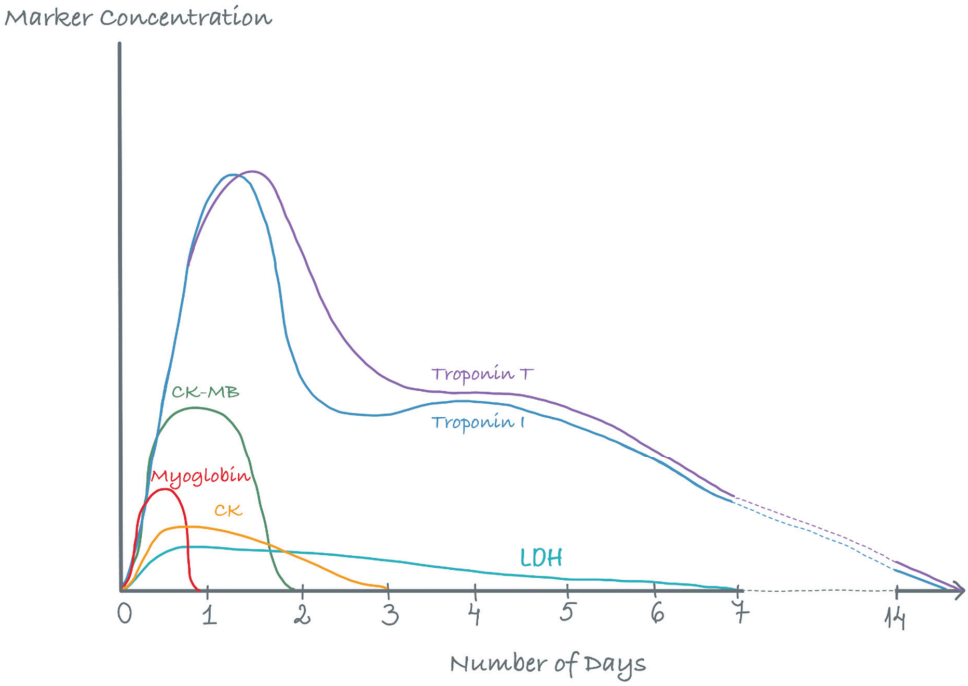


Figure 1. Time evolution of biomarker concentrations in AMI

1. Cardiac troponin

Troponin (Tn) is a regulatory complex consisting of 3 proteins located at regular intervals in the thin filaments of striated muscles (composed of actin, tropomyosin and troponin). The three individual proteins are: TnT (tropomyosin-bound subunit, 37kDa), TnI (actin-bound inhibitory subunit, 24kDa), and TnC (Ca2+-binding subunit, 18kDa). In the relaxed muscle state, TnI is bound to actin, blocking its binding to myosin. The triggering of muscle contraction is accomplished by Ca^{2+} binding to TnC, causing a conformational change that detaches TnI from actin, allowing the interaction between actin and myosin to take place.

Unlike the other markers, the forms of Tn in skeletal and cardiac muscles differ. For **TnC**, the forms found in type 2 fibers of skeletal and myocardial muscles are identical, making it impossible to use it as a differential marker. **TnI** has a cardiospecific form (cTnI=cardiac TnI), as well as different forms in type 1 and type 2 striated muscle fibers, each encoded by a separate gene. **TnT** has distinct forms in myocardium (cTnT=cardiac TnT), fast-twitch and slow-twitch skeletal muscle, but cTnT has been detected in fetal skeletal muscle and diseased skeletal muscle. In any case, post-translational modifications produce detectable differences between cTnT produced in the myocardium and cTnT produced in diseased skeletal muscle, so current generation commercial assays (ELISA) should have near 100% myocardial specificity. However, mild increases in circulating cTnT, using even newer assays, have been reported in patients with muscular dystrophy and renal failure who have no other evidence of heart disease.

In cardiac myocytes, cTnT and cTnI are predominantly bound to muscle fibers as described above, and the bound form is slowly released within 1-2 weeks after myocardial infarction. Thus, although cTnI and cTnT are relatively small proteins that are rapidly eliminated, their plasma level decreases slowly after cardiac injury.

A small fraction of cTn in myocardial cells is found free in the cytoplasm (on average 6% for cTnT and 2.5% for cTnI). The free fraction is released early from the affected myocardial cells and allows its detection in a time interval similar to CK-MB, cTn reaching a maximum approximately 24 hours after myocardial infarction.

Due to the slow release of the bound cTn fraction, the rapid decrease in circulating cTn immediately after reaching the maximum is followed by a plateau or even a slight secondary increase. It is important that such an increase is not interpreted as a marker of another infarction. Circulating cTn decreases to normal values after 5-10 days, depending on the size of the infarct.

2. Creatine kinase (CK)

CK is a dimeric enzyme consisting of 2 catalytic subunits (polypeptide chains with different structure, having a molecular weight of approx. 40 kDa each) called M (muscle) and B (brain). The 3 isoenzymes resulting from the association of the two subunits are: CK-BB (CK1, containing 2 B chains), CK-MB (CK2, containing a B chain and an M chain), CK-MM (CK3, containing 2 M chains).

CK is spread throughout the body, having the highest concentrations in the muscles and brain, although practically CK from the brain does not cross the blood-brain barrier to reach the plasma. In the brain and smooth muscle, the dominant isoenzyme is CK-BB.

In the normal heart, a percentage of 15-20% of CK is represented by CK-MB, the rest being CK-MM, which, however, is not uniformly distributed, a greater amount being found in the right heart compared to the left heart. CK-MB accounts for 0-1% of CK in skeletal muscle type 1 fibers and 2-6% in type 2 fibers. Also, increased production of CK-MB compared to CK-MM production is observed during muscle regeneration process.

The serum concentration of CK and CK-MB begins to increase 3-8 hours after the occurrence of AMI, and the amount released is proportional to the necrosis mass. The enzyme reaches a maximum at 24 hours and returns to the normal value after 48-72 hours (Figure 1).

The determination of the CK-MB fraction is carried out by immunometric techniques, being a quantitative determination (the enzymatic activity is not determined). Considering that CK-MB is not entirely specific to the myocardium (it also exists in striated muscles), more useful in the laboratory diagnosis of AMI is the ratio between CK-MB (expressed quantitatively as concentration) and total CK activity (measured as enzyme activity U/L), called relative index or relative percentage, because different measurement units are used for the two components in the ratio. A higher index is characteristic of AMI, and CK-MB must exceed the upper limit of the reference range both quantitatively (>5 ng/ml) and in percentage by the index calculation (>6%). Also, for an accurate diagnosis, the evolution of the enzyme concentration over time must be taken into account (Figure 1).

Subjected to electrophoresis, two CK isoenzymes (CK-MB and CK-MM) present in turn different molecular species called isoforms, which arise under the action of serum carboxypeptidases that hydrolyze the terminal lysine residues (Liz) of the M subunit. Thus CK-MB can have one Lys residue hydrolyzed (CK-MB1) or none (CK-MB2), while CK-MM can have one Lysine residue lysed (CK-MM2), two Liz residues (CK -MM1) or none (CK-MM3). This modification that gives rise to CK isoforms occurs only after the release of enzymes into the blood following tissue damage. Thus, in AMI, native, tissue isoforms without hydrolyzed lysine residues of CK (CK-MB2 and CK-MM3) are released into the circulation, requiring several hours to transform into CK-MB1 and CK-MM1/CK-MM2 isoforms.

Consequently, a higher ratio of CK-MB2/CK-MB1 and CK-MM3/CK-MM1 indicates that a recent release of enzymes into the circulation has occurred. This increase in the ratio of isoforms is observed shortly before serum CK-MB exceeds the upper limit of the reference range, so that, in the first 3-4 hours after the onset of symptoms, this determination can increase the sensitivity of the diagnosis of AMI. Testing can be done with a fast electrophoresis system that will remain dedicated to this type of analysis.

3. *LDH (lactate dehydrogenase)*

LDH is a Zn enzyme, with a molecular weight of 135 kDa, having a tetrameric structure involving two subunits, H (heart) and M (muscle). By combining the 2 subunits, 5 isoenzymes result: LDH1 (HHHH), LDH2 (HHHM), LDH3 (HHMM), LDH4 (HMMM) and LDH5 (MMMM). The LDH1 isoenzyme is relatively abundant in the myocardium, and the LDH5 isoenzyme is more widespread in the striated muscles.

It is a less specific myocardial necrosis marker that contributes, along with ASAT, to the late diagnosis of AMI, its plasma kinetics being slow (Figure 1). Its disadvantage is the very weak specificity for myocardium, it can also be released from skeletal muscle, lung, kidney, liver, erythrocytes. The specificity of LDH is increased by determining the LDH1 isoenzyme activity.

LDH1 activity increases in 8-12 hours after the onset of necrosis, reaches a maximum in 48-72 hours and returns to normal in 8-14 days after AMI. Because the half-life is prolonged, LDH1 is useful for the diagnosis of late-presenting patients. The increase in the LDH1/LDH2 ratio over 0.75, determined by the increase in LDH1 concentration compared to LDH2 (normal LDH2>LDH1), has a sensitivity and specificity of over 90% for myocardial necrosis.

Currently, it is a rare determination compared to the markers of choice (cTn, CK-MB).

4. Myoglobin

Myoglobin is a hemoprotein that binds oxygen in skeletal muscles and myocardium. There is only one shape common to both types of muscles. Lacking cardiac specificity, myoglobin's utility derives from its kinetics (Figure 1).

With a molecular weight of only 18 kDa, it diffuses out of affected cells faster than other proteins. Increased serum concentrations appear 1-3 hours after the first symptoms of AMI, faster than in the case of troponin or other markers. Myoglobin is eliminated mainly by glomerular filtration. The half-life is approximately 4 hours, but is longer if renal function is impaired. Characteristically, the myoglobin level reaches a maximum about 6-9 hours after AMI and returns to normal values after 24 hours. In healthy individuals, myoglobin levels depend on muscle mass and muscle activity, similar to the CK pattern.

Plasma levels are higher in men than in women. The myoglobin level increases with age, reflcting the decrease in glomerular filtration rate. The daily variation is approximately 10-15%.

Despite the lack of specificity of myoglobin for the myocardium, as a test for AMI it offers quite high clinical specificity (95%) when patients with renal failure or those suspected of muscle diseases are excluded.

5. ASAT/AST/GOT (Aspartate aminotransferase)

It is one of the first enzyme markers that was used for the diagnosis of AMI, currently being replaced by much more specific markers, previously presented. The enzyme is released following myocardial necrosis, but is not specific.

Its activity can also increase in diseases of liver cytolysis (stasis liver), lung diseases (pulmonary thromboembolism), muscle diseases and traumas, gangrene, hemolytic anemias, acute pancreatitis.

USEFUL BIOMARKERS IN THE DIFFERENTIAL DIAGNOSIS OF CARDIAC ISCHEMIA

D-Dimer

During the coagulation process, thrombin converts fibrinogen to soluble fibrin by proteolytically removing both fibrinopeptides A and fibrinopeptides B. Soluble fibrin spontaneously polymerizes, and the D regions are covalently cross-linked, a process that is catalyzed by factor XIII. Fibrin is ultimately degraded via the fibrinolytic pathway. Plasmin cleaves fibrin cross-links and releases fibrin degradation products in the form of two fragmented D molecules cross-linked with 200 kDa GM (D-dimer).

Elevated levels of circulating D-dimers have been described in patients with pulmonary embolism, deep vein thrombosis and disseminated intravascular coagulation.

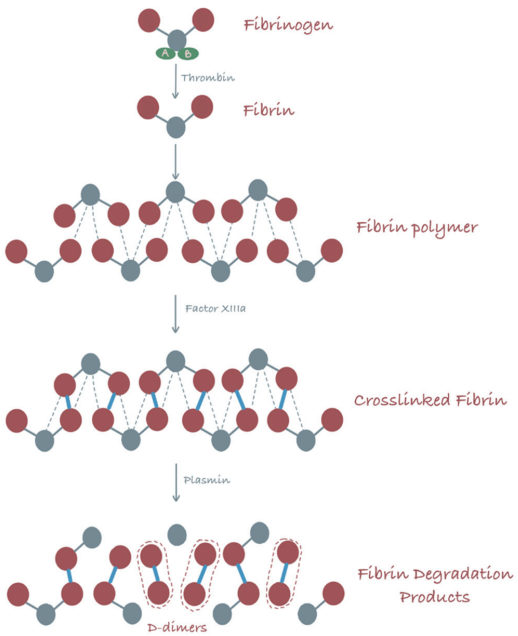


Figure 2. D-dimer production

B-type natriuretic peptide (BNP) is a member of a class of hormones that regulate blood pressure. In humans, the heart (ventricular myocardium) is the main source of circulating BNP. The molecule is released into the blood in response to increased heart pressure. Different studies have demonstrated that elevated levels of circulating BNP are found in the early stages of chronic heart failure. The level of BNP in the blood continues to rise as chronic heart failure progresses. In addition, BNP has been shown to be useful as a prognostic indicator in patients with acute coronary syndromes as well as an initial test to rule out HF in patients with dyspnea.

EXPERIMENTAL WORK

Determination of the biochemical parameters of the cardiovascular system using the Triage Profiler SOB Panel

Alere Triage Profiler SOB Panel

Alere Triage Profiler SOB Panel (Shortness of Breath = Dyspnea) is an immunofluorescence test intended for use with Alere Triage measuring devices, for the quantitative determination of the following plasma parameters:

- Creatin kinase MB;
- Myoglobin;
- Troponin I;
- BNP;
- D-dimer.

This test is used for:
- AMI diagnosis;
- evaluation of the prognosis of patients with ACS;
- diagnosis and assessment of the degree of HF;
- evaluation of the prognosis of patients with HF;
- evaluation of patients with suspected ICD or thromboembolic events (pulmonary embolism).

Biological specimens used: whole blood or plasma anticoagulated with EDTA.

The principle of the test procedure

The test procedure involves adding a few drops of whole blood or EDTA anticoagulant plasma to the sample port on the test device. The specimen reacts with fluorescent antibody conjugates and flows through the test device by capillary action. The complexes of each fluorescent antibody conjugate with the antigen are captured in different areas, specific for each analyte. The test device is inserted into the Alere Triage meter, which is programmed to perform the analysis after the specimen has reacted with the reagents in the test device.

The analysis is based on the quantitative determination of fluorescence by the measuring device within a measuring area of the test device. The concentration of the analyte in the specimen is directly proportional to the detected fluorescence. Results are displayed on the meter screen in approximately 20 minutes after adding the specimen. All results are stored in the memory of the measuring device to be displayed or printed when needed. If connected, the measuring device can transmit the results to the computer system of the laboratory or hospital.

Reagents

The test device contains:
- Murine monoclonal antibodies against CK-MB, myoglobin, troponin I, D-dimer and BNP;
- Murine polyclonal antibodies against CK-MB, myoglobin and BNP;
- Goat polyclonal antibodies against troponin I;
- Fluorescent dye;
- Stabilizers

Test procedure

- Whole blood samples and test devices are kept in the refrigerator.
- Before testing, they must be allowed to reach room temperature~15min.
- The test device is removed from the individual foil only when applying the sample.

Step I - Adding the biological sample collected from the patient (blood)

1. Open the protective film of the test device.
2. Place the test device on a horizontal, flat surface.
3. Before application, the whole blood sample is gently mixed by inverting the tube several times to avoid hemolysis.
4. Using the transfer pipette, fully squeeze the large (upper) bulb and insert the tip into the whole blood sample.
5. Slowly release the bulb. The barrel of the transfer pipette should be completely filled, with some liquid flowing into the small (lower) bulb (should fill <1/4 of the volume of the lower bulb).
6. Insert the tip of the transfer pipette into the sample port of the test device and fully squeeze the large bulb. The entire volume of liquid in the barrel of the transfer pipette must flow into the sample port. The contents of the specimen in the small (lower) bulb should not be evacuated.
7. Remove the tip of the transfer pipette from the sample port, then release the large (upper) bulb.
8. Discard the transfer pipette.
9. Allow the whole blood sample to absorb completely (approx. 15 min) before inserting the test device into the machine.

Step II - Run test

1. Insert the device into the socket and turn it on from the ON button;
2. Wait approx. 2 min for initialization.
3. From the main screen, select Run Test (using the up/down arrows) and press Enter.
4. Select Patient Sample (using the up/down arrows) and press Enter.
5. Enter the patient identification number and press Enter.
6. Confirm correct number entry by selecting Confirm Patient ID and pressing Enter. If the number was not entered correctly, select Correct Patient ID, press Enter and repeat the previous step.
7. Holding the test device by the edges, insert it into the measuring device slowly until you hear a *click* and press Enter.
8. The test device must be inserted into the measuring device within max. 30 minutes from the moment of adding the sample.
9. The results are printed automatically in 15 min and a sound signal (beep) is heard. A blocked result indicates that the result was invalid and the test must be repeated;
10. Remove the test device from the device and throw it away.
11. Turn off the device from the ON button.

Normal values (reference values):

- CK-MB: 0.1 - 4.3 ng/mL;
- MYO: 0.1 - 107 ng/mL;
- TN-I: 0.01 - 0.40 ng/mL;
- BNP: 0.1 - 100 pg/mL;
- D-DIM: 0.1- 600 ng/mL.

CLINICAL CASES

1. Patient: **AB, sex: M, 55 years old**

The patient presents with the following symptoms: severe chest pain with radiation to the left shoulder and left arm, shortness of breath (dyspnea), cold sweats, nausea, anxiety. The doctor recommends performing the following biochemical analysis:

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	130 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	30 U/l	F:10-22 U/l M:10-29 U/l	**Total Proteins**	6.2 g/dl	6.2-8 g/dl
ASAT	125 U/l	F:10-21 U/l M:10-25 U/l	**Hemoglobin**	14.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	40 mg/dl	15-45 mg/dl	**hsCRP**	43 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3 mg/l high CV risk
Creatinine	1.0 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	50 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	100 ml/min	95-150 ml/min	**Cystatin C**	0.99 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	23 mEq/l	22-27 mEq/l
Amylase	68 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	200 mg/dl	140- 200 mg/dl	**K+**	4.5 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	20 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	210 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	300 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CK-MB	113 U/l	2-10 U/l	**GGT**	22 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	200 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.025	1.015- 1.025
pH	5.5	6.5
Proteins	+/-	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

Compare these values with the normal values, and establish a possible diagnosis.

2. A 55-year-old man arrived at the Emergency Department complaining of intense chest pain that had persisted for the past hour. He had a prior history of exertional angina for two years. What tests would you request from the biochemistry laboratory?

3. A 66-year-old man had been experiencing central chest pain during exertion for several months. In the afternoon before his admission, he had an unusually severe episode of chest pain that occurred at rest and persisted for approximately an hour. Upon admission, his physical examination revealed no abnormalities, and his ECG appeared normal. Troponin levels were clearly elevated. Comment on these results.

4. A football player which was out of the field for the last 3 months, restarted playing, but collapsed in the 74th minute of a match. Tests show that his CK was elevated at 8200 U/L (reference range 30-200 U/L), and CK-MB was 10% of the total CK. Troponin levels were within the reference range and the ECG was normal. Comment on these results.

Lab Notes

Use this space for handwritten notes during the practical session.

L6. HIGH SENSITIVITY C-REACTIVE PROTEIN DETERMINATION

LEARNING OBJECTIVES

Explain the role of C-reactive protein (CRP) as an acute-phase inflammatory marker.

Distinguish between standard CRP and high-sensitivity CRP (hsCRP) measurements.

Describe the principle of hsCRP determination with ELISA technique.

Interpret hsCRP levels in the context of cardiovascular risk stratification.

KEY POINTS

CRP is an acute-phase protein synthesised by the liver in response to inflammation, infection, or tissue injury, mediated by interleukin-6.

hsCRP measures CRP at concentrations below 10 mg/L with high analytical sensitivity and is an independent predictor of future cardiovascular events.

hsCRP cardiovascular risk categories: < 1 mg/L = low risk; 1–3 mg/L = intermediate risk; > 3 mg/L = high risk (values above 10 mg/L suggest acute inflammation).

ELISA (the principle of this practical session) is the standard method for hsCRP measurement in automated clinical chemistry analysers.

GENERAL ASPECTS

C-reactive protein (CRP) a clinically significant „acute phase protein" whose concentration increases dramatically (up to one thousand-fold) following an inflammatory event and falls rapidly, returning to normal values within a few days of the end of the inflammation. This protein is a β-globulin, originally named after a property of serum of acutely ill patients, which caused the precipitation of a cell wall component called C-polysaccharide (fraction C) from pneumococcal extracts. It was initially thought that CRP might be a pathogenic secretion as it was elevated in people with a variety of illnesses including cancer, however, discovery of hepatic synthesis demonstrated that it is a native protein.

CRP consists of five subunits having 105,000 Da. CRP is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes) - Figure 1. CRP binds strongly to certain lipids, particularly phospholipids/phosphocolins. CRP is involved in the body's response to foreign compounds. CRP increases in infection and inflammatory disease processes (up to 20- to 30- fold) and is widely measured as an alternative or adjunct to the less sensitive erythrocyte sedimentation rate (ESR).

HIGH-SENSIVITY C-REACTIVE PROTEIN

High-sensitivity CRP (hsCRP) is the same protein but is named for the newer, monoclonal antibody–based test methodologies that can detect CRP at levels below 1 mg/L. The hsCRP test determines risk of cardiovascular diseases. Using the hsCRP assay, levels of less than 1, 1 to 3, and greater than 3 mg/L correspond to low-, moderate-, and high-risk groups for future cardiovascular events. Some clinical studies have shown that a slightly increased CRP level in the range of 3 - 10 mg/L is associated with an increased risk of the manifestation of cardiovascular complications.

Primarily, there is an increased risk of ischemic heart disease, but the risk of ischemic cerebrovascular accident and atherosclerosis in other locations also rises. Clinical studies performed on large sets have proved that people with slightly increased CRPs had higher cardiovascular morbidity and mortality rates. This relationship is valid not only in primary prevention, for previously healthy people without cardiovascular complications, but also in secondary prevention, for patients with cardiovascular diseases.

However, CRP is not a risk factor that induces atherosclerosis, it is just an indicator of risk (like other inflammatory markers). The CRP level is increased in people with major causal risk factors such as smoking, hypertension, obesity, metabolic syndrome, diabetes mellitus, etc. The CRP level decreases if a person manages to reduce the cholesterol level, lose weight, induce a regression of diabetes (overweight or obesity reduction), etc. In addition, a slight increase in CRP may not always be associated with an elevated risk of cardiovascular complications: the interpretation of a CRP result between 3 and 10 mg/L should be with caution and individually, as a slight increase in CRP may be due to any inflammation in the body and may not have relationship to cardiovascular diseases (chronic diseases of the intestine, lungs, joints, etc.), or can be the result of a starting or subsiding acute inflammation.

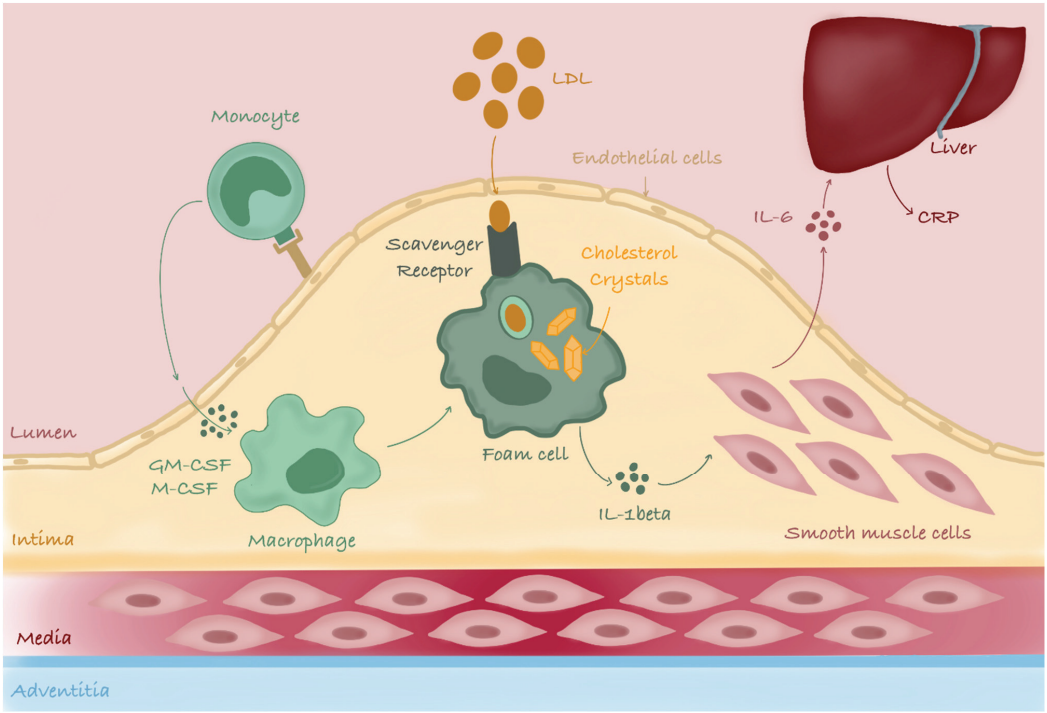


Figure 1. Immune response in atherosclerosis

Atherosclerosis development is initiated by LDL retention. Lipoprotein accumulation in the subendothelial space leads to increase of adhesion molecules on the endothelial surface as well as monocyte recruitment to the forming lesion. Endothelial cells generate macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), which cause monocytes to move into the subendothelial space and differentiate into macrophages. Smooth muscle cells could also transdifferentiate into macrophage-like cells. Further, foam cells are formed when macrophages take up lipoproteins via scavenger receptors. Cholesterol crystals are formed in these cells and activate the domains-containing protein 3 inflammasome, leading to the production of IL-1β, which stimulates smooth muscle cells to release IL-6. Both IL-1β and IL-6 have proinflammatory effects. In addition, circulating IL-6 may trigger the liver to produce CRP. Patients with atherosclerotic cardiovascular disease have increased levels of CRP.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) METHOD

ELISA stands for enzyme-linked immunosorbent assay, also called as enzyme immunoassay (EIA). An ELISA, like other types of immunoassays, utilizes antibodies to detect a certain antigen using specific antibody-antigen interactions. In an ELISA assay, the antigen is immobilized to a solid surface, either directly or by the use of a capture antibody that is immobilized on the surface. The antigen is then bound to a detection antibody, which is conjugated with an enzyme or a fluorophore.

An ELISA is generally performed in a multi-well plate (96- or 384-wells), which provides a solid surface to immobilize the antigen, facilitating the separation of the antigen from the rest of the molecules in the sample.

- ***Direct ELISA***

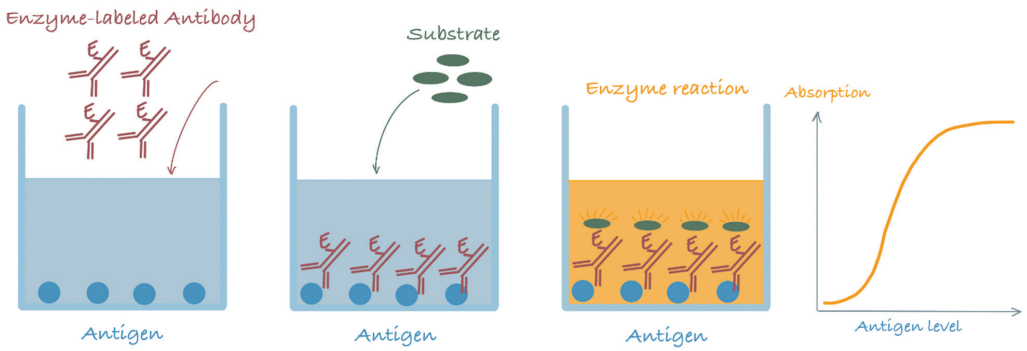


Figure 2. Direct ELISA

In direct ELISA, the antigen is immobilized on a solid surface (usually a microplate), and a single enzyme-linked antibody specific to the antigen is added for detection. This method is quick and simple but offers lower sensitivity because it lacks a signal amplification step.

- ***Indirect ELISA***

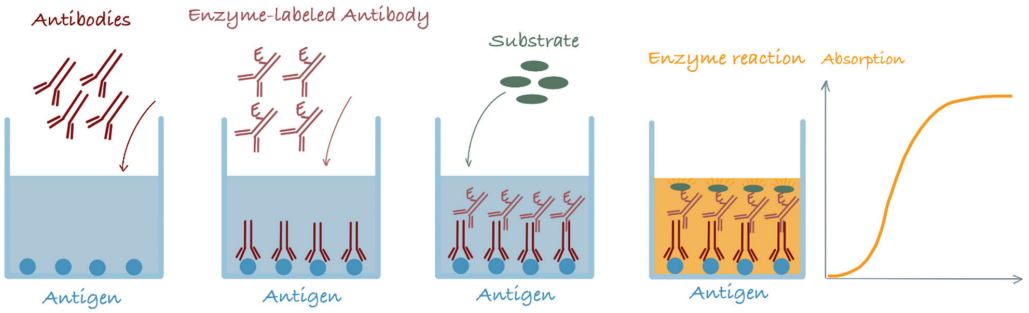


Figure 3. Indirect ELISA

Here, the antigen is immobilized, and a primary antibody binds to it, followed by an enzyme-linked secondary antibody that recognizes the primary one. This approach provides higher sensitivity due to amplification but involves more steps and potential cross-reactivity.

- ***Competitive ELISA***

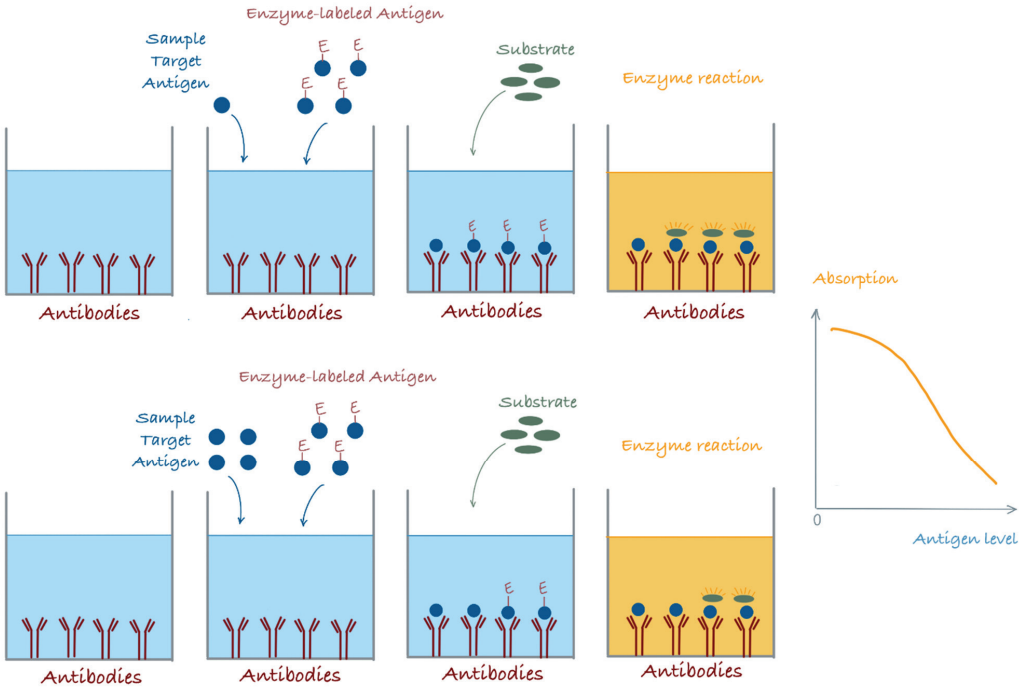


Figure 4. Competitive ELISA

In competitive ELISA, the sample antigen competes with a labelled antigen for a limited number of antibody binding sites. The signal intensity is inversely proportional to the antigen concentration - the higher the antigen in the sample, the lower the detected signal.

- ***Sandwich ELISA***

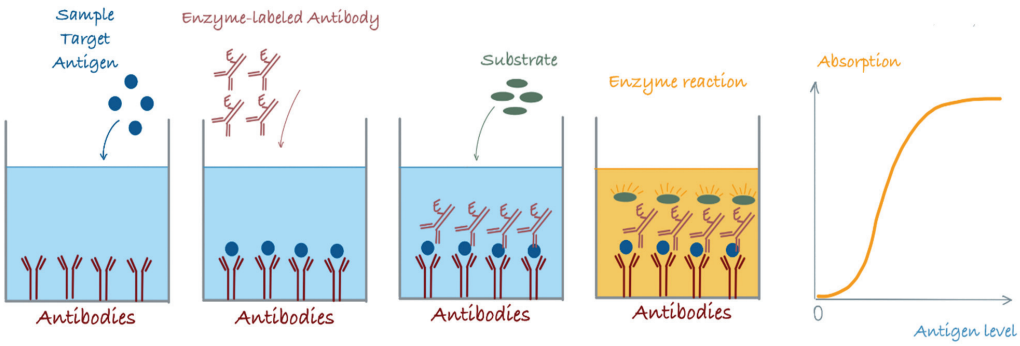


Figure 5. Sandwich ELISA

In this format, the antigen is “sandwiched” between two antibodies - a capture antibody bound to the plate and a detection antibody that recognizes a different epitope. It is highly specific and sensitive, ideal for detecting complex samples.

EXPERIMENTAL WORK

Determination of hsCRP in human serum using the ELISA test

Principle of the Assay

The hsCRP ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the on the CRP molecule. This mouse monoclonal anti-CRP antibody is used for solid phase immobilization (on the microtiter wells). A goat anti-CRP antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45-minute incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A tetramethylbenzidine (TMB) reagent is added and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl changing the color to yellow. The concentration of CRP is directly proportional the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

Assay Procedure

1. **Patient serum and control serum should be diluted 100 fold prior to use.**
2. Secure the desired number of coated wells in the holder.
3. Dispense **10 µL** of DILUTED serum into appropriate wells.
4. Dispense **100 µL** of CRP Enzyme Conjugate Reagent into each well.
5. Thoroughly mix for 30 seconds. It is very important to mix completely.
6. Incubate at room temperature (18 °C – 25 °C) for 45 minutes.
7. Remove the incubation mixture by flicking plate contents into a waste container. Rinse and flick the microtiter wells 5 times with deionized or distilled water. DO NOT USE TAP WATER.
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense **100 µL** TMB solution into each well. Gently mix for 5 seconds.
10. Incubate at room temperature for 20 minutes.
11. Stop the reaction by adding **100 µL** of Stop Solution to each well.
12. Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely**.
13. Read absorbance at 450 nm with a microtiter well reader **within 15 minutes**.

Expected Values

It is recommended that each laboratory establish its own normal range based on the patient population. However, based on published literature healthy individuals are expected to have CRP values as follows:

Adult serum: **<1 mg/L -** low risk of developing cardiovascular disease;
1-3 mg/L - medium risk;
3-10 mg/L - high risk;
>10 mg/L - evaluation of the patient for the presence of other inflammatory pathologies, acute infections.

Quantitative ELISA Kit Principle

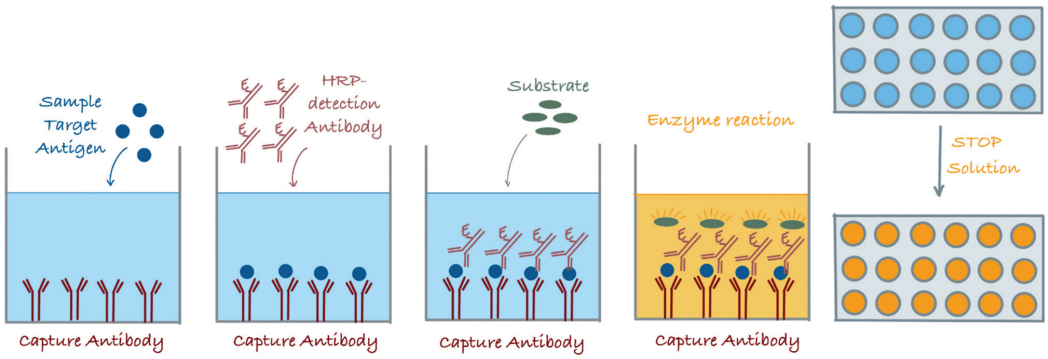


Figure 6. ELISA Kit principle

CASE STUDY

1. A patient underwent a routine cholecystectomy and was monitored postoperatively for inflammation and infection using C-reactive protein (CRP) levels.

   Clinical Course:

   - Days 1-3 Post-Op: The patient initially followed a normal post-surgical CRP response, with levels peaking around 100 mg/L, consistent with typical post-operative inflammation.
   - Day 5: The patient developed a fever, and CRP levels were abnormally high (exceeding 100 mg/L). This raised concerns for possible infection.
   - Day 6: The patient's condition worsened, prompting further investigation. The surgical wound was re-opened, revealing an abscess, which was subsequently drained. CRP levels peaked above 200 mg/L, reflecting an ongoing inflammatory response.
   - Days 7-8: Following intervention, the patient showed clinical improvement. CRP levels declined, suggesting resolution of infection and effective recovery.

   Comment on these results, indicating the importance of CRP determination.

2. The medical history of a 70-year-old woman included longstanding systolic hypertension, which was managed with an antihypertensive drug, as well as a lifelong smoking habit. She was thin (BMI = 23 kg/m^2) and a strong family history of early-onset cardiovascular disease. Her lipid profile was within optimal ranges:

   - Total cholesterol: 214 mg/dL (5.54 mmol/L)
   - LDL-C: 102 mg/dL (2.64 mmol/L)
   - HDL-C: 80 mg/dL (2.07 mmol/L)

   Her high-sensitivity C-reactive protein (hsCRP) levels-measured twice over a two-week span-were elevated at 7.6 mg/L and 7.3 mg/L, respectively.

   Comment on these results, indicating also the risk for cardiovascular diseases.

Lab Notes

Use this space for handwritten notes during the practical session.

L7. CLINICAL IMPLICATIONS OF ALKALINE PHOSPHATASE (ALP)

LEARNING OBJECTIVES

Describe the physiological role and tissue distribution of alkaline phosphatase (ALP) and its isoenzymes.

Identify clinical conditions associated with elevated or decreased ALP levels.

Correlate specific ALP isoenzyme patterns with bone, liver, and other pathologies.

Perform and interpret an ALP activity determination.

KEY POINTS

ALP is a membrane-bound enzyme present in bone (osteoblasts), liver (biliary epithelium), intestine, kidney, and placenta.

Elevated ALP is mostly associated with bone disease (Paget's disease, metastases, hyperparathyroidism) and hepatobiliary disease (cholestasis).

ALP isoenzyme fractionation differentiates tissue sources: bone isoenzyme is heat-labile; liver isoenzyme is heat-stable.

In cholestasis, ALP elevation is typically accompanied by elevated GGT; in bone disease, GGT remains normal, aiding differential diagnosis.

GENERAL ASPECTS

Alkaline Phosphatase (ALP) is a generic name for a group of enzymes that catalyze the release of phosphate group from a series of substrates and have maximum activity at alkaline pH, comprised between 9.0-10.5. The natural substrate and *in vivo* role of ALP are unknown. Individuals with hereditary ALP deficiency excrete large amounts of phosphoethanolamine (phosphocolamine), suggesting that this might be the natural substrate. ALP is attached to cell membranes, possibly playing a role in transmembrane transport.

ALP is encoded by **4 genes for 4 isozymes**:

- Intestinal
- Placental
- Germ cell
- Non-specific form

Germ cell isozyme is present in testes and thymus, and the non-specific one contains enzymes from liver, bone and kidney. The liver and bone isoforms differ in sialic acid content.

In adults, approximately half of the serum ALP activity is of hepatic origin and the other half of bone origin. Only a small part is of intestinal origin.

The primary importance of measuring alkaline phosphatase is to check the possibility of bone disease or liver disease.

However, ALP can be elevated also in physiological conditions.

ELEVATIONS OF ALP IN PHYSIOLOGICAL CONDITIONS

Alkaline phosphatase levels can be elevated in several normal, non-pathological states.

One of the most common is late pregnancy, during which the placenta produces its own isoenzyme, leading to serum ALP levels that may rise up to twice the normal range.

Children and adolescents also frequently exhibit elevated ALP due to rapid bone growth and increased osteoblastic activity during periods of skeletal development.

Alkaline phosphatase (ALP) levels can show physiological elevation after a heavy meal, particularly a high-fat one, due to the increase in the intestinal isoenzyme. This is a normal, temporary response and is more pronounced in individuals with blood types O and B. Because of this, fasting is typically required for accurate ALP blood test results.

These physiological elevations are generally mild to moderate and should be distinguished from pathological causes to avoid misinterpretation in clinical settings.

ALP: INVESTIGATIONS OF BONE METABOLISM

Clinicians investigating patients with bone diseases need to know to what extent bone is being broken down and formed. Therefore, biochemical markers of bone resorption and bone formation are useful in assessing the extent of disease, as well as for treatment monitoring. As first line tests to diagnose patients with calcium disorders and bone diseases, measurements of calcium, alkaline phosphatase, followed by PTH, magnesium, urine calcium excretion, 25-hydroxycholecalciferol, urinary hydroxyproline and osteocalcin are required.

Bone is continuously remodeled at discrete sites in the skeleton in order to maintain the integrity of the tissue (Figure 1). During this process, old bone is resorbed by osteoclasts and replaced with new osteoid secreted by osteoblasts. First osteoclasts are activated and the resorption phase takes approximately 10 days. Following resorption, unclassified macrophage-like cells are found at the remodeling site in the intermediate, or reversal phase. Osteoblast precursors are then recruited, which proliferate and differentiate into mature osteoblasts, before secreting new bone matrix. In the last step, the matrix mineralizes to generate new bone and this completes the remodeling process.

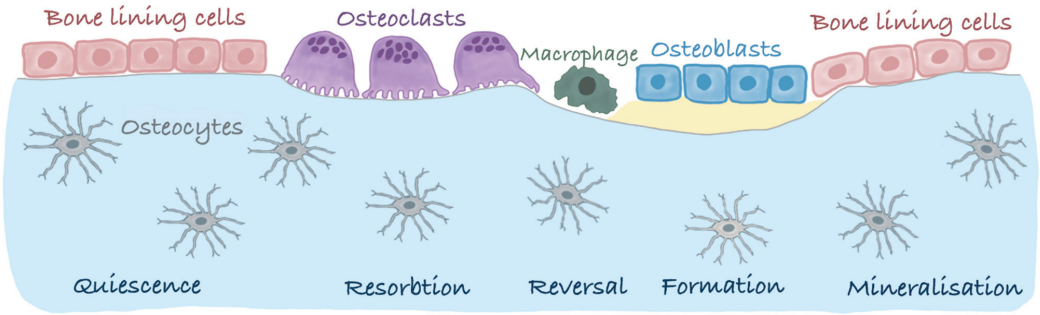


Figure 1. Bone remodelling process

Alkaline phosphatase profiles in different bone diseases

The activity of the enzyme alkaline phosphatase has traditionally been used as an indicator of bone turnover. The osteoblasts that lay down the collagen framework and the mineral matrix of bone have high activity of this enzyme. Increased osteoblastic activity is indicated by an elevated alkaline phosphatase activity in serum specimen. Indeed, children who have active bone growth compared with adults have higher 'normal' alkaline phosphatase activity in serum. However, alkaline phosphatase is also produced by the cells lining the bile canaliculi and is a marker for cholestasis. The bone isoenzyme of alkaline phosphatase may be measured, but there is need for a more specific and more sensitive marker.

1. Paget's disease

Paget's disease generally appears in elderly patients and is characterized by increased osteoclastic activity, which leads to increased bone resorption. Increased osteoblastic activity repairs resorbed bone, but the new bone is reformed in a disorganized way. The clinical sign is almost always bone pain, which can be particularly severe. Serum alkaline phosphatase is high, and urinary hydroxyproline excretion is elevated. These provide a way of monitoring the progress of the disease.

2. Osteomalacia and rickets

Osteomalacia is the name given to defective bone mineralization in adults. Rickets is characterized by defects of bone and cartilage mineralization in children.

Vitamin D deficiency was once the most common reason for rickets and osteomalacia. Vitamin D status can be assessed by measurement of the main circulating metabolite, 25-hydroxycholecalciferol, in a serum specimen. The metabolism of vitamin D is shown below. In severe osteomalacia due to vitamin D deficiency, serum calcium will fall, and there will be an appropriate increase in PTH secretion. Serum alkaline phosphatase activity will also be elevated.

Vitamin D and/or calcium deficiency leads to a decrease in the level of ionized calcium (Ca^{2+}), resulting in an increase in Parathormone (PTH). PTH increases tubular reabsorption of calcium to correct the serum calcium into the normal range. However, in severe calcium and vitamin D deficiency, the serum calcium is below normal. In addition, PTH causes phosphorus loss via the urine, resulting in a decrease in serum HPO_4^{2-}. An inadequate calcium-phosphorus product ($Ca^{2+} \times HPO_4^{2}$) leads to a defect in bone mineralization that causes rickets in children and osteomalacia in adults. There are various inherited and acquired disorders that can disrupt calcium and phosphorus metabolism that can also result in defective mineralization of the skeleton.

There are 3 inherited syndromes that cause vitamin D resistance.

- Vitamin D-dependent rickets type 1 (DDR-1) is caused by a mutation in the gene for the enzyme 1-alpha-hydroxylase, which prevents the conversion of the inactive form of vitamin D to its active form, $1,25(OH)_2D$.
- Vitamin D-dependent rickets type 2 (DDR-2) results from a mutation in the vitamin D receptor (VDR) gene, causing the body to be unable to properly recognize/use the active form $1,25(OH)_2D$ that is produced.
- A genetic defect that results in the overproduction of hormone response element-binding protein disrupts the interaction of $1,25(OH)_2D$ with its VDR, resulting in DDR-3.

There are both inherited and acquired disorders that cause severe hypophosphatemia and reduce renal production of the active vitamin D form, $1,25(OH)_2D$. The inherited disorders X-linked hypophosphatemic rickets and autosomal dominant hypophosphatemic rickets result from increased production or decreased degradation of phosphatonins, such as FGF23. Tumor-induced osteomalacia is an acquired condition caused by excessive production of FGF23 by certain tumors, leading to phosphaturia and decreased renal synthesis of $1,25(OH)_2D$.

- **Hypophosphatasia**

This disease is a form of rickets or osteomalacia that results from a deficiency of alkaline phosphatase.

3. Bone metastases

In bone metastases, calcium levels are high, phosphate levels also increase and PTH is usually decreased. Alkaline phosphatase levels are usually elevated.

Therefore, Osteoblastic/Bone Conditions associated with increased ALP activity:

- Paget's Disease
- Over-activity of the Parathyroid glands (Primary Hyperparathyroidism, Secondary Hyperparathyroidism from kidney disease, osteomalacia, malabsorption)
- Rickets - Vitamin D deficiency
- Healing fractures - or tissue regeneration can transiently raise ALP as a result of enhanced osteoblastic or tissue repair activity
- Rapid bone growth (such as after a fracture, bone cancers like osteogenic sarcoma, Osteomalacia, and Paget's Disease)
- Treated osteoporosis
- Hyperthyroidism due increased bone turonover

ALP: INVESTIGATIONS OF LIVER DISEASE

Since the mucosal cells that line the bile system of the liver are the source of alkaline phosphatase, the free flow of bile through the liver and down into the biliary tract and gallbladder are responsible for maintaining the proper level of this enzyme in the blood. When the liver, bile ducts or gallbladder systems are not functioning properly or are blocked, this enzyme is not excreted through the bile and alkaline phosphatase is released into the blood stream. Thus, the serum alkaline phosphatase is a measure of the integrity of the hepatobiliary system and the flow of bile into the small intestine.

Therefore, increased levels of alkaline phosphatase may occur in congestion or obstruction of the biliary ducts, or the duct leading from the gallbladder through the pancreas that empty into the duodenum. Any of these organs (liver, gallbladder, pancreas, or duodenum) may be involved. Increases in alkaline phosphatase activity in liver disease are the result of increased synthesis of the enzyme by cells lining the bile canaliculi, usually in response to cholestasis, which may be either intra- or extrahepatic.

1. Extrahepatic biliary obstruction

Gallstones can partially or fully block the bile duct. Such a blockage is known as extrahepatic obstruction. If the blockage is complete, both bilirubin and alkaline phosphatase are raised. There is little or no urobilinogen in urine. Stools will be pale in color. When the obstruction is removed, the stools regain their color and urine again becomes positive for urobilinogen.

2. Intrahepatic biliary obstruction

Intrahepatic biliary obstruction is much more difficult to diagnose than extrahepatic obstruction. The bile canaliculi can become blocked due to cirrhosis, liver cancer or infection. This leads to an increased concentration of conjugated bilirubin in serum.

In hepatocellular damage, obstruction may be secondary to damage to the hepatocytes by infection or toxins, rather than direct involvement/damage of the biliary tract. The most common causes of acute jaundice seen in adults are viral hepatitis and paracetamol poisoning. In these cases, not only are the bilirubin and alkaline phosphatase levels raised, but AST and ALT are elevated indicating hepatocellular damage.

Cholestasis, even of short duration, results in an increased enzyme activity to at least twice the upper end of the reference interval. High alkaline phosphatase activity may also occur in cirrhosis.

3. Other causes of liver congestion/cholestasis associated with increased ALP activity:

- Oral contraceptives
- Obstructive pancreatitis
- Hepatitis/Mononucleosis/CMV
- Parasites
- Malignancy involving liver

INCREASED ALP ACTIVITY IN NON-BONE/NON-LIVER CONDITIONS

Although alkaline phosphatase (ALP) activity is most often elevated in bone and liver disorders, increased serum ALP can also arise from several non-hepatic, non-skeletal conditions. These elevations may result from enzyme release by other tissues, such as the intestine, placenta, or kidneys, or from systemic physiological or pathological processes. Understanding these alternative sources is essential to avoid misinterpretation of ALP results and to correctly identify the underlying cause of the elevation.

- Inflammatory and systemic disorders:
  - Amyloidosis
  - Sarcoidosis
  - Rheumatoid arthritis
  - Systemic infections (e.g., sepsis)
  - Herpes zoster (shingles)
  - Granulation tissue formation
- Gastrointestinal conditions:
  - Inflammatory bowel disease (ulcerative colitis, Crohn's disease)
  - Peptic or intestinal ulcers
- Malignant and ischemic conditions:
  - Certain cancers (Hodgkin's lymphoma, gynecologic malignancies)
  - Acute tissue damage in the heart or lungs (myocardial or pulmonary infarctions)

DECREASED SERUM ALKALINE PHOSPHATASE

While much attention is given to elevated alkaline phosphatase (ALP) levels, abnormally low serum ALP can also provide important clinical information. Decreases in ALP activity may reflect nutritional deficiencies, genetic disorders, endocrine abnormalities, or certain systemic conditions. Recognizing these causes is essential for proper interpretation of laboratory results.

- Nutritional deficiencies:
  - Zinc deficiency
  - Vitamin C deficiency (scurvy)
  - Folic acid deficiency
  - Vitamin B6 insufficiency
  - Malnutrition with low protein intake or impaired absorption (including hypochlorhydria)
- Endocrine disorders:
  - Hypothyroidism
  - Insufficient parathyroid gland function
- Metabolic and other causes:
  - Excessive vitamin D intake
  - Low phosphorus levels (hypophosphatasia)
  - Celiac disease
  - Pernicious anemia

EXPERIMETAL WORK

Serum Alkaline Phosphatase (ALP) determination

Principle

p-Nitrophenyl phosphate will be hydrolyzed by alkaline phosphatase (from the patient's serum) into p-nitrophenol, a yellow-colored compound whose absorption is measured spectrophotometrically, the intensity of the color being directly proportional to the activity of the enzyme (ALP) in the serum.

$$p-nitrophenyl\ phosphate\ +\ H_2O \xrightarrow{ALP} p-nitrophenol\ +\ phosphate$$

Reagents

1. R1 = 1M diethanolamine buffer solution, pH=9.8: dissolve 100mg MgCl2 and 95.6ml diethanolamine, with a density of 1.1g/ml, in distilled H2O, bring the pH to 9.8 with 1M HCl solution, then complete with distilled H2O, in a volumetric flask up to 1 liter.

2. R2 = Substrate p-nitrophenylphosphate (pNPP), 10mM: dissolve 64.2mg of pNPP in 20ml of diethanolamine buffer solution. pNPP = 371,12g/mol.

Mix 5 volumes of R1+1 volume of R2 (**working reagent isalready prepared = RL = Working reagent**)!!

3.Serum or plasma

4. Other materials: Automatic pipettes of 20-200 µl and 100-1000 µl. Spectrophotometer, cuvettes, timer.

Procedure

1. Turn on the spectrophotometer and bring it to the wavelength λ=450nm.

2. The spectrophotometer is calibrated against air.

3. **Prepare the Sample directly in the cuvette as follows:** 1000 µL RL + 20 µL serum and homogenize with the tip of the 20 µL automatic pipette.

4. When the serum is added, the timer starts.

5. Read the absorbance values at 450 nm at **1** minute and **3** minutes (the absorbance will increase in value).

Calculation

Calculate the medium rate of the reaction as **ΔA / min**. The activity of enzyme is calculated with:

$$a\ =\ \Delta A\ /\ min\ x\ 5757\ =\ (A3-A1)/2\ x\ 5757\ (U\ /\ L)$$

where 5757 - is a quantity that depends on the molar absorption coefficient of pNPP and the volume of the sample.

Normal values: Children: 10 - 400 U / L
Adults: 60 - 180 U / L

CASE STUDY

1. Patient: **MJ, sex: M, 58 years old.**

The patient presents with the following symptoms: fatigue, jaundice, abdominal discomfort, abdominal weight gain, bloating, swelling, gingival bleeding. The doctor recommends performing the biochemical analysis below. Compare these values with the normal values and establish a possible diagnosis.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	60 mg/dl	75-115 mg/dl	**Direct Bilirubin**	1.0 mg/dl	<0.2 mg/dl
HbA1c	6 %	4-6 %	**Total Bilirubin**	3.9 mg/dl	<1 mg/dl
ALAT	40 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	4.1 g/dl	6.2-8 g/dl
ASAT	45 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	14.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	40 mg/dl	15-45 mg/dl	**hsCRP**	12 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	1.0 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.05 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	100 ml/min	95-150 ml/min	**Cystatin C**	0.99 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	23 mEq/l	22-27 mEq/l
Amylase	99 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	130 mg/dl	140- 200 mg/dl	**K+**	5.0 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	40 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	298 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	150 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	6 U/l	2-10 U/l	**GGT**	122 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	700 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.015	1.015- 1.025
pH	5.5	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	+(2mg/dl)	normal
bilirubin	+	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

2. Patient: **JI, sex: F, 20 years old.**

The patient presents with the following symptoms: fever, anorexia, fatigue, loss of appetite, nausea, vomiting, jaundice, dark urine. The doctor recommends performing the biochemical analysis below. Compare the values with the normal values and establish a possibile diagnosis.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia à jeun (fasting blood glucose)	75 mg/dl	75-115 mg/dl	**Direct Bilirubin**	3.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	9.8 mg/dl	<1 mg/dl
ALAT	2150 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	4.0 g/dl	6.2-8 g/dl
ASAT	2035 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	13.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	45 mg/dl	15-45 mg/dl	**hsCRP**	100 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3 mg/l high CV risk
Creatinine	0.9 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.05 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	100 ml/min	95-150 ml/min	**Cystatin C**	1.0 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	29 mEq/l	22-27 mEq/l
Amylase	100 U/l	50-100 U/l	**Na+**	122 mEq/l	135-145 mEq/l
Total Cholesterol	150 mg/dl	140- 200 mg/dl	**K+**	2.5 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	55 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	75 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	632 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	6 U/l	2-10 U/l	**GGT**	150 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	500 U/l	F: <240 U/l; M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.030	1.015- 1.025
pH	6.0	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	+++ (6mg/dl)	normal
bilirubin	++	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

3. A 65-year-old male presented to the orthopedic clinic with severe pain in his right leg and pelvis. Radiographic imaging revealed characteristic signs affecting his femur, pelvis, and skull. Laboratory tests showed normal serum parameters except for a markedly elevated alkaline phosphatase level of 2700 U/L (reference range: 30–130 U/L). The patient was subsequently started on bisphosphonate therapy to manage the disease. Comment on these results.

Lab Notes

Use this space for handwritten notes during the practical session.

L8. CLINICAL IMPLICATIONS OF SERUM α-AMYLASE, LIPASE AND GAMMA-GLUTAMYLTRANSFERASE

LEARNING OBJECTIVES

Explain the origin, function, and clinical significance of serum alpha-amylase in pancreatic and salivary gland disorders.

Explain the origin, function, and clinical significance of serum lipase in pancreatic disorders.

Describe the diagnostic role of gamma-glutamyltransferase (GGT) in liver and biliary tract disease.

Interpret combined lipase, amylase and GGT results in the differential diagnosis of abdominal and hepatobiliary conditions.

Perform and interpret the determination of serum amylase and GGT activity.

KEY POINTS

Alpha-amylase cleaves α-1,4-glycosidic bonds in starch; it is produced by the exocrine pancreas and the salivary glands.

Serum amylase > 3× the upper limit of normal is a key criterion for acute pancreatitis; lipase measurement improves specificity.

Serum lipase is a highly sensitive and specific indicator for acute pancreatitis.

GGT is a membrane-bound enzyme highly sensitive to hepatobiliary disease and excessive alcohol intake; it is elevated in cholestasis, alcoholic liver disease, and drug-induced hepatotoxicity.

Combined elevation of GGT and ALP suggests cholestasis; isolated GGT elevation points to alcohol excess or enzyme induction by drugs.

AMYLASE

Amylase (AMS) is a digestive enzyme predominantly secreted by the pancreas and salivary glands, being present in other tissues at minimal levels. Amylase was initially described in the early 1800s and is one of the pioneering enzymes to undergo scientific investigation.

The primary role of amylases is to break down the glycosidic bonds within starch molecules, transforming complex carbohydrates into simpler sugars. Amylase enzymes are categorized into 3 main classes (alpha-, beta-, and gamma amylases) each targeting distinct segments of the carbohydrate molecule.

Alpha amylase is present in humans, animals, plants, and microbes, whereas beta amylase is primarily found in microbes and plants. Gamma amylase, on the other hand, can be located in both animals and plants.

In 1908, a study by Wohlgemuth identified the presence of amylase in urine, paving the way for its application as a diagnostic laboratory test. Amylase is a frequently ordered standard diagnostic test, often combined with lipase, particularly when acute pancreatitis is suspected in patients.

Tissue Source

The pancreas and the salivary glands are the major tissue sources of serum AMS. Lesser concentrations are found in skeletal muscle and the small intestine and fallopian tubes. AMS is a small enzyme, with a molecular weight of 50,000 - 55,000 Da. Because of its small size, it is readily filtered by the renal glomerulus and also appears in the urine. Digestion of starches begins in the mouth with the hydrolytic action of salivary AMS. Salivary AMS activity, however, is of short duration because, on swallowing, it is inactivated by the acidity of the gastric contents. Pancreatic AMS then performs the major digestive action of starches once the polysaccharides reach the intestine.

Diagnostic Significance

The diagnostic significance of serum and urine AMS measurements is in the diagnosis of acute pancreatitis. Disorders of tissue other than the pancreas can also produce elevations in AMS levels. Therefore, an elevated AMS level is a nonspecific finding. However, the degree of elevation of AMS is helpful, to some extent, in the differential diagnosis of acute pancreatitis. In addition, other laboratory tests (e.g., measurements of urinary AMS levels, AMS clearance studies, AMS isoenzyme studies, and measurements of serum lipase levels), when used in conjunction with serum AMS measurement, increase the specificity of AMS measurements in the diagnosis of acute pancreatitis.

In acute pancreatitis, serum AMS levels begin to rise 2 to 12 hours after the onset of an attack, peak at 24 h, and return to normal levels within 3 to 5 days. Values generally range from 250 to 1,000 Somogyi units per dL (2.55ULN). Values can reach much higher levels. Other disorders causing an elevated serum AMS level include salivary gland lesions, such as mumps and parotitis, and other intra-abdominal diseases, such as perforated peptic ulcer, intestinal obstruction, cholecystitis, ruptured ectopic pregnancy, mesenteric infarction, and acute appendicitis. In addition, elevations have been reported in renal insufficiency and diabetic ketoacidosis.

Serum AMS levels in intra-abdominal conditions other than acute pancreatitis are usually less than 500 Somogyi units per dL.

Much interest has been focused recently on the possible diagnostic use of AMS isoenzyme measurements. Serum AMS is a mixture of a number of isoenzymes that can be separated on the basis of differences in physical properties, most notably with electrophoresis, although chromatography and isoelectric focusing also have been applied. In normal human serum, two major bands and as many as four minor bands may be seen. The bands are designated as P-type and S-type isoamylase.

-P isoamylase is derived from pancreatic tissue;

-S isoamylase is derived from salivary gland tissue, as well as the fallopian tube and lung.

The isoenzymes of salivary origin (S1, S2, S3) migrate most quickly, whereas those of pancreatic origin (P1, P2, P3), are slower (Figure 1). The most commonly observed fractions are P2, S1, and S2.

In acute pancreatitis, there is typically an increase in P-type activity, with P3 being the most predominant isoenzyme. However, P3 also has been detected in cases of renal failure and, therefore, is not entirely specific for acute pancreatitis. S-type isoamylase represents approximately two-thirds of AMS activity of normal serum, whereas P-type predominates in normal urine.

LIPASE

The lipases belong to the alpha/beta-hydrolase fold superfamily of enzymes. They work by employing chymotrypsin-like hydrolysis, which uses a histidine base, a serine nucleophile, and aspartic acid. Lipase is an enzyme that breaks down triglycerides into free fatty acids and glycerol by catalyzing the hydrolysis of the ester bonds in triglycerides. Lipases are present in pancreatic secretions and participate in fat digestion and metabolism. They play an essential role in lipid transport and serve individual functions in several tissues, including hepatic lipase in the liver, hormone-sensitive lipases in the adipocytes, lipoprotein lipase in the endothelial cells, and pancreatic lipase in the small intestine.

High levels of serum lipase may be indicative of pancreatitis. In the case of acute pancreatitis, diagnosis is based on the lab results with two of the three criteria. The criteria used for diagnosis include acute epigastric pain radiating to the back, increased serum amylase, or increased lipase levels, which are up to three times the upper limit of normal serum lipase levels. The latter is a more specific diagnostic marker than amylase or imaging with CT or MRI. 20% of the patients with acute pancreatitis can have normal serum amylase. Serum lipase is used as main biomarker in diagnosing acute pancreatitis. Moreover, serum lipase is normal in salivary gland problems and is more rarely elevated in abdominal conditions such as perforated ulcer.

Normal values: 10-60 U/L

γ-GLUTAMYLTRANSFERASE

γ-Glutamyltransferase/γ-Glutamyltranpepdidase (GGT/γ-GT) is an enzyme involved in the transfer of the γ-glutamyl residue from γ-glutamyl peptides to aminoacids, H_2O, and other small peptides. In most biologic systems, glutathione serves as the γ -glutamyl donor.

The specific physiologic function of GGT has not been clearly established, but it is suggested that GGT is involved in peptide and protein synthesis, regulation of tissue glutathione levels, and the transport of amino acids across cell membranes.

GGT activity is found primarily in the kidney, brain, prostate, pancreas, and liver.

Clinical applications of assay, however, are confined mainly **to evaluation of liver and biliary system disorders.**

Because of the effects of alcohol on GGT activity, elevated GGT levels may indicate **alcoholism**, particularly chronic alcoholism. Generally, enzyme elevations in persons who are alcoholics or heavy drinkers range from two to three times above the normal range, although higher levels have been observed. GGT assays are useful in monitoring the effects of abstention from alcohol and are used as such by alcohol treatment centers. Levels usually return to normal within 2 to 3 weeks after cessation but can rise again if alcohol consumption is resumed. Because of the susceptibility to enzyme induction, any interpretation of GGT levels must be done with consideration of the consequent effects of drugs and alcohol.

GGT levels could be also elevated in other conditions, such as acute pancreatitis, diabetes mellitus, and myocardial infarction.

GGT activity is useful in differentiating the source of elevated Alkaline Phosphatase (ALP) level because GGT levels are normal in skeletal disorders and during pregnancy. It is particularly useful in evaluating hepatobiliary involvement in adolescents because ALP activity will invariably be elevated as a result of bone growth.

Recommendations for determining GGT

GGT is the most sensitive indicator for the detection of alcoholism, being the enzyme whose increase exceeds the other liver enzymes currently dosed. In alcoholics, the serum level of GGT can reach values 50 times above the normal value, the degree of increase depending both on the amount of alcohol consumed and especially on the prolonged persistence of consumption. It also plays a role in monitoring abstinence from alcohol.

In hepato-biliary diseases, GGT correlates with alkaline phosphatase (ALP) levels. The increases are not specific and can also be associated with pancreatic, cardiac, renal diseases, diabetes. GGT testing is also useful for diagnosing liver disease in the presence of bone disease, pregnancy or childhood when ALP levels increase while GGT remains at normal levels.

Clinical significance

In interpreting the elevated GGT values, in addition to the level of GGT activity, the serum level of GGT in relation to aminotransferases, i.e. the GGT/AST or GGT/ALT ratio (in jaundice patients this ratio measures the intensity of cholestasis in relation to cell membrane damage) and the level of GGT in relation to the other cholestasis enzymes, respectively ALP, are taken into account.

1. Alcoholism

2. Liver problems:

- In **acute viral hepatitis**, the increase in GGT is lower than that of other enzymes (GGT/AST = 0.1 - 0.2), but returns to normal last GGT/AST=1;
- In **chronic active, viral or autoimmune hepatitis**, increases can exceed 7 times upper limit of normal (GGT/AST = 1 - 3);
- In **acute alcoholic hepatitis** GGT/AST > 6;
- In **liver cirrhosis**, GGT/AST increases are on average 2 times in cirrhosis and about 10 times in alcoholic cirrhosis;
- In **primary biliary cirrhosis**, GGT increases in parallel with ALP, before the appearance of jaundice, increases can reach up to 13 times the upper limit of normal;
- In **fatty liver of alcoholic etiology** GGT is about 2 times the ULN and persists increased for a long time after discontinuation of consumption, and in non-alcoholic fatty liver the slight increase in aminotransferases, more commonly than GGT;

3. Cholestasis syndrome:

- GGT and ALP increase in approximately the same proportion in mechanical cholestasis and viral, as opposed to drug-induced cholestasis in which GGT increases much more than ALP; on average, the increases exceed 6 times the upper limit of normal;
- In **extrahepatic cholestasis** GGT increases> 10 times, GGT/AST = 3 - 6 in recent obstruction and GGT/AST > 6 in long-term obstruction;
- In **intrahepatic cholestasis** (acute hepatitis, pregnancy, medications, Hodgkin's disease, parenteral nutrition, bile duct atresia, etc.) GGT increases are smaller: in pregnancy GGT does not increase as much as ALP, and children with benign recurrent cholestasis have normal GGT levels despite the presence of jaundice;

4. Tumors:

- In **primary liver tumors, liver metastases**, the evolution of GGT is parallel to that of ALP and the increases can exceed 14 times the upper limit of normal; GGT is increased in 90% of patients with metastases, and dynamic determinations can monitor the response to chemotherapy;
- In **chronic liver congestion**: GGT can increase up to 5 times, and in acute liver congestion (portal vein thrombosis), the increase is small compared to that of transaminases and LDH.

5. Medication: anticonvulsant medication (increases greater than 3 times the upper limit of normal are no longer due to treatment)

6. Isolated increases in GGT: fatty liver, subclinical biliary obstruction, congestive heart failure.

7. Other causes of GGT growth: acute pancreatitis (5 times higher than normal); acute myocardial infarction; acute renal failure, nephrotic syndrome, renal graft rejection (moderate increases); diabetes mellitus (slightly increased); brain tumors and hemorrhages (slight growths); neoplasms (moderate growths: malignant melanoma, breast cancer, lung cancer).

EXPERIMENTAL WORK

1. Determination of α-amylase

Principle

Colorimetric test with 2-chloro-4-nitrophenyl maltotrioside (CNP-G3) as direct substrate. Color is released directly as a result of a α-cleavage at the aglycone:

$$2-chloro-4-nitrophenyl\ maltotrioside \xrightarrow{\alpha-amylase} chloro-nitrophenol + glucose$$

Reagents

1. MES buffer: (pH=6), 100mmol/L
2. NaCl, 350 mmol/L
3. Ca-Acetate, 6 mmol/L
4. PotassiumThiocyanate, 900 mmol/L
5. CNP-G3

Procedure

Pipette into the test tube as follows:

Reagents, µl	Sample
Working reagent	1000
Serum	10

Incubate 1 minute at 37ºC, then read absorbance and start stopwatch at the same time. Repeat readings after exactly 1, 2 and 3 minutes. Determine the change of absorbance per minute (ΔA/min) and use this for calculation.

Calculation:

$$\text{Amylase activity} = \Delta A/\text{min} \times 15408(\text{U/L})$$

Normal values: up to 120 U/L

2. γ-Glutamyltransferase determination

Gamma-glutamyltransferase (GGT) is used in the diagnosis and monitoring of hepatobiliary diseases. GGT levels are also elevated in other conditions, such as acute pancreatitis, diabetes mellitus, and myocardial infarction. Gamma-glutamyltransferase is also a sensitive screening test for occult alcoholism.

Principle:

$$L-g-Glutamyl-3-carboxy-4-nitroanilide + Glycylglycine \xrightarrow{GGT} L-g-glutamyl-glycylglycine + 5-amino-2-nitrobenzonate$$

Gamma-glutamyltransferase transfers the g-glutamyl group of L-g-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The amount of 5-amino-2-nitrobenzonate liberated is proportional to the GGT activity and can be determined photometrically.

Reagents:
R1: TrisGlycylglycin buffer pH 8.25, 100 mmol/l
R2: L-g-Glutamyl-3-carboxy-4-nitroanilide, 2.9 mmol/l

Procedure

Pipette into the test tube as follows:

Reagents, µl	Sample
Working reagent	1000
Serum	100

Mix, read initial absorbance at 405 nm again stair, and start stop watch at the same time. Repeat readings after exactly 1, 2 and 3 minutes. Determine them change of absorbance per minute (ΔA/min) and use this for calculation.

Calculation

$$\text{GGT activity} = \Delta A/\text{min} \times 1158\ (\text{U/L})$$

Normal values:
Men: 8-61 U/L
Women: 5-40 U/L

CASE STUDY

1. Patient: **DE, sex: M, 45 years old**.

The patient presents with the following symptoms: voluntary alcohol intake, headache, anxiety. The doctor recommends performing the following the analysis below. Establish a possible diagnosis.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	100 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	29 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	6.9 g/dl	6.2-8 g/dl
ASAT	23 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	16.3 g/100ml	F: 12-16 g/dl M: 14-18 g/dl
Urea	39 mg/dl	15-45 mg/dl	**hsCRP**	8 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	1.0 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.09 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	112 ml/min	95-150 ml/min	**Cystatin C**	0.86 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	25 mEq/l	22-27 mEq/l
Amylase	77 U/l	50-100 U/l	**Na+**	140 mEq/l	135-145 mEq/l
Total Cholesterol	220 mg/dl	140- 200 mg/dl	**K+**	4.9 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	35 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	280 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	155 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	9 U/l	2-10 U/l	**GGT**	90 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	220 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.015	1.015- 1.025
pH	6.5	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

2. Patient: **KL, sex: M, 63 years old.**

The patient presents with the following symptoms: malaise, fever, nausea, vomiting, sharp pain in the upper abdomen, abdominal cramps. The doctor recommends performing the following biochemical analysis

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia à jeun (fasting blood glucose)	196 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	1.3 mg/dl	<1 mg/dl
ALAT	250 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	6.6 g/dl	6.2-8 g/dl
ASAT	170 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	14.6 g/100ml	F: 12-16 g/dl M: 14-18 g/dl
Urea	45 mg/dl	15-45 mg/dl	**hsCRP**	128 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	0.9 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.09 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	123 ml/min	95-150 ml/min	**Cystatin C**	0.96 mg/l	0.49-1.13 mg/l
Uric acid	5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	23 mEq/l	22-27 mEq/l
Amylase	1298 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	150 mg/dl	140- 200 mg/dl	**K+**	4.5 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	41 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 μUI/ml
Triacylglycerols	200 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	476 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	9 U/l	2-10 U/l	**GGT**	134 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	470 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.025	1.015- 1.025
pH	5.5	6.5
Proteins	negative	negative
Glucose	+/-(100mg/dl)	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

Compare these values with the normal values, and establish a possible diagnosis

3. A 58-year-old male (BMI=30) with a history of hypertension and moderate alcohol consumption presented to the emergency department with sudden-onset severe epigastric pain radiating to his back, accompanied by nausea and vomiting. The pain began approximately four hours after a heavy meal and progressively worsened. The patient appeared distressed due to pain. Abdominal examination revealed tenderness in the epigastric region without rebound tenderness or guarding. Bowel sounds were present but diminished. His blood pressure: 145/90 mmHg; heart rate: 110 bpm; respiratory rate: 22 breaths per minute; temperature: 37.8°C. The abdominal ultrasound revealed gallstones within the gallbladder without evidence of cholecystitis.

The laboratory investigations were as follows:

- Serum Amylase: 980 U/L (reference interval: 25–125 U/L)
- Serum Lipase: 760 U/L (reference interval: 18–180 U/L)
- Alanine Aminotransferase (ALT): 190 U/L (reference interval: 7–40 U/L)
- Aspartate Aminotransferase (AST): 220 U/L (reference interval: 10–40 U/L)
- Alkaline Phosphatase (ALP): 150 U/L (reference interval: 30–120 U/L)
- Total Bilirubin: 2.5 mg/dL (reference interval: 0.1–1.2 mg/dL)
- C-Reactive Protein (CRP): 115 mg/L (reference interval: <5 mg/L)

Comment on these results and establish a possible diagnosis.

Lab Notes

Use this space for handwritten notes during the practical session.

L9. BIOCHEMICAL INVESTIGATIONS FOR THE ENDOCRINE SYSTEM. THYROID HORMONE ANALYSIS

LEARNING OBJECTIVES

Describe the synthesis, transport, regulation, and physiological actions of thyroid hormones (T3 and T4).

Explain the hypothalamic-pituitary-thyroid (HPT) axis and its negative feedback mechanisms.

Select and interpret appropriate thyroid function tests: TSH, free T4, free T3, and thyroid antibodies.

Distinguish between hypothyroidism and hyperthyroidism based on clinical features and biochemical findings.

KEY POINTS

The thyroid gland primarily secretes T4 (thyroxine); peripheral deiodination converts T4 to the biologically active T3 (tri-iodothyronine) in target tissues.

The HPT axis: TRH (hypothalamus) → TSH (pituitary) → T3/T4 (thyroid); elevated T3/T4 suppresses TSH via negative feedback.

TSH is the most sensitive first-line test for thyroid dysfunction: suppressed TSH indicates hyperthyroidism; elevated TSH indicates hypothyroidism.

Primary hypothyroidism (most often Hashimoto's thyroiditis): high TSH, low fT4, anti-TPO antibodies. Graves' disease (hyperthyroidism): low TSH, high fT4, stimulating TSH-receptor antibodies.

GENERAL ASPECTS

A hormone is a chemical released by one or more cells that affects cells in other parts of the organism. Only a small amount of hormone is required to alter cell metabolism. It is essentially a chemical messenger that transports a signal from one cell to another. All multicellular organisms produce hormones; plant hormones are also called phytohormones. Hormones in animals are often transported in the blood. Cells respond to a hormone when they express a specific receptor for that hormone. The hormone binds to the receptor protein, resulting in the activation of a signal transduction mechanism that ultimately leads to cell type-specific responses.

Endocrine hormone molecules are secreted (released) directly into the bloodstream, while exocrine hormones are secreted directly into a duct, and from the duct they either flow into the bloodstream or they flow from cell to cell by diffusion in a process known as paracrine signaling.

Investigation of an endocrine gland looks about for the presence of clinical symptoms of hyper- or hypofunction of the referred endocrine gland.

For the evaluation of the endocrine function, the following measurements are needed either alone or in association.

- plasma concentration of the hormone
- urine excretion of either the hormone or its catabolic products
- secretion dynamics of the hormone
- the hormone reserve and hormone regulation through dynamic tests
- the level of the hormone receptors
- the selective effects of the hormone on target tissues

Measurements of the plasma concentration of the hormones

The hormone levels in the plasma are very small, between 1 nmol/l and 1 µmol/l for the steroid hormones and between 1pmol/l and 1 nmol/l for the protein and peptide hormones. Despite of so small concentrations, it is possible to measure hormones concentrations in the “jungle” of plasma proteins by using high-performance, specific and sensitive assays.

Biochemical investigation of the endocrine system takes into account the following:

- for the hormones with a constant level for one day (thyroxin, etc.) one sample is sufficient to determine the hormone sample;
- for the hormone with a pulsate secretion, like testosterone, LH, there is a need to measure the hormone concentration in multiple samples taken in different moments of the day or in a mixture from multiple samples taken with an interval of 20-30 min.
- for the hormones with a circadian rhythm (e.g. cortisol) the interpretation of obtain values is done according to the hour of sample drawing. For women, the plasma levels of gonadotrophins, estrogen and progesterone would be interpreted according to the ovarian phases and there would be needed determinations in precise days of the menstrual cycle.
- For the hormone that needs a plasma transporter (thyroid hormones and steroids), the free hormone is responsible for the hormone action and there are assays that permit the measurements of either the total or the free hormone.
- For healthy people the normal ranges of hormones concentrations in the plasma are large and the levels of the pairs of hormones and their upstream regulators (e.g. TSH and the thyroid hormones, LH and testosterone, etc.) would reflect better the clinical state. For example, if the testosterone is at its inferior plasma concentration limit and LH is increased the patient has a testicular insufficiency, but if the LH is normal, then the testicular function would be considered as normal.

The measurement of urine levels of hormones and their metabolites

For the hormones with a fluctuant secretion (cortisol for example) urine determinations could have some advantages over the determinations from isolates blood samples. The determinations are taken from urine collected in 24 hours.

Disadvantages for the urine measurements of the hormones:

- urinary secretion depends on the kidney function so, the interpretation would be according to the creatinine level.
- There are hormones which are eliminated through the bile
- The urine levels could be modified by the medication
- There are hormones with different tissue origins but with common urinary metabolites. For example, urine 17-ketosteroids rise from the adrenal gland or from gonads so they have a reduced value for the interpretation of the testicular function.

The rate of hormone secretion and production

A radioactive hormone is injected and the dilution of the hormone because of the endogenous hormone production in a precise period is measured.

The receptors and hormones antibodies assays

The measurement of hormone receptors is needed for diagnose of the diseases associated with hormone resistance. DNA techniques as sequencing are used for the detections of mutations in the protein's structures. The presence in some cases of the hormone antibodies (Graves' disease for e.g.) can be performed using immunoassays.

The effects of the hormones at the target tissues would be the ideal methods.

Modern methods for the hormone's measurements

The four major techniques used for endocrine measurements are as follows:

- Antibody-based immunologic assays, of which there are two subcategories:
  - competitive immunoassays
  - immunometric (sandwich) assays
- Chromatographic assays
- Mass spectrometry
- Nucleic acid-based assays

THYROID HORMONES

The thyroid gland produces two key hormones: thyroid hormones and calcitonin. Thyroid hormones are essential for regulating metabolism, neurological development, and various bodily functions. Calcitonin, secreted by parafollicular C cells, plays a role in calcium homeostasis.

Located in the lower anterior neck, the thyroid gland has a butterfly shape, consisting of two lobes on either side of the trachea connected by the isthmus, a thin band of thyroid tissue crossing the trachea. Positioned behind the thyroid gland are the parathyroid glands, which control serum calcium levels, and the recurrent laryngeal nerves, which innervate the vocal cords. These anatomical relationships are particularly significant during thyroid surgery, as damage to these structures can result in hypocalcemia or a permanently hoarse voice.

Thyroxine (T4) and tri-iodothyronine (T3) are known as the 'thyroid hormones'. Synthesis starts in the thyroid gland by iodination and coupling of two tyrosine molecules which are attached to a complex protein called thyroglobulin. T4 has four iodine atoms and T3 has three.

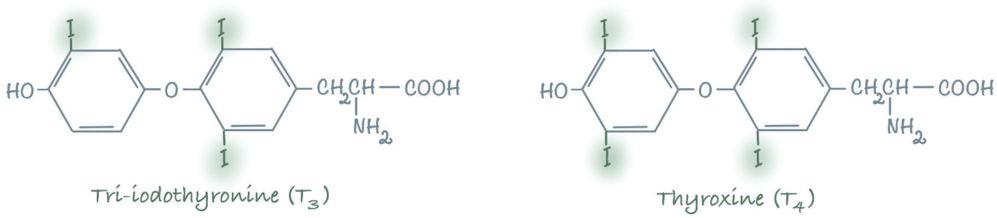


Figure 1. Chemical structures of the thyroid hormones

Thyroid hormone synthesis

Follicular cells of the thyroid gland actively transport iodide ions from the blood into the cytosol. These cells also synthesize thyroglobulin, which is a large glycoprotein. Thyroglobulin is produced in the rough endoplasmic reticulum, modified by the attachment of carbohydrate molecules in the Golgi apparatus, and then packaged into secretory vesicles. These vesicles transport thyroglobulin to the follicle lumen through exocytosis. Thyroglobulin contains several tyrosine residues, which will later undergo iodination. In the diet, iodine is ingested as iodide, which must first be oxidized to iodine before it can bind to the tyrosine residues of thyroglobulin. This oxidation occurs as iodide crosses the cell membrane into the follicle lumen. Once converted to iodine, it binds to tyrosine residues within thyroglobulin.

- Attachment of one iodine molecules to tyrosine produces monoiodothyrosine (MIT).
- Attachment of two iodine molecules results in di-iodothyrosine (DIT).
- Coupling of two DIT molecules forms thyroxine (T4).
- Coupling of MIT and DIT produces tri-iodothyronine (T3).

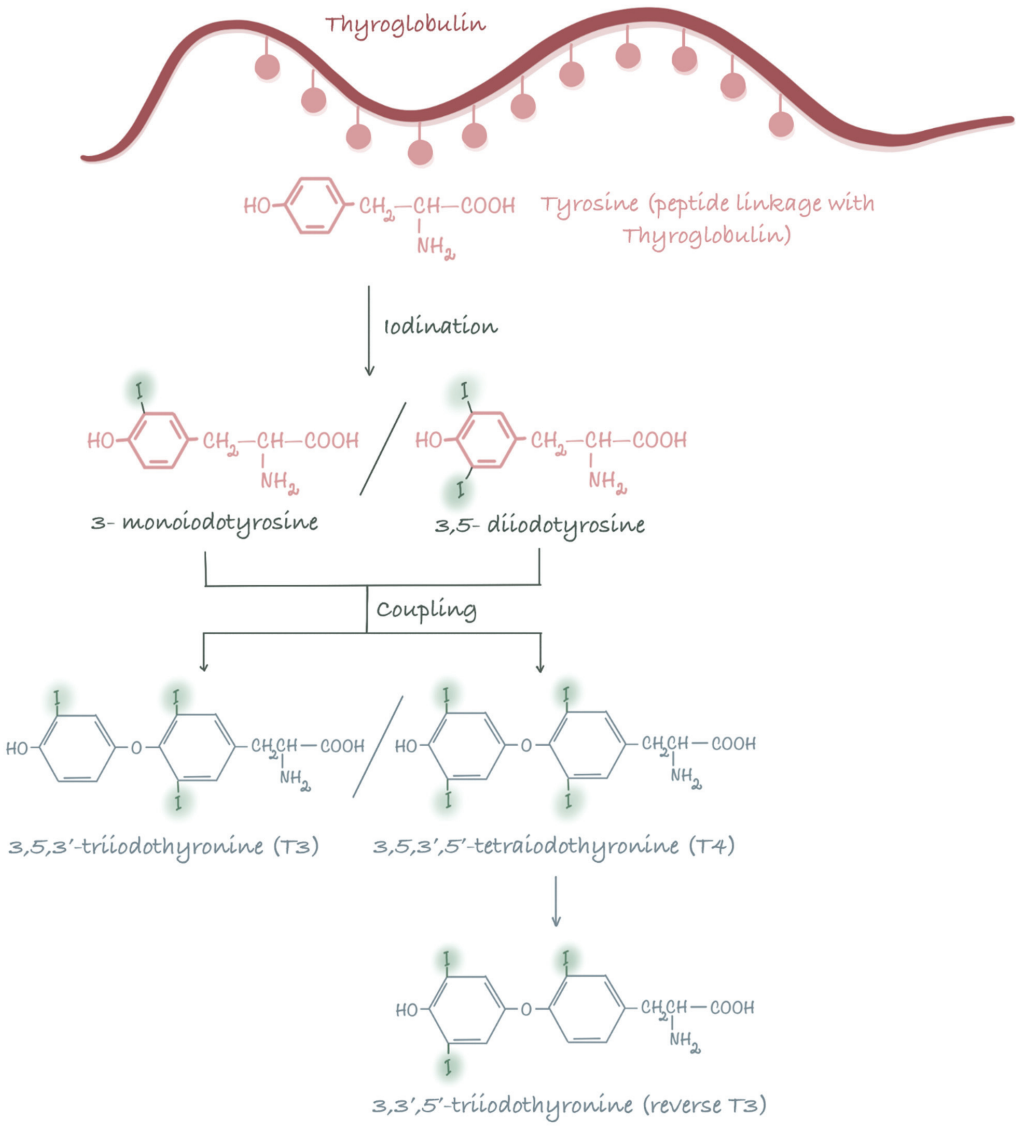


Figure 2. Thyroid hormone synthesis

The iodinated thyroglobulin, now containing T3 and T4, is stored in the colloid within the follicle. The processes of iodide oxidation, tyrosine iodination, and coupling reactions are all catalyzed by the enzyme thyroid peroxidase (TPO). Under the influence of thyroid-stimulating hormone (TSH), which is secreted by the anterior pituitary, colloid droplets re-enter follicular cells via pinocytosis and fuse with lysosomes. The lysosomal enzymes then break down thyroglobulin through proteolytic digestion, releasing T3 and T4 for secretion into the bloodstream.

The thyroid gland primarily secretes thyroxine (T4), with a plasma concentration of approximately 100 nmol/L. In peripheral tissues, particularly the liver and kidneys, T4 undergoes deiodination, converting about two-thirds of it into tri-iodothyronine (T3), which circulates at a much lower concentration of around 2 nmol/L.

Most cells can uptake T4 and convert it into the more biologically active T3 through the action of deiodinase enzymes D1 or D2**.** It is T3 that binds to thyroid hormone receptors, initiating the physiological effects of thyroid hormones. Alternatively, T4 can be metabolized by deiodinase D3, producing reverse T3 (rT3), which is biologically inactive. By adjusting the balance between T3 and rT3 production, tissues can fine-tune their local thyroid hormone activity. However, the precise mechanisms underlying this regulation are not yet fully understood**.**

Thyroid hormone synthesis regulation

The hypothalamic-pituitary-thyroid (HPT) axis consists of thyrotropin-releasing hormone (TRH), thyroid-stimulating hormone (TSH), and thyroid hormones (T3 and T4).

- TRH, a tripeptide secreted by the hypothalamus, stimulates the anterior pituitary to produce TSH, a large glycoprotein hormone.
- TSH then acts on the thyroid gland, promoting the synthesis and release of thyroid hormones.
- TSH production is regulated through negative feedback, depending on the levels of circulating unbound thyroid hormones.

Key Points for Thyroid Disease Investigation:

- Excessive thyroid hormone production leads to suppressed TSH levels.
- Insufficient thyroid hormone secretion results in elevated TSH levels, as the pituitary attempts to stimulate the thyroid.

Understanding these fundamental mechanisms is crucial for the correct interpretation of thyroid function test results in diagnosing primary thyroid disorders.

Binding in plasma

In plasma, more than 99.95% of thyroxine (T4) is transported bound to proteins. The main transport proteins are:

- Thyroxine-binding globulin (TBG) - carries ~70% of T4
- Albumin - transports ~25% of T4
- Transthyretin (prealbumin) - binds ~5% of T4

Similarly, over 99.5% of triiodothyronine (T3) is bound to the same proteins. However, it is the unbound (‘free’) T4 and T3 that exert biological effects, including regulating feedback to the pituitary and hypothalamus. Changes in binding protein concentrations can affect thyroid hormone test results, making interpretation more challenging. For example, during pregnancy, fluctuations in TBG levels can alter total hormone measurements, despite normal free T4 and T3 levels.

Thyroid hormone action

T3 binds to its nuclear receptor on thyroid hormone-responsive genes, initiating the production of messenger RNA, which subsequently leads to protein synthesis that regulates metabolism and development. The effects of thyroid hormones include promoting tissue growth, supporting brain maturation, increasing heat production and oxygen consumption, and enhancing the expression of β-adrenergic receptors. Clinically, individuals with excessive thyroid hormone levels (thyrotoxicosis) exhibit symptoms of increased metabolic activity, such as tachycardia and tremors, whereas those with hypothyroidism experience reduced metabolic activity, leading to symptoms like edema and

constipation. In hypothyroidism, lipid metabolism is impaired, often leading to elevated serum cholesterol levels. This occurs due to reduced cholesterol breakdown, caused by the downregulation of LDL receptors in the liver, leading to decreased sterol excretion via the gut. Concerning the effects on tissue maturation, the most striking impact is seen in congenital hypothyroidism. If untreated within the first 3 months of life, it can lead to irreversible brain damage. Hypothyroid children may exhibit delayed skeletal development, short stature, and late puberty.

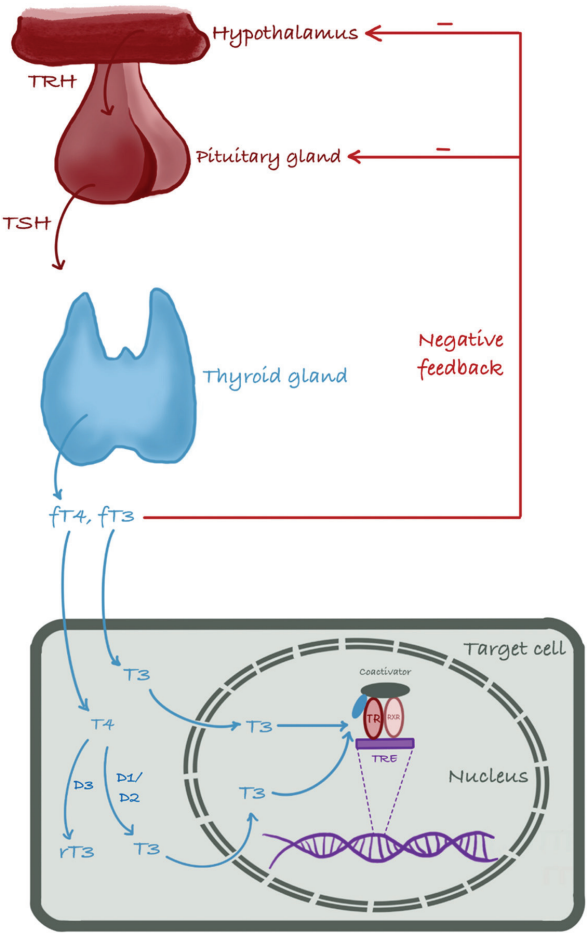


Figure 3. Thyroid hormone synthesis regulation

THYROID FUNCTION TESTS

Traditionally, biochemical measurements in the diagnosis of thyroid disease have been known as thyroid function tests. TSH and some estimate of T4 status (either total T4 or free T4) are the first-line tests.

1. TSH (Thyroid-Stimulating Hormone)

Advances in technology have significantly improved the measurement of TSH, aiding in the diagnosis and monitoring of thyroid disorders. Early TSH assays struggled to detect low hormone concentrations, with detection limits overlapping the lower end of the normal range in healthy individuals. Modern, highly sensitive TSH assays can now measure much lower levels, providing a clearer indication of whether TSH secretion is truly reduced.

Due to its log-linear relationship with TRH, TSH is highly sensitive to thyroid imbalances, making it the primary test used in many laboratories for assessing thyroid function. However, there is one key exception: in hypopituitarism, TSH measurement is unreliable for diagnosing primary thyroid disease or monitoring replacement therapy. For example, after a hypophysectomy, TSH becomes undetectable, and T4 levels must be assessed instead to determine the adequacy of treatment.

2. Total T4 or Free T4

When thyroxine replacement or anti-thyroid therapy (e.g., carbimazole) is initiated or adjusted, TSH levels may take several weeks to normalize. During this period, it is essential to monitor T4 levels to assess thyroid function, particularly during anti-thyroid treatment, as patients may rapidly develop severe hypothyroidism if left unmonitored.

3. Total T3 or Free T3

In some cases, evaluating T3 levels alongside T4 is beneficial. In hyperthyroidism, T3 levels often rise more significantly than T4, making T3 measurement useful for detecting thyrotoxicosis at an early stage. Some patients may experience T3 toxicosis, where only T3 is elevated, while T4 remains within the normal range.

4. Thyroid Antibodies

The presence of autoantibodies against thyroid tissue antigens can assist in diagnosing and monitoring autoimmune thyroid disorders.

Anti-thyroid peroxidase (anti-TPO) antibodies are particularly useful in identifying hypothyroidism, such as Hashimoto’s thyroiditis.

Stimulating thyroid receptor antibodies help diagnose thyrotoxicosis, such as Graves' disease.

THYROID DISORDERS

From a clinical perspective disorders of thyroid function can be classified into two broad categories: hyperfunction states where thyroid hormones are produced in excess, referred to as hyperthyroidism, and hypofunction states where there is a deficiency of thyroid hormones, referred to as hypothyroidism.

HYPOTHYROIDISM

Hypothyroidism usually develops slowly and clinical biochemistry has an important role to play in diagnosis.

The **clinical features** of hypothyroidism include:

- lethargy and tiredness
- slow speech and mental function
- cold intolerance
- weight gain
- dryness and coarsening of skin and hair
- hoarse voice
- slow relaxation of muscles and tendon reflexes
- many other associated signs including anemia, dementia, constipation, bradycardia, muscle stiffness, carpal tunnel syndrome, subfertility and galactorrhea, periorbital and facial edema.

Causes

Over 90% of cases of hypothyroidism appear due to:

- autoimmune destruction of the thyroid gland (Hashimoto's disease)
- radioiodine or surgical treatment of hyperthyroidism.

Less common causes:

- transient hypothyroidism resulting from medications like lithium carbonate
- TSH deficiency, which may occur as part of panhypopituitarism
- congenital defects, such as defects in the biosynthesis of T4 and T3, or end-organ resistance of target tissues to their effects.
- severe iodine deficiency.

Diagnosis

Hypothyroidism results from a deficiency of thyroid hormones. Primary hypothyroidism, caused by the failure of the thyroid gland itself, is one of the most frequently encountered endocrine disorders. A high TSH level is typically diagnostic. Secondary hypothyroidism, due to inadequate TSH secretion by the pituitary gland, is far less common. While isolated TSH deficiency is rare, dysfunction of the hypothalamic–pituitary–thyroid axis can occur due to various pituitary diseases or damage. If clinical signs suggest broader pituitary involvement beyond hypothyroidism, further evaluation of pituitary function, including a TRH test, may be necessary.

HYPERTHYROIDISM

Thyrotoxicosis occurs when tissues are exposed to excessive levels of thyroid hormones. The term 'hyperthyroidism' specifically refers to an overactive thyroid gland, but thyrotoxicosis can also result from excessive intake of T4 or, in rare cases, from increased pituitary stimulation of the thyroid.

The **clinical features** of hyperthyroidism include:

- weight loss despite normal appetite
- sweating and heat intolerance
- increased irritability
- fatigue
- palpitation – sinus tachycardia or atrial fibrillation
- agitation and tremor
- generalized muscle weakness; proximal myopathy
- angina and heart failure
- diarrhea
- oligomenorrhoea and subfertility
- goiter
- eyelid retraction and lid lag

Causes

Hyperthyroidism can result from:

- Graves' disease (diffuse toxic goitre)
- toxic multinodular goitre
- solitary toxic adenoma
- thyroiditis
- exogenously administered iodine and iodine-containing drugs, e.g. amiodarone
- excessive T4 and T3 ingestion.

Graves' disease is the most common cause of hyperthyroidism and is an autoimmune disorder in which antibodies targeting the TSH receptor on thyroid cells mimic the effects of the pituitary hormone. As a result, the usual regulatory mechanisms for T4 synthesis and secretion are disrupted. The high levels of thyroid hormones in the blood completely suppress pituitary TSH secretion.

While eyelid retraction in Graves' disease is primarily due to elevated thyroid hormone levels, not all eye-related symptoms arise from this mechanism. Instead, the thyroid and orbital muscles may share a common antigen, triggering an immune response from circulating autoantibodies. This inflammatory process can lead to severe exophthalmos, which may even develop in patients with normal thyroid function (euthyroid individuals).

Diagnosis

The diagnosis of primary hyperthyroidism is confirmed by detecting a suppressed TSH concentration alongside elevated thyroid hormone levels. In particular, when modern, highly sensitive assays show undetectable TSH in a symptomatic patient, it strongly indicates primary hyperthyroidism. However, biochemical confirmation of suspected hyperthyroidism can sometimes be challenging. Total T4 levels in serum do not always accurately reflect metabolic status due to variations in thyroid hormone-binding proteins. For instance, during pregnancy, high estrogen levels

stimulate the liver to produce more thyroid-binding globulin (TBG), leading to elevated total T4 despite normal free T4 levels. Similarly, congenital TBG deficiency can cause misleading results when screening for thyroid hormones, even in the absence of thyroid disease. Although rare, TBG deficiency is less commonly encountered than increased TBG levels.

Free T4 assays are now routinely used as primary tests for thyroid dysfunction. Because TSH secretion is highly responsive to changes in free T4, many laboratories rely solely on TSH measurements for thyroid disease screening. Free T4 testing is especially useful in cases where binding proteins are altered, such as in pregnancy, women taking oral contraceptives, and patients with nephrotic syndrome. In some individuals with clinical signs of hyperthyroidism, total T4 levels may fall within the reference range, but further testing reveals an elevated T3 concentration-this condition is known as 'T3 toxicosis.' In these cases, TSH remains undetectable.

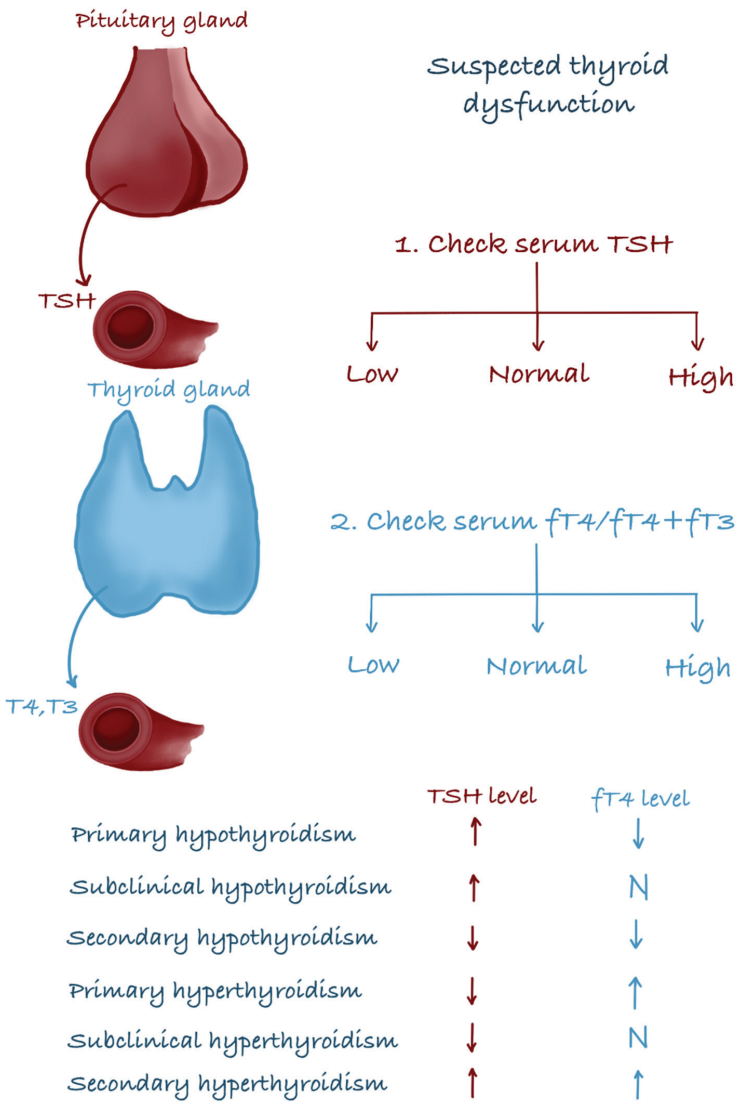


Figure 4. Diagnosis of thyroid disorders

EXPERIMENTAL WORK

Quantitative determination of thyroid stimulating hormone (TSH) concentration in human serum (ELISA TSH)

Initial laboratory tests for any patient with suspected thyroid disease should include an immunometric TSH assay. If the physician is reasonably confident that a functional disorder of the thyroid is present, an FT4 should be included as an initial test to confirm the presence and assess the degree of the abnormality inferred from the TSH result. There is rarely reason to measure total T3 in the initial evaluation unless the patient is receiving liothyronine or liotrix. The assay is useful in the diagnosis of thyroid or pituitary disorders.

Introduction

The determination of serum or plasma levels of thyroid stimulating hormone (TSH or thyrotropin) is recognized as a sensitive method in the diagnosis of primary and secondary hypothyroidism. TSH is secreted by the anterior lobe of the pituitary gland and induces the production and release of thyroxine (T4) and triiodothyronine (T3) from the thyroid gland. It is a glycoprotein with a molecular weight of approximately 28,000 daltons, consisting of two chemically different subunits, alpha and beta. Although the concentration of TSH in the blood is extremely low, it is essential for the maintenance of normal thyroid function. The release of TSH is regulated by a TSH-releasing hormone (TRH) produced by the hypothalamus. The levels of TSH and TRH are inversely related to the level of thyroid hormone. When there is a high level of thyroid hormone in the blood, less TRH is released by the hypothalamus, so less TSH is secreted by the pituitary. The opposite action will occur when there is decreased thyroid hormone in the blood. This process is known as a negative feedback mechanism and is responsible for maintaining the proper blood levels of these hormones. TSH and the pituitary glycoproteins: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG), have identical alpha chains. The beta chains are distinct but do contain regions with identical amino acid sequences. These regions of homology can cause considerable cross-reactivity with some polyclonal TSH antisera. The use of a monoclonal antibody in **ELISA TSH** test eliminates this interference, which could result in falsely elevated TSH values in either menopausal or pregnant females, a population whose evaluation of thyroid status is clinically significant.

Principle

The **ELISA TSH** test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the intact TSH molecule. Mouse monoclonal anti-TSH antibody is used for solid phase immobilization (microtiter wells), and goat anti-TSH antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the TSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 60-minute or overnight incubation at room temperature, the solid phase is washed with water to remove unbound labeled antibodies. A solution of 3,3',5,5'-tetramethylbenzidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the resulting yellow color is measured spectrophotometrically at 450 nm. The concentration of TSH is directly proportional to the color intensity of the test sample.

Reagents

1. Antibody-Coated Wells (1 plate, 96 wells). Microtiter wells coated with mouse monoclonal anti-TSH.

2. Enzyme Conjugate Reagent (1 bottle, 13 mL). Contains goat anti-TSH conjugated to horseradish peroxidase.

3. Reference Standard Set (1 mL/vial). Contains 0, 0.5, 2.0, 5.0, 10.0 and 25.0 µIU/mL TSH in equine serum with preservatives. Lyophilized.

4. TMB Reagent (1 bottle, 11 mL). Contains 3,3',5,5'-tetramethylbenzidine (TMB) stabilized in buffer solution.

5. Stop Solution (1N HCl) (1 bottle, 11 mL). Contains diluted hydrochloric acid.

Materials required but not provided

1. Distilled or deionized water

2. Precision pipettes: 50 µl, 100 µl, 200 µl, and 1 ml

3. Disposable pipette tips

4. Microtiter well reader capable of reading absorbance at 450nm.

5. Orbital motion microtiter well shaker

6. Absorbent paper

7. Graph paper (semi-log, etc.)

Instrumentation

A microtiter well reader with a bandwidth of 10nm or less and an optical density range of 0 to 2 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

Specimen collection and preparation

1. Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only. Avoid grossly hemolytic, lipemic, or turbid samples.

2. Specimens should be capped and may be stored for up to 48 hours at 2-8°C prior to assaying. Specimens held for a longer time should be frozen only once at -20°C prior to assay. Thawed samples should be inverted several times prior to testing.

Reagent preparation

1. All reagents should be allowed to reach room temperature (18-25°C) before use.

2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.

3. Reconstitute each lyophilized standard with 1.0 mL dH20. Allow the reconstituted material to stand for at least 20 minutes. Reconstituted standards should be stored sealed at 2-8°C, and are stable at 2-8°C for at least 30 days.

4. Specimens expected to have a TSH concentration greater than 25μIU/mL should be diluted 1:10 with TSH-free serum (Zero calibrator).

Procedural notes

1. Manual pipetting: It is recommended that no more than 32 wells be used for each assay run. Pipetting of all standards, samples, and controls should be completed within 3 minutes.

2. Automated pipetting: A full plate of 96 wells may be used in each assay run. However, it is recommended that pipetting of all standards, samples, and controls be completed within 3 minutes.

3. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.

4. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

Assay procedure

1. Secure the desired number of coated wells in the holder.

2. Dispense 100μl of standards, specimens, and controls into appropriate wells.

3. Dispense 100μl of Enzyme Conjugate Reagent into each well.

4. Thoroughly mix for 30 seconds. It is very important to have complete mixing.

5. Incubate at room temperature (18-25°C) for 60 minutes (1 hour).

6. Remove the incubation mixture by flicking plate contents into a waste container.

7. Rinse and flick the microtiter wells 5 times with distilled or dionized water. (Please do not use tap water).

8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.

9. Dispense 100μl of TMB Reagent into each well. Gently mix for 10 seconds.

10. Incubate at room temperature, for 20 minutes.

11. Stop the reaction by adding 100μl of Stop Solution to each well.

12. Gently mix for 30 seconds. *Ensure that all the blue color changes completely to yellow.*

13. Read absorbance at 450nm with a microtiter plate reader within 15 minutes.

Calculation of results

1. Calculate the mean absorbance value (OD450) for each set of reference standards, controls and samples.

2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in μIU/ml, with absorbance on the

vertical (y) axis and concentration on the horizontal (x) axis.

3. Using the mean absorbance value for each sample, determine the corresponding concentration of TSH in μlU/ml from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

4. Any diluted samples must be further corrected by the appropriate dilution factor.

Normal values:

	TSH (μlU/ml)
Adults	
21-54 years	0.4-4.2
55-87 years	0.5-8.9
Pregnancy	
1st Trimester	0.3-4.5
2nd Trimester	0.5-4.6
3rd Trimester	0.8-5.2

CASE STUDY

1. Patient: **IK, sex: F, 40 years old**.

The patient presents with the following symptoms: weight gain in the past 6 months, fatigue, weakness, always feeling cold, decreased intellectual performance, irregular menstrual cycles, dysuria, pollakiuria. The doctor recommends performing the analysis below.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	100 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	25 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	7.1 g/dl	6.2-8 g/dl
ASAT	21 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	10.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	25 mg/dl	15-45 mg/dl	**hsCRP**	16 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	0.7 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.03 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	102 ml/min	95-150 ml/min	**Cystatin C**	1.00 mg/l	0.49-1.13 mg/l
Uric acid	5.5 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	25 mEq/l	22-27 mEq/l
Amylase	75 U/l	50-100 U/l	**Na+**	138 mEq/l	135-145 mEq/l
Total Cholesterol	296 mg/dl	140- 200 mg/dl	**K+**	5.1 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	45 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	42 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	306 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	0.5 pg/ml	2.2-5.5 pg/ml
LDH	236 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	0.2 ng/dl	0.7-2.2 ng/dl
CKMB	20 U/l	2-10 U/l	**GGT**	31 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	100 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.025	1.015- 1.025
pH	7.5	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	+(25/µl)	negative
Red blood cells	negative	negative

Compare these values with the normal values and establish a possible diagnosis.

2. Patient: **LM, sex: F, 19 years old**

The patient presents with the following symptoms: general weakness, nervousness, anxiety, irritability, sweating, clammy and red skin, hand tremors, weight loss. The doctor recommends performing the following biochemical analysis:

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	69 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	19 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	6.6 g/dl	6.2-8 g/dl
ASAT	15 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	11.6 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	20 mg/dl	15-45 mg/dl	**hsCRP**	6 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	0.6 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.06 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	103 ml/min	95-150 ml/min	**Cystatin C**	0.99 mg/l	0.49-1.13 mg/l
Uric acid	8 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	26 mEq/l	22-27 mEq/l
Amylase	68 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	101 mg/dl	140- 200 mg/dl	**K+**	4.5 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	51 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	0.03 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	70 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	16.0 pg/ml	2.2-5.5 pg/ml
LDH	151 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	8.3 ng/dl	0.7-2.2 ng/dl
CKMB	6 U/l	2-10 U/l	**GGT**	11 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	201 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.010	1.015- 1.025
pH	5.0	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	+(15mg/dl)	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	negative	negative
Red blood cells	negative	negative

Compare these values with the normal values and establish a possible diagnosis.

3. Patient: **EF, sex: F, 68 years old.**

The patient presents with the following symptoms: weight loss in the past six months, anxiety, sweating, headache, thirst, nausea and vomiting, oliguria, abdominal pain, lower limb edema. The doctor recommends performing the following biochemical analysis:

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia a jeun (fasting blood glucose)	80 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	15 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	6.4 g/dl	6.2-8 g/dl
ASAT	19 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	14.9 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	126 mg/dl	15-45 mg/dl	**hsCRP**	10 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	3.2 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.06 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	50 ml/min	95-150 ml/min	**Cystatin C**	6.22 mg/l	0.49-1.13 mg/l
Uric acid	13 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	15 mEq/l	22-27 mEq/l
Amylase	89 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	189 mg/dl	140- 200 mg/dl	**K+**	7.2 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	45 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	0.10 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	130 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	10.0 pg/ml	2.2-5.5 pg/ml
LDH	155 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	5.1 ng/dl	0.7-2.2 ng/dl
CKMB	9 U/l	2-10 U/l	**GGT**	20 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	155 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.010	1.015- 1.025
pH	7.0	6.5
Proteins	++(100mg/dl)	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	negative	negative
Leucocytes	++(75/µl)	negative
Red blood cells	++(50/µl)	negative

Compare these values with the normal values, and establish a possible diagnosis

Lab Notes

Use this space for handwritten notes during the practical session.

L10. LABORATORY ASSESSMENT OF SEXUAL HORMONES

LEARNING OBJECTIVES

Classify the main categories of sexual hormones (protein and steroid) and describe their physiological roles.

Explain the regulation of the hypothalamic-pituitary-gonadal (HPG) axis.

Interpret laboratory measurements of FSH, LH, hCG, oestrogens, progesterone, and testosterone in clinical contexts.

Identify conditions associated with abnormal levels of reproductive hormones.

KEY POINTS

Protein sexual hormones (FSH, LH, hCG, prolactin) act via membrane receptors and second-messenger systems; steroid hormones (oestrogens, progesterone, testosterone) act via intracellular nuclear receptors.

The HPG axis: GnRH (hypothalamus) → FSH and LH (anterior pituitary) → sex steroids (gonads); negative feedback by oestrogens and progesterone regulates the cycle.

FSH promotes follicular growth in women and spermatogenesis in men; LH triggers ovulation and sustains the corpus luteum; hCG maintains the corpus luteum in early pregnancy.

Oestrogen, progesterone, and testosterone levels vary with sex, age, and menstrual cycle phase; results must always be interpreted with reference to the appropriate physiological range.

GENERAL ASPECTS

Sexual hormones are of two types: ***proteins*** and ***steroids***.

The steroids have a molecular weight of about 300 daltons, presenting thus a small size. Because of their size and liposolubility (soluble in fat), they can easily diffuse through target cells, having consequently intra-cellular receptors. While the protein hormones weigh more than 5.000 daltons and therefore cannot penetrate the cells, they need membrane receptors. The steroids can circulate in the blood stream, only if bound to non-specific proteins like albumin, or to specific proteins like SHBG (sex hormone binding globulin). On the other hand, the protein hormones are hydro-soluble and circulate freely in the blood.

THE PROTEIN HORMONES

A. The gonadotropins

These hormones are produced by different tissues (pituitary and placenta) and their main function is gonadic regulation (ovaries and testicles). The gonadotropins include:

- FSH (folliculo-stimulating hormone),
- LH (luteinizing hormone) and
- hCG (human chorionic gonadotropin).

A1. Folliculo-stimulating hormone (FSH)

FSH is a glycoprotein produced by the pituitary gland and has a molecular weight of about 30.000 daltons. Morphologically, it is a heterodimer composed by two different sub-units: a and b. The a sub-unit (89 amino acids) is common to all gonadotropins and also to TSH (thyrotrophic hormone). The b sub-unit (118 amino acids) is specific for FSH.

The main function of FSH is to promote and sustain the ovarian follicular growth in women and the spermatogenesis in men. FSH stimulates also the synthesis of its own receptor on the granulosa and Sertoli cells and the LH receptor on granulosa cells. It stimulates the aromatase activity inside the granulosa cells (the enzyme converting the androgens into estrogens).

FSH synthesis and secretion by the pituitary gland is controlled by different regulators, like: GnRH (gonadotropin releasing hormone of hypothalamic origin), ovarian estrogens, activine and inhibine (both of gonadic origin).

A2. Luteinizing hormone (LH)

LH is a glycoprotein with a molecular weight of approximately 30.000 daltons, and produced by the pituitary gland. Like FSH, LH is also present as a heterodimer with two different sub-units: a and b.

The principal functions of LH are:

1. promoting the androgen synthesis in the thecal cells of the ovaries and in the interstitial cells of the testicles,

2. inducing ovulation (by stimulating the cascade of proteolytic enzymes leading to the rupture of basement membrane of the follicle) and

3. maintaining the corpus luteum during the menstrual cycle.

LH synthesis and secretion from the pituitary gland is controlled by different regulators, like GnRH (gonadotropin releasing hormone of hypothalamic origin) and ovarian estrogens and progesterone.

In women, LH and FSH levels can help to differentiate between primary ovarian failure (failure of the ovaries themselves or lack of ovarian development) and secondary ovarian failure (failure of the ovaries due to disorders of either the pituitary or the hypothalamus). Increased levels of LH and FSH are seen in primary ovarian failure (e.g., Turner's syndrome, 17-alpha-hydroxylase deficiency, radiation, chemotherapy, thyroid disease, ovarian tumor). When a woman enters menopause and her ovaries stop working, LH levels will rise.

Low levels of LH and FSH are seen in secondary ovarian failure and indicate a problem with the pituitary or hypothalamus.

In men, high LH levels indicate primary testicular failure. This can be due to developmental defects in testicular growth or to testicular injury (e.g., Klinefelters syndrome, radiation, chemotherapy).

The results for the test for LH response to GnRH can help differentiate between primary dysfunction (failure of the ovaries or testes) and secondary disorder (a problem involving the pituitary or hypothalamus). Once the baseline level of LH has been measured, a dose of GnRH is given by injection. A subsequent increase in the level of LH indicates that the pituitary responded to the GnRH and points to a disorder involving the ovaries or testes. A reduced level of LH shows that the pituitary did not respond to the GnRH and suggests a disease involving the pituitary or hypothalamus.

In young children, high levels of LH and FSH and/or development of secondary sexual characteristics at an unusually young age are an indication of precocious puberty. This is much more common in girls than in boys. In delayed puberty, LH and FSH levels can be normal or below what is expected for a youth within the age range of puberty. The test for LH response to GnRH may need to be performed along with other testing to diagnose the reason for the delayed puberty.

A3. Human chorionic gonadotropin (hCG)

hCG is a glycoprotein with a molecular weight of about 43.000 daltons, produced by the syncytiotrophoblast. It is a heterodimer composed by two different sub-units: a and b. The specific b sub-unit contains 145 amino acids and can be distinguished from the b sub-unit of LH only by 30 amino acids in the C terminal part of the molecule.

The whole molecule of hCG is also called holo-hCG, in order to be distinguished from total hCG, currently measured in the labs, (holo-hCG + free b sub-unit). The free b sub-unit circulates also in the blood. A particular form of hCG, called "nicked" hCG, is a holo-hCG or a free b sub-unit, where the bond between the 46th and the 47th amino acid is broken. This gives rise to a particular tri-dimensional form of the molecule, making it often difficult to be recognized by the antibodies used for its measurement. The immunological recognition of "nicked" forms is especially important when hCG is measured to determine the risk of a mother to carry a trisomy 21 baby (known as the "double test or triple test"), as in this chromosomal pathology, the "nicked" forms increase significantly.

The function of hCG is essential to maintain the corpus luteum of pregnancy and its progesterone secretion. But it has also an anti-gonadotrophic effect, as it inhibits the secretion of LH and FSH. hCG is said to be a "steroidogenic" hormone, not only because it favors the secretion of progesterone by the corpus luteum, but also because it stimulates the steroid secretion from fetal gonads. The regulation of hCG synthesis and secretion is provided by a trophoblastic GnRH.

B. Prolactin (Prl)

Human prolactin is a non-glycosylated protein, which contains a simple polypeptide chain of 198 amino acids. It is structurally similar to hPL (human placental lactogen) and to growth hormone (GH). In the circulation, prolactin can appear in its monomeric (little prolactin) or polymeric form (big or big-big prolactin), as well. The quantitative relationship between these different forms varies according to physiologic and pathologic conditions and according to the antibody used for their measurement.

Prolactin is essentially of pituitary origin, but the stromal cells of the endometrium produce prolactin during the secretory phase, as well. This hormone is considered as a marker of decidualisation.

The principal biological function of prolactin in women is to control breast development and lactation.

The role of prolactin in men and of endometrial prolactin in women is not yet known. If progesterone is the main regulator of endometrial prolactin, the pituitary prolactin is essentially controlled by dopamine (called also PIF or prolactin inhibiting factor). However, TRH (thyroid releasing hormone) and VIP (vasoactive intestinal peptide) are capable of stimulating the release of pituitary prolactin.

Men and non-pregnant women will normally have only small amounts of prolactin in their blood. Prolactin levels do, however, need to be evaluated based on the time of day that they are collected. The levels will vary over a 24-hour period, rising during sleep and peaking in the morning.

High levels of prolactin (hyperprolactinemia) are normal during pregnancy and after childbirth while the mother is nursing. High levels are also seen with:

- Anorexia nervosa

- Drugs: Estrogen, tricyclic antidepressants, and drugs that block the effect of dopamine (a brain chemical that regulates and inhibits the production of prolactin) such as: tranquilizers, some hypertension drugs, and some drugs that are used to treat gastroesophageal reflux

- Hypothalamic diseases

- Hypothyroidism

- Kidney disease

- Nipple stimulation (moderate increase)

- Other pituitary tumors and diseases

- Polycystic ovary syndrome

- Prolactinomas

Levels of prolactin that are below normal are not usually treated but may be indicative of a more general hypopituitarism (decreased pituitary function and decreased hormone production). Low levels may also be caused by drugs such as dopamine.

THE STEROID HORMONES

A. Estrogens

Estrogens are of three types: estrone (E1), estradiol (E2) and estriol (E3).

At equal concentrations, E2 has a stronger biological effect than E1 which is more powerful than E3. E2 can be reversibly converted to E1 and E1-sulfate. This sulfate is quantitatively the most important metabolite in the circulation. The enzymatic conversion of estrogens occurs in the liver. Estrogens are excreted in the urine as glucuronides or sulfates

Estradiol (E2)

In women of reproductive age, E2 is essentially produced by the enzymatic conversion of androgens (androstenedione and testosterone). The androgens are produced by the thecal cells under the influence of LH and their conversion in E2 occurs in the granulosa cells of the follicle, through the enzyme aromatase. Aromatase activity depends on FSH levels. Thus, a harmonious secretion of E2 is dependent on the two pituitary gonadotropins.

In the post-menopausal women, the low level of E2 is provided by peripheral (liver, fat and muscular tissues) conversion (aromatisation) of androgens secreted by the adrenal glands.

In men, 20% of circulating E2 is provided by a Sertoli cell production and 80% comes from the peripheral conversion of androgens.

The principal functions of E2 in women are the mitotic effect on the uterine mucosa and on the breast, the feed-back (positive and negative) on pituitary gonadotropins and its role in bone mineralization.

Estrone (E1)

In women of reproductive age, E1 is mainly produced from the enzymatic conversion of androstenedione, which is secreted under the influence of LH by the thecal cells. The aromatase activity depends on FSH. In the menopausal women and in men, E1 and its sulfate represent the main circulating estrogens.

The biological function of E1 is still speculative, but it could be related to the regulatory effect that the conversion of E1 into E2 has on the degree of estrogenisation.

Estriol (E3)

In women of reproductive age, the very low concentrations of E3 are produced by hepatic hydroxylation of E1 and E2. During pregnancy, E3 is produced in large quantities from the feto-placental unit. As 17-hydroxylase is lacking in the placenta and 3-b-hydroxy-dehydrogenase is absent in the fetus, the E3 production is dependent on a feto-placental collaboration. The mechanism is as follows, placental pregnenolone (the precursor of progesterone) is reduced to dehydroepiandrosterone sulfate (DHEAS) in the fetal adrenal glands. DHEAS returns to the placenta, where it is transformed into androstenedione and then into E3. The E3 concentrations strongly increase during pregnancy, reflecting thus the feto-placental co-operation. For this reason, the level of E3 has been used for a long time to assess high-risk pregnancies.

The biological role of E3 remains still unknown. Estrogen fraction test is done to evaluate sexual maturity, menstrual problems, and fertility problems in females. This test may also be used to test for tumors that excrete estrogen. In pregnant women it aids in determining fetal-placental health. Estrogen fraction is used in men to evaluate gynecomastia, or feminization syndromes.

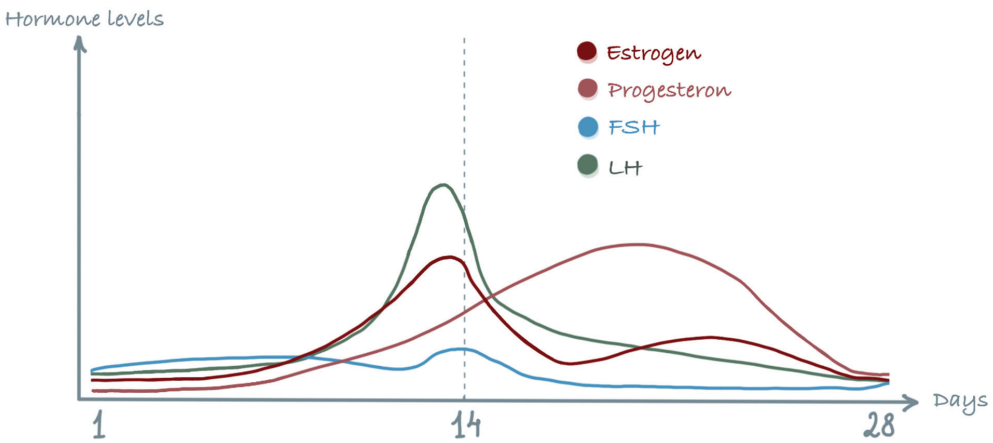


Figure 1. Hormone evolution during a menstrual cycle

B. Progesterone (P4)

In non-pregnant women of reproductive age, P4 is essentially of ovarian origin, the participation of the adrenal cortex is negligible. In the middle of the menstrual cycle, it is the LH peak which induces biochemical and phenotypical changes of granulosa cells, called also "the process of luteinisation". This process makes the granulosa cells capable to produce progesterone. Thus, progesterone, which becomes detectable from midcycle onwards, is essentially produced by the corpus luteum. In the beginning of pregnancy (< 12 weeks), P4 is also produced by the corpus luteum; after 12 - 14 weeks of pregnancy the synthesis and production of P4 are exclusively of placental origin

The biological role of P4 is to transform the uterine mucosa, already stimulated by E2, in a secretory mucosa, which can receive a fertilized ovum. On the other hand, progesterone inhibits uterine contractions. Progesterone synthesis in the corpus luteum is stimulated by LH and hCG. The regulation of progesterone production in the placenta is not yet known, but it is thought to be partly dependent on hCG.

Progesterone assay test is ordered to evaluate women who are having difficulty becoming pregnant or maintaining a pregnancy, and to monitor high-risk pregnancies.

C. Testosterone (T)

In women of reproductive age, T is produced by the thecal cells which surround the follicle. This androgen (T) serves as a substrate for the synthesis of E2, but it is also detected in circulation, even in very low concentrations. In men testosterone is produced by Leydig cells, but the contribution of adrenal androgens cannot be neglected, especially in certain pathologies of the new born. The biological role of T in women is to favor follicular atresia (a follicle which has a diminished capacity of aromatisation cannot aromatase all androgens becomes atretic). In men, T provides the appearance of secondary sex characteristics (voice, pilosity) and controls gonadotropins secretion.

Testosterone levels are ordered to evaluate:

- ambiguous sex characteristics
- precocious puberty
- virilizing syndromes in the female
- infertility in the male
- rare tumors of the ovary and testicle

Overproduction of testosterone caused by testicular, adrenal, or pituitary tumors in the young male may result in precocious puberty.

Overproduction of testosterone in females, caused by ovarian and adrenal tumors, can result in masculinization, the symptoms of which include cessation of the menstrual cycle (**amenorrhea**) and excessive growth of body hair (**hirsutism**).

When reduced levels of testosterone in the male indicate underactivity of the testes (**hypogonadism**), testosterone stimulation tests may be ordered.

Preparation for the tests

The progesterone and testosterone tests require a blood sample; it is not necessary for the patient to restrict food or fluids before the test.

Testosterone specimens should be drawn in the morning, as testosterone levels are highest in the early morning hours.

The estrogen fraction test can be performed on blood and/or urine.

Normal values

Estrogen levels vary in women, ranging from 24-149 picograms per ml of blood. In men, the normal range is between 12-34 picograms per ml of blood.

Progesterone levels vary from less than 150 nanograms per deciliter (ng/dl) of blood to 2,000 nanograms in menstruating women. During pregnancy, progesterone levels range from 1,500-20,000 ng/dl of blood.

Testosterone values vary from laboratory to laboratory, but can generally be found within:

- Men. 300-1,200 ng/dl
- Women. 30-95 ng/dl
- Prepubertal children:
  - Less than 100 ng/dl (boys),
  - Less than 40 ng/dl (girls).

Abnormal results

Increased levels of estrogen are seen in feminization syndromes:

- when a male begins to develop female secondary sex characteristics
- during precocious puberty
- when children develop secondary sexual characteristics at an abnormally early age
- because of ovarian, testicular, or adrenal tumor
- during normal pregnancy, cirrhosis, and increased thyroid levels (hyperthyroidism)

Decreased levels of estrogen are found in the following conditions:

- a failing pregnancy
- during menopause
- anorexia nervosa
- primary and secondary hypogonadism
- Turner's syndrome, seen in females with one missing X chromosome (45 X)

Increased levels of progesterone are seen:

- during ovulation and pregnancy
- with certain types of ovarian cysts
- with a tumor of the ovary known as a choriocarcinoma

Decreased levels of progesterone are seen:

- in toxemia of pregnancy
- with a threatened abortion
- during placental failure
- after fetal **death**
- with amenorrhea
- due to ovarian dysfunction

Increased levels (male) of testosterone are found in:

- sexual precocity
- the viral infection of encephalitis
- tumors involving the adrenal glands
- testicular tumors
- excessive thyroid production (hyperthyroidism)
- testosterone resistance syndromes.

Increased levels (females) of testosterone are found:

- ovarian tumors
- adrenal tumors
- in the presence of excessive hair growth of unknown cause (hirsutism).

Decreased levels (male) of testosterone are seen in:

- Klinefelter syndrome (males 47 XXY, 48 XXXY)
- a chromosomal deficiency
- primary and secondary hypogonadism
- Down syndrome
- surgical removal of the testicles
- cirrhosis.

Commercial tests

FSH - Follicular Stimulating Hormone Tests

FSH, or follicle-stimulating hormone, is excreted by the pituitary gland. During the follicular phase of the menstrual cycle, FSH fosters the growth and maturation of a woman's eggs. If a woman experiences infertility or approaches menopause, it may be a sign of a low ovarian egg supply - and this is indicated by a high level of FSH. With infertility issues, the FSH hormone is released in higher-than-normal amounts to stimulate the ovaries into producing a mature egg and more estrogen. The FSH Urine Test is a test kit for the determination of FSH (human Follicular Stimulating Hormone) concentration in urine specimens.

Saliva ovulation predictors (Saliva fertility tests)

When a woman is about to ovulate, her saliva begins to form a distinct fern-like pattern due to an increase in the level of salt and estrogen. This visible ferning pattern begins to appear around 3 to 4 days prior to ovulation. Ovulation microscopes - or saliva fertility tests - allow you to predict ovulation by viewing the changes in the make up of your saliva prior to ovulation.

With the saliva-based ovulation fertility tests, just add a drop of saliva to the lens and let the sample dry. In five minutes, view the sample through the microscope.

EXPERIMENTAL PART

1. Quantitative determination of luteinizing hormone in human serum (ELISA LH)

Introduction

This assay is useful in the diagnosis and treatment of gonadal dysfunction.

Luteinizing hormone (LH) is produced in both men and women from the anterior pituitary gland in response to luteinizing hormone-releasing hormone (LH-RH or Gn-RH), released by the hypothalamus. LH, also called interstitial cell-stimulating hormone (ICSH) in men, is a glycoprotein with a molecular weight of approximately 30,000 daltons. It is composed of two non-covalently associated dissimilar amino acid chains, alpha and beta. The alpha chain is similar to that found in human thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). The differences between these hormones lie in the amino acid composition of their beta subunits, which account for their immunological differentiation. The basal secretion of LH in men is episodic and has the primary function of stimulating the interstitial cells (Leydig cells) to produce testosterone. The variation in LH concentrations in women is subject to the complex ovulatory cycle of healthy menstruating women, and depends upon a sequence of hormonal events along the gonado-hypothalamic-pituitary axis. The decrease in progesterone and estradiol levels from the preceding ovulation initiates each menstrual cycle. As a result of the decrease in hormone levels, the hypothalamus increases the secretion of gonadotropin-releasing factors (GnRF), which in turn stimulates the pituitary to increase FSH production and secretion. The rising FSH levels stimulate several follicles during the follicular phase; one of these will mature to contain the egg. As the follicle develops, estradiol is secreted slowly at first, but by day 12 or 13 of a normal cycle, increases rapidly. LH is released as a result of this rapid estradiol rise because of direct stimulation of the pituitary and increasing GnRF and FSH. These events constitute the pre-ovulatory phase. Ovulation occurs approximately 12 to 18 hours after the LH reaches a maximum level. After the egg is released, the corpus luteum is formed which secretes progesterone and estrogen, the feedback regulators of LH. The luteal phase rapidly follows this ovulatory phase, and is characterized by high progesterone levels, a second estradiol increase, and low LH and FSH levels. Low LH and FSH levels are the result of the negative feedback effects of estradiol and progesterone on the hypothalamic-pituitary axis. After conception, the developing embryo produces hCG, which causes the corpus luteum to continue producing progesterone and estradiol. The corpus luteum regresses if pregnancy does not occur, and the corresponding drop in progesterone and estradiol levels results in menstruation. The hypothalamus initiates the menstrual cycle again as a result of these low hormone levels. Patients suffering from hypogonadism show increased concentrations of serum LH. A decrease in steroid hormone production in females is a result of immature ovaries, primary ovarian failure, polycystic ovary disease, or menopause. LH secretion is not regulated in these cases. A similar loss of regulatory hormones occurs in males when the testes develop abnormally or anorchia exists. High concentrations of LH may also be found in primary testicular failure and Klinefelter syndrome, although LH levels will not necessarily be elevated if the secretion of androgens continues. Increased concentrations of LH are also present during renal failure, cirrhosis, hyperthyroidism, and severe starvation. A lack of secretion by the anterior pituitary may cause lower LH levels. As may be expected, low levels may result in infertility in both males and females. Low levels of LH may also be due to the decreased secretion of GnRH by the hypothalamus. Low LH values may therefore indicate some dysfunction of the pituitary or hypothalamus, but the actual source of the problem must be confirmed by other tests. In the differential diagnosis of hypothalamic, pituitary, or gonadal dysfunction, assays of LH concentration are routinely performed in conjunction with FSH assays as their roles are closely interrelated. Furthermore, the hormone levels are used to determine menopause, pinpoint ovulation, and monitor endocrine therapy.

Principle of the assay

The ELISA LH is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a mouse monoclonal anti-α-LH antibody for solid phase (microtiter wells) immobilization, and a mouse monoclonal anti-β-LH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45 minutes incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of Tetramethylbenzidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the resulting yellow color is measured spectrophotometrically at 450 nm. The concentration of LH is directly proportional to the color intensity of the test sample.

Reagents and materials provided

1. Antibody-Coated Wells (96 wells). Microtiter wells coated with mouse anit-α-LH antibody.

2. Enzyme Conjugate Reagent (13 ml). Contains mouse monoclonal anti-LH conjugated to horseradish peroxidase.

3. Reference Standard Set. Contains 0, 5, 15, 50, 100, and 200 mIU/ml human LH in bovine serum with preservatives.

4. TMB Reagent (11 ml). Contains 3,3',5,5'-tetramethylbenzidine (TMB) stabilized in buffer solution.

5. Stop Solution - 1N HCl (11 ml). Contains diluted hydrochloric acid.

Materials required but not provided

1. Distilled or deionized water

2. Precision pipettes: 0.05, 0.1, 0.2, and 1 ml

3. Disposable pipette tips

4. Microtiter well reader capable of reading absorbance at 450nm

5. Vortex mixer or equivalent

6. Absorbent paper

7. Linear graph paper

Instrumentation - see TSH

Specimen collection and preparation - see TSH

Assay procedure

1. Secure the desired number of coated wells in the holder.
2. Dispense 50µl of standards, specimens, and controls into appropriate wells.
3. Dispense 100µl of Enzyme Conjugate Reagent into each well.
4. Thoroughly mix for 30 seconds. It is very important to have complete mixing.
5. Incubate at room temperature (18-25°C) for 45 minutes.
6. Remove the incubation mixture by flicking plate contents into a waste container.

7. Rinse and flick the microtiter wells 5 times with distilled or deionized water.
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 100µl of TMB Reagent into each well. Gently mix for 5 seconds.
10. Incubate at room temperature, in the dark, for 20 minutes.
11. Stop the reaction by adding 100µl of Stop Solution to each well.
12. Gently mix for 30 seconds. Ensure that all of the blue color changes completely to yellow.
13. Read absorbance at 450nm with a microtiter plate reader within 15 minutes.

Calculation of results

1. Calculate the average absorbance value (A_{450}) for each set of reference standards, controls and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in mIU/ml on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of LH in mlU/ml from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4. Any diluted samples must be further corrected by the appropriate dilution factor.

Expected values

Each laboratory should establish its own normal range based on patient population.

Adult	**mIU/ml**
Male	1.24-7.8
Female	
Follicular phase	1.68-15
Ovulatory peak	21.9-56.6
Luteal phase	0.61-16.3
Postmenopausal	14.2-52.3

2. Urinary measurement of the human chorionic gonadotropin (HCG)

Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced in pregnancy that is made by the developing embryo soon after conception and later by the syncytiotrophoblast (part of the placenta). Its role is to prevent the disintegration of the corpus luteum of the ovary and thereby maintain progesterone production that is critical for a pregnancy in humans.

hCG may have additional functions; for instance, it is thought that hCG affects the immune tolerance of the pregnancy. Early pregnancy testing, in general, is based on the detection or measurement of hCG. Because hCG is produced also by some kinds of tumor, hCG is an important tumor marker, but it is not known whether this production is a contributing cause or an effect of tumorigenesis.

As structure, hCG is a glycoprotein, heterodimeric, with an α subunit identical to that of luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and β subunit that is unique to hCG.

Human chorionic gonadotropin interacts with the LHCG receptor and promotes the maintenance of the corpus luteum during the beginning of pregnancy, causing it to secrete the hormone progesterone.

It has also been hypothesized that hCG may be a placental link for the development of local maternal immunotolerance. It has also been suggested that hCG levels are linked to the severity of morning sickness in pregnant women. Because of its similarity to LH, hCG can also be used clinically to induce ovulation in the ovaries as well as testosterone production in the testes. Human chorionic gonadotropin also plays a role in cellular differentiation/proliferation and may activate apoptosis.

hCG measurements

Levels of hCG may be measured in the blood or urine. Most commonly, this is done as a pregnancy test, intended to indicate the presence or absence of an implanted embryo. Testing for hCG may also be done when diagnosing or monitoring germ cell tumors and gestational trophoblastic disease.

Most tests employ a monoclonal antibody, which is specific to the β-subunit of hCG (β-hCG). This procedure is employed to ensure that tests do not make false positives by confusing hCG with LH and FSH. (The latter two are always present at varying levels in the body, whereas the presence of hCG almost always indicates pregnancy.)

The urine test may be a chromatographic immunoassay or any of several other test formats, home-, physician's office-, or laboratory-based. Published detection thresholds range from 20 to 100 mIU/ml, depending on the brand of test. Early in pregnancy, more accurate results may be obtained by using the first urine of the morning when hCG levels are highest. When the urine is dilute (specific gravity less than 1.015), the hCG concentration may not be representative of the blood concentration, and the test may be falsely negative.

The serum test, using 2-4 mL of venous blood, is typically a chemiluminescent or fluorimetric immunoassay that can detect βhCG levels as low as 5 mIU/ml and allows quantification of the βhCG concentration. The ability to quantify the βhCG level is useful in the monitoring germ cell and trophoblastic tumors, follow up care after miscarriage, and in diagnosis of and follow-up care after treatment of ectopic pregnancy. The lack of a visible fetus on vaginal ultrasound after the βhCG levels have reached 1500 IU/ml is strongly indicative of an ectopic pregnancy.

Gestational trophoblastic disease like Hydatidiform moles ("molar pregnancy") or Choriocarcinoma may produce high levels of βhCG (due to the presence of syncytialtrophoblasts-part of the villi that make up the placenta) despite the absence of an embryo. This, as well as several other conditions, can lead to elevated hCG readings in the absence of pregnancy.

hCG levels are also a component of the *triple test*, a screening test for certain fetal chromosomal abnormalities/birth defects.

Reference levels

The following is a list of serum hCG levels. (LMP is the last menstrual period.)

- 3 weeks since LMP: 5 - 50 mIU/ml
- 4 weeks since LMP: 5 - 426 mIU/ml
- 5 weeks since LMP: 18 - 7,340 mIU/ml
- 6 weeks since LMP: 1,080 - 56,500 mIU/ml

- 7 – 8 weeks since LMP: 7, 650 - 229,000 mIU/ml
- 9 – 12 weeks since LMP: 25,700 - 288,000 mIU/ml
- 13 – 16 weeks since LMP: 13,300 - 254,000 mIU/ml
- 17 – 24 weeks since LMP: 4,060 - 165,400 mIU/ml
- 25 – 40 weeks since LMP: 3,640 - 117,000 mIU/ml
- Non-pregnant females: <5.0 mIU/ml
- Postmenopausal females: <9.5 mIU/ml

Pregnancy test strip for urine

The appearance and rapid increase in the concentration of hCG in the mother's urine makes it a good marker for confirming pregnancy. The concentration of hCG in urine increases steadily to a circulation peak of as much as 50,000 mIU/ml between the eighth and eleventh weeks.

Principle

hCG Urine Test is a chromatographic immunoassay which uses specific antibodies to selectively identify hCG in urine with a high degree of sensitivity. Elevated levels of hCG as low as 20 mIU/ml can be detected within 3 minutes.

Urine is added to the test kit and allowed to migrate through the absorbent device. The labeled antibody-dye conjugate binds to the hCG in the specimen forming an antibody-antigen complex. This complex binds to the anti-hCG antibody in the test zone and produces a purple color band when the hCG concentration is equal to or greater than 20 mIU/ml. In the absence of hCG, no band is formed in the test zone. The reaction mixture continues flowing through the absorbent device past the test and control zones. Unbound conjugate binds to the reagents in the control zone, producing a purple color band, demonstrating that the reagents and the test kit are functioning correctly.

Urine collection and storage

(1) First morning urine typically contains the highest concentration of hCG and is therefore the best sample for performing the urine test. However, any urine specimen may be used.

(2) Collect the urine specimen in a clean glass or plastic container. Do not use preservatives.

(3) If the specimen is not used immediately following collection, but is to be used within 48 hours it should be refrigerated (2 to 8^{o}C), and brought back to room temperature (4 to 30^{o}C) before testing.

Procedure

1. Remove the test strip from its foil pouch.
2. Holding the strip vertically, carefully dip it into the specimen. Do not immerse the strip past the maximum line (Figure 1).
3. The strip can be removed from the specimen when red-dye begins to migrate through the Result Window.
4. Interpret test results at 3 to 5 minutes. Do not interpret test results after 5 minutes.

Interpretation of results

(1) NEGATIVE: If there is only one purple color band in the result window, this indicates that the specimen does not contain a detectable level of hCG and should be interpreted as a negative result (Figure 2).

(2) POSITIVE: If there are two purple color bands in the result window, this indicates that the specimen contains hCG and should be interpreted as positive result (Figure 2).

(3) INVALID: If there is no purple color band in the result window, the test result is invalid. The control band will not appear if an insufficient volume of specimen is added into the test kit. Proper procedures may not have been followed in performing the test or deterioration of the test kit may have occurred. Repeat the test procedure using a new test kit.

Limitations of the procedure

1. In addition to pregnancy, hCG has been found in patients with both gestation and non-gestation trophoblastic diseases. These conditions should be ruled out when interpreting hCG levels to establish a pregnancy diagnosis.
2. Although the test is very accurate in detecting pregnancy a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. Consult with a physician if unexpected or inconsistent results are obtained.
3. A normal pregnancy cannot be distinguished from an ectopic pregnancy based solely on hCG levels. Also, a spontaneous miscarriage may cause confusion in interpreting test results.
4. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
5. A negative result obtained from a urine specimen collected from a mother in very early pregnancy may be due to an extremely low concentration of hCG. In such cases, the test should be repeated on a fresh specimen obtained two days later.
6. If a urine sample is too dilute, it may not contain a representative urinary hCG concentration. If a negative result is obtained with a low specific gravity specimen and pregnancy is still suspected, obtain a first morning urine specimen and retest.

Normal values

- < 5 mIU/ml: healthy men or non-pregnant women.

Urine hCG levels during pregnancy are estimated to be:

- 10 - 30 mIU/ml: 7-10 days post conception.
- 37.000 - 50.000 mIU/ml: 8-11 weeks after last menstrual period.

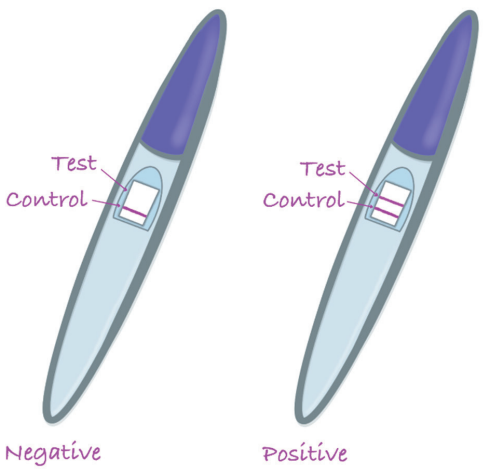


Figure 2. Urine pregnancy test

Lab Notes

Use this space for handwritten notes during the practical session.

L11. INVESTIGATION OF THE RENAL FUNCTION. DETERMINATION OF CYSTATIN C

LEARNING OBJECTIVES

Identify the principal laboratory markers used to assess renal function: urea, creatinine, and cystatin C.

Explain the advantages of cystatin C as a marker of glomerular filtration rate (GFR) compared to creatinine.

Calculate and interpret creatinine clearance as an estimate of GFR.

Interpret renal function test results in the context of acute and chronic kidney disease.

KEY POINTS

Glomerular filtration rate (GFR) is the primary index of renal function;

Serum creatinine is the most widely used renal marker, but its level is influenced by muscle mass, diet, and tubular secretion, reducing its reliability in early CKD.

Cystatin C is freely filtered at the glomerulus and completely reabsorbed and catabolised by the proximal tubule; it is not secreted, making it a more reliable GFR marker than creatinine.

The combination of serum cystatin C and creatinine-based equations (e.g., CKD-EPI) provides the most accurate GFR estimation and is recommended for CKD staging.

GENERAL ASPECTS

The nephron is the functional unit of the kidney. The main functions of the kidneys include:

- regulation of water, electrolyte and acid–base balance
- excretion of the products resulting from protein and nucleic acid metabolism (e.g. urea, creatinine and uric acid).

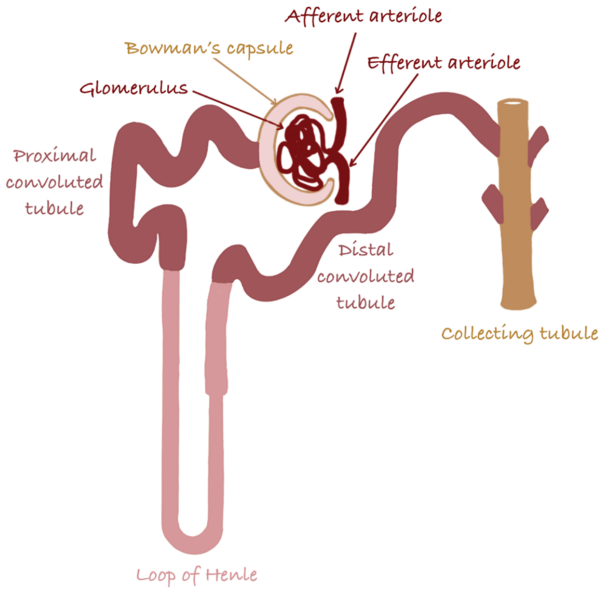


Figure 1. The nephron

The kidneys also function as endocrine organs, synthesizing various hormones and being regulated by other hormones. Arginine vasopressin (AVP) plays a role in maintaining water balance, while aldosterone influences sodium reabsorption in the nephron. Parathyroid hormone facilitates the reabsorption of calcium in the tubules, the excretion of phosphate, and the synthesis of 1,25-dihydrocholecalciferol (the active form of vitamin D). Renin, produced by juxtaglomerular cells, stimulates the formation of angiotensin I, which ultimately leads to the production of aldosterone. Erythropoietin is also formed, and it stimulates synthesis of red blood cells.

Renal function will be discussed in terms of glomerular and tubular function.

GLOMERULAR FUNCTION

Urea

Blood urea nitrogen was the first endogenous substance measured in serum or plasma to assess renal function. It is a major by-product of protein metabolism, and 90% of urea is cleared by the kidneys. Urea is freely filtered by the glomerulus and not secreted by the tubules. A large portion (40-70%) is passively reabsorbed from the renal tubules; thus, its concentration will underestimate glomerular filtration rate (GFR) in settings of decreased renal perfusion because some of the urea that is filtered will return to the bloodstream. Its concentration in the blood can vary with diet, hepatic function, and numerous disease states.

Serum creatinine

Filtration of plasma at the glomeruli represents the first step in urine formation. The GFR can be explained as the volume of plasma from which a given substance is completely cleared by glomerular filtration per unit time. In a healthy adult, this is approximately 140 mL/min, but can fluctuate enormously with body size, therefore is usually normalized. Typically, it is corrected to a body surface area of 1.73 m^2 (so the units are mL/min/1.73 m^2).

Traditionally, serum creatinine has been used as an accessible measure of glomerular function.

Creatine is produced in the liver and kidneys from arginine and glycine and transported to the skeletal muscles and brain where it is transformed into a macroergic compound (creatine phosphate). Daily, in the muscles, creatine phosphate dehydrates spontaneously, non-enzymatically to creatinine in a proportion of 1-2%. The total amount of creatinine produced daily depends on muscle mass.

Creatinine circulates in the plasma unbound to proteins, is freely filtered and excreted in the urine without reabsorption. A small part (less than 10%) of urinary creatinine is tubularly secreted.

Serum creatinine is not a highly sensitive marker of renal function, as its concentration typically remains within the reference range until the GFR has declined by more than 50%. Therefore, the detection of a progressive rise in serum creatinine values in the same individual, even when results remain within the normal interval, can be an early indicator of declining renal function. In healthy individuals, physiological variations in creatinine levels over time are much smaller than the width of the reference interval, making such trends clinically significant.

Several extrarenal factors can also influence serum creatinine levels, including muscle mass, dietary intake, and physical activity. Therefore, men generally display higher values than women due to their greater muscle mass; elder persons have a lower creatinine level that a younger person. Diet can also play a role: a meat-rich meal may transiently increase serum creatinine concentrations by up to 30% because of the direct absorption of creatinine from ingested meat. Similarly, short-term elevations may be observed after intense physical exercise.

Creatinine clearance

Creatinine clearance is defined as the volume of plasma (expressed in milliliters) from which creatinine is completely removed by the kidneys each minute. To account for differences in body size, the result is commonly normalized to a standard body surface area, calculated from the patient's weight and height. Because creatinine is filtered at the glomerulus with minimal tubular reabsorption, this measurement is widely used as an estimate of the glomerular filtration rate (GFR). It is especially useful in patients with suspected or established kidney disease, or in those receiving medications with potential nephrotoxic effects, such as certain antibiotics.

For an accurate determination, the test requires a 24-hour urine collection, along with a blood sample to measure serum creatinine. Calculation of creatinine clearance:

$$C_{cr}(ml/min) = \frac{U \times V}{P \times 1440}$$

where: U = creatinine concentration in urine (mg / ml)

V = urine volume (ml in 24 hours)

P = creatinine concentration in serum (or plasma) (mg / ml)

1440 = minutes in 24 hours

Although creatinine clearance is more sensitive than serum creatinine for detecting a reduced GFR, the method is inconvenient for patients, prone to imprecision, and has largely been replaced by prediction equations that estimate GFR.

Estimated GFR (eGFR)

The relatively poor inverse relationship between serum creatinine and GFR can be enhanced by considering various confounding factors such as age, sex, ethnicity, and body weight. The equation created by Cockcroft and Gault in the 1970s, along with the four-variable formula developed later from the Modification of Diet in Renal Disease (MDRD) Study, are the most commonly widely used as prediction formulas.

While prediction equations represent an improvement on serum creatinine and creatinine clearance, they still only provide estimates of GFR and must be interpreted carefully. For instance, they tend to be less accurate in individuals with relatively normal GFR levels. This is why numerous hospital laboratories refrain from reporting a specific result when GFR exceeds 60 mL/min/1.73 m^2. Other groups of patients in whom eGFR may be less reliable include those with atypical body shapes or masses, such as individuals with muscle wasting or amputees. Lastly, research indicates that the GFR estimated by the MDRD formula can be influenced by meat consumption.

It is important to place the limitations of eGFR discussed in the earlier section into the correct context. For instance, the four-variable MDRD formula provides a more accurate estimation of GFR than using serum creatinine alone, as it accounts for various confounding factors. The Cockcroft-Gault formula requires weight in addition to age, sex, and creatinine to be utilized. Consequently, the MDRD formula, which includes age, sex, and ethnicity but excludes weight, is much simpler to apply. This is one of the key reasons for the widespread use of the MDRD equation. Nevertheless, the Cockcroft–Gault formula continues to be commonly used for calculating medication dosages.

Cystatin C

Cystatin C is a low-molecular-weight protein produced by nucleated cells. It is a cysteine proteinase inhibitor. It is freely filtered by the glomerulus, reabsorbed, and catabolized by the proximal tubule. Produced at a constant rate, levels remain stable if kidney function is normal.

Cystatin C, like creatinine, correlate inversely with GFR. However, unlike creatinine, the concentration of cystatin C is independent of weight and height, muscle mass, age (>1 year) or sex and is largely unaffected by intake of meat or non-meat-containing foods.

Cystatin C has been recently proposed as a new sensitive endogenous serum marker for the glomerular filtration rate. When the rate at which the fluid filtrate is formed, is reduced, indicating decreased kidney function, blood levels of substances removed by them (such as cystatin C) increase and are an indication of kidney function. A rise in cystatin C is often detectible before there is a measureable decrease in GFR or increase in creatinine.

Cystatin C may be used as an alternative to creatinine and creatinine clearance to screen for and monitor kidney dysfunction. While there are growing data and literature supporting the use of cystatin C, there is still a degree of uncertainty about when and how it should be used. It may be especially useful in those cases where creatinine measurement is not appropriate, such as in patients with cirrhosis, obesity, or malnutrition or who have a reduced muscle mass. In addition to kidney dysfunction, it has been associated with an increased risk of cardiovascular disease and heart failure in the elderly.

Cystatin C can be measured by immunoassay methods.

Several **other markers** can be utilized to assess clearance, but they are often too expensive and labor-intensive for widespread use: their application is mostly confined to research or specialized nephrology environments, such as evaluating potential kidney donors. These markers include inulin, iothalamate, iohexol, and radioisotopic markers like 51Cr-EDTA. The latter is frequently employed in pediatric oncology departments to evaluate kidney function before determining chemotherapy dosages.

Proteinuria

Proteinuria can be classified in: glomerular proteinuria, tubular proteinuria, overflow proteinuria, Tamm-Horsfall proteinuria. Concerning the glomerular function, we include glomerular proteinuria, which is its 'leakiness'. This is presented separately at Biochemical analysis of urine (L4).

Note! The glomerular filtration rate, like the heart and respiration rates, fluctuates throughout the day. A change in the GFR of up to 20% between two consecutive creatinine clearances may not indicate any real change in renal function.

RENAL TUBULAR FUNCTION

The glomeruli play a crucial role in efficiently filtering waste products and toxins from the body. To prevent the loss of essential substances such as water, sodium, glucose, and amino acids, tubular reabsorption must be equally effective. For instance, of the 180 liters of fluid that enter the glomerular filtrate daily, over 99% is reabsorbed. Unlike the glomerular filtration rate (GFR), which is a standard measure of kidney function, there are no simple tests to quantitatively assess tubular function.

Some tubular function disorders are inherited; for example, certain individuals cannot lower their urine pH below 6.5 due to defective hydrogen ion secretion. However, renal tubular damage is more commonly a secondary consequence of other conditions or injuries. In fact, any cause of acute renal failure may lead to renal tubular dysfunction.

In order to investigate tubular function, some of the following tests can be performed

Osmolality measurements in plasma and urine

The renal tubules carry out a wide range of complex functions. However, in clinical practice, urine osmolality is often used as a general indicator of tubular function. This is because the most commonly affected tubular function in disease is the ability to concentrate urine. When the tubules and collecting ducts function properly and antidiuretic hormone (ADH) is present, they effectively reabsorb water. This ability can be evaluated by measuring urine concentration, typically through osmolality. Urine osmolality is then compared to plasma osmolality. A urine osmolality of 600 mmol/kg or higher generally indicates intact tubular function. Conversely, if urine osmolality is similar to plasma osmolality (urine-to-plasma osmolality ratio ~1), it suggests that the tubules are not adequately reabsorbing water.

Urine pH and the acid load test

Urine pH measurements can be a helpful initial step in diagnosing Renal Tubular Acidosis (RTA), a condition commonly associated with hyperchloremic metabolic acidosis. RTA can be classified as follows:

- Type I. - Inherited or acquired defective hydrogen ion secretion in the distal tubule
- Type II. - Reduced capacity of bicarbonate reabsorption in the proximal tubule.
- Type III. - Is a pediatric variant of type I RTA
- Type IV. - Bicarbonate reabsorption by the renal tubule is impaired due to aldosterone deficiency, aldosterone receptor defects, or drugs which inhibit aldosterone action.

To diagnose RTA, the first step is to confirm the presence of persistent, unexplained metabolic acidosis. If RTA is suspected after ruling out other conditions, a fresh urine sample should be tested for pH, as delayed analysis can lead to falsely high readings due to bacterial activity. Normally, metabolic acidosis triggers increased acid excretion, resulting in a urine pH below 5.3; if the urine is not sufficiently acidic, further testing is needed. An acid load test with ammonium chloride may be used to assess urine acidification, but it should be avoided in severely acidotic or liver disease patients due to potential side effects. Instead, a furosemide test is preferred, as it induces acidic urine through sodium exchange mechanisms. In both tests, failure to produce urine with a pH below 5.3 supports the diagnosis of RTA.

Specific proteinuria

Protein in urine has already been noted as a sign of leaky glomeruli. β2-microglobulin and α1-microglobulin are small proteins that pass through the glomeruli and are typically reabsorbed by tubular cells. Elevated levels of these proteins in urine serve as a sensitive marker of renal tubular cell damage.

Glycosuria

The presence of glucose in urine despite normal blood glucose levels typically indicates a tubular defect that impairs glucose reabsorption. In this case, the renal threshold (the tubules' capacity to reabsorb glucose) has been exceeded, leading to a benign condition known as renal glycosuria. Glycosuria can also occur alongside other tubular function disorders, such as Fanconi syndrome, which is characterized by generalized tubular defects, including renal tubular acidosis, aminoaciduria, and tubular proteinuria.

Aminoaciduria

Amino acids in the glomerular filtrate are typically reabsorbed in the proximal tubules. However, excessive amounts may appear in urine if their plasma concentration surpasses the renal threshold or if there is a specific defect in tubular reabsorption. This impairment can result from the inherited metabolic disorder cystinuria or, more commonly, from acquired renal tubular damage.

Specific tubular defects

The Fanconi syndrome

Fanconi syndrome refers to a condition characterized by generalized tubular defects, including renal tubular acidosis, aminoaciduria, and tubular proteinuria. It can result from heavy metal poisoning, exposure to toxins, or inherited metabolic disorders such as cystinosis.

Renal stones

Renal stones (calculi) cause severe pain and discomfort and are a common cause of urinary tract obstruction. Chemical analysis of these stones is essential for understanding their formation. The main types of renal stones include:

- Calcium phosphate: may be a consequence of primary hyperparathyroidism or renal tubular acidosis.
- Magnesium, ammonium and phosphate: are often associated with urinary tract infections.
- Oxalate: may be a consequence of hyperoxaluria.
- Uric acid: may be a consequence of hyperuricaemia
- Cystine: these are rare and a feature of the inherited metabolic disorder cystinuria

EXPERIMENTAL WORK

Determination of Cystatin C in human serum using the ELISA test (Enzyme-linked immunosorbent assay)

Principle of the Assay

In the Human Cystatin C ELISA, standards, quality controls and samples are incubated in microtitrate plate wells precoated with polyclonal anti-human cystatin C antibody. After 30 minutes incubation and washing, polyclonal anti-human cystatin C antibody, conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 30 minutes with captured cystatin C. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of cystatin C. A standard curve is constructed by plotting absorbance values against concentrations of cystatin C standards, and concentrations of unknown samples are determined using this standard curve.

Assay Procedure

1. Pipet **100 µL** of samples into the appropriate wells
2. Incubate the plate at room temperature (ca. 25 °C) for **15 minutes**, shaking at ca. 300 rpm on an orbital microplateshaker.
3. Wash the wells **3-times** with Wash Solution (0.30 mL per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µL** of Conjugate Solution into each well.
5. Incubate the plate at room temperature (ca. 25 °C) for **15 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. Incubation without shaking is an alternative that requires extended incubation with substrate – see step 8 below.
6. Wash the wells **3-times** with Wash Solution (0.35 mL per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µL** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
8. Incubate the plate for **10 minutes** at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20 °C. Do not shake with the plate during the incubation.
9. Stop the colour development by adding **100 µL** of Stop Solution.
10. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 - 650 nm).

The absorbance should be read within 5 minutes following step 9.

Normal values:

Children:

0-3 months: 0,81-2,32 mg/L
4-11 months:0,65-1,49 mg/L
1-17 years : 0,5-1,27 mg/L

Adults: 0.49-1,13 mg/L

CASE STUDY

1. Patient: **GH, sex: F, 75 years old**.

The patient presents with the following symptoms: anorexia, frequent urination (small quantities especially at night), dysuria, hypertension. The doctor recommends performing the analysis below. Establish a possible diagnosis.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia à jeun (fasting blood glucose)	80 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	5 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	15 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	5.0 g/dl	6.2-8 g/dl
ASAT	19 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	8.5 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	149 mg/dl	15-45 mg/dl	**hsCRP**	19 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	4.3 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.06 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	9 ml/min	95-150 ml/min	**Cystatin C**	6.99 mg/l	0.49-1.13 mg/l
Uric acid	15 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	12 mEq/l	22-27 mEq/l
Amylase	89 U/l	50-100 U/l	**Na+**	120 mEq/l	135-145 mEq/l
Total Cholesterol	189 mg/dl	140- 200 mg/dl	**K+**	7.3 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	45 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	130 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	155 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	9 U/l	2-10 U/l	**GGT**	20 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	135 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.002	1.015- 1.025
pH	7.0	6.5
Proteins	+++(1000mg/dl)	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	+	negative
Leucocytes	+++(500/µl)	negative
Red blood cells	++(50/µl)	negative

2. Patient: **EF, sex: F, 65 years old**.

The patient presents with the following symptoms: overweight, chills, distal interphalangeal joint pain and swelling, itchy skin (pruritus), dysuria, hypogastric pain. The doctor recommends performing the analysis below. Establish a possible diagnosis.

Biochemical examination of blood

Denomination	Result	Reference range	Denomination	Result	Reference range
Glycemia à jeun (fasting blood glucose)	81 mg/dl	75-115 mg/dl	**Direct Bilirubin**	0.2 mg/dl	<0.2 mg/dl
HbA1c	4.9 %	4-6 %	**Total Bilirubin**	0.9 mg/dl	<1 mg/dl
ALAT	19 U/l	F: 10-22 U/l M:10-29 U/l	**Total Proteins**	7.2 g/dl	6.2-8 g/dl
ASAT	22 U/l	F: 10-21 U/l M:10-25 U/l	**Hemoglobin**	12.9 g/dl	F: 12-16 g/dl M: 14-18 g/dl
Urea	39 mg/dl	15-45 mg/dl	**hsCRP**	12.0 mg/l	<1 mg/l low CV risk 1-3 mg/l medium CV risk >3mg/l high CV risk
Creatinine	1.0 mg/dl	F<0.9 mg/dl M<1,1 mg/dl	**cTnI**	0.08 ng/ml	0.01-0.40 ng/ml
Creatinine Clearance	100 ml/min	95-150 ml/min	**Cystatin C**	1.0 mg/l	0.49-1.13 mg/l
Uric acid	12 mg/dl	F: 2.5-7 mg/dl M: 3-9 mg/dl	**Alkaline Reserve**	26 mEq/l	22-27 mEq/l
Amylase	89 U/l	50-100 U/l	**Na+**	144 mEq/l	135-145 mEq/l
Total Cholesterol	310 mg/dl	140- 200 mg/dl	**K+**	4.5 mEq/l	3.8-5.4 mEq/l
HDL Cholesterol	20 mg/dl	F:45-65 mg/dl M:35-55 mg/dl	**TSH**	3.1 mU/ml	0.27-4.20 µUI/ml
Triacylglycerols	380 mg/dl	F: 40-140 mg/dl M:60-165mg/dl	**fT3**	2.9 pg/ml	2.2-5.5 pg/ml
LDH	203 U/l	F:135-215 U/l M:135-225 U/l	**fT4**	1.8 ng/dl	0.7-2.2 ng/dl
CKMB	9 U/l	2-10 U/l	**GGT**	10 U/l	F: 5-40 U/l M: 8-61 U/l
			Alkaline phosphatase	146 U/l	F: <240 U/l M:<270 U/l

Biochemical examination of urine

Denomination	Result	Reference range
Density	1.025	1.015- 1.025
pH	8.0	6.5
Proteins	negative	negative
Glucose	negative	negative
Ketone bodies	negative	negative
UBG	normal	normal
bilirubin	negative	negative
Nitrites	+	negative
Leucocytes	++(75/µl)	negative
Red blood cells	negative	negative

Lab Notes

Use this space for handwritten notes during the practical session.

L12. CLINICAL ENZYMOLOGY. KINETIC DETERMINATION OF CK-MB IN SERUM

LEARNING OBJECTIVES

Explain the principle of kinetic (rate) enzyme activity determination.

Describe the clinical significance of CK-MB as a specific cardiac biomarker in myocardial infarction diagnosis.

Understand the relationship between CK-MB activity, CK-MB mass, and their respective diagnostic time windows.

Perform and calculate the kinetic determination of CK-MB activity in serum.

KEY POINTS

Kinetic (rate) enzyme assays measure the rate of substrate consumption or product formation over time, reporting enzyme activity in U/L or nkat/L.

CK (creatine kinase) exists as three isoenzymes: CK-MM (predominantly in skeletal muscle), CK-MB (myocardium and skeletal muscle), and CK-BB (brain).

CK-MB rises 4–6 hours after myocardial infarction, peaks at 12–24 hours and returns to normal within 48–72 hours; it is used to detect re-infarction after troponin elevation.

A CK-MB/total CK ratio > 5% strongly suggests myocardial origin; CK-MB mass (immunoassay) is more sensitive than CK-MB activity for early AMI diagnosis.

SERUM ENZYMES IN DISEASE

Enzymes are assessed both in terms of activity and mass using immunochemical techniques. Enzymes can be divided into two groups. Some, such as coagulation cascade enzymes, have a defined function in blood. Others emerge in the blood incidentally, and their measurement is useful in diagnosis. Damage to the tissues of origin, or proliferation of the cells from which these enzymes originate, will result in an increase in their activity in the plasma. It is worth noting that increases in serum enzyme activity are only roughly proportional to the degree of tissue damage.

The following enzymes have been shown to have diagnostic value:

- Alanine aminotransferase (ALT): an indicator of hepatocellular damage.
- Aspartate aminotransferase (AST): an indicator of hepatocellular damage, or a marker of muscle damage.
- Creatine kinase (CK): a marker of muscle damage and acute MI.
- Alkaline phosphatase (ALP): increases in cholestatic liver disease and a marker of osteoblast activity in bone disease.
- Acid phosphatase (ACP): increases in prostate cancer and osteoclast activity
- γ-Glutamyl transpeptidase: a sensitive but non-specific marker of liver disease.
- Amylase: an indicator of cell damage in acute pancreatitis.
- Lipase: an indicator of cell damage in acute pancreatitis.
- Lactate dehydrogenase (LDH): non-specific enzyme, increased in tissue damage
- Cholinesterase: normally involved in neuromuscular conduction, incidentally breaks down suxamethonium (succinyl-choline), a muscle-relaxing drug used in anaesthesia. Patients with abnormal cholinesterase may be unable to metabolize the drug normally and as a result suffer prolonged paralysis after anaesthesia. This condition is called scoline apnea. Cholinesterase determiations are also useful in the diagnosis of poisoning with pesticides that inhibit cholinesterase.

Isoenzyme determination

Certain enzymes are present in the plasma in two or more molecular forms. These forms are referred to as isoenzymes and, despite their distinct structures, they carry out the same catalytic role. Various isoenzymes may originate from distinct tissues, and their specific identification can provide insights into the location of the disease. A specific isoenzyme of creatine kinase (CK-MB) is beneficial for the early identification of myocardial infarction. Cardiac muscle has a higher proportion of this isoenzyme compared to skeletal muscle, and elevated CK-MB levels suggest that a myocardial infarction has taken place.

EXPERIMENTAL WORK

Determination of CK-MB activity in serum

Principle

Antibodies, which are incorporated to the enzyme reagent bind to and inhibit the activity of the M subunit of CK-MB. The remaining activity of the B subunit is measured according to the following reaction:

$$Creatine - phosphate + ADP \xrightarrow{Creatine\ Kinase} Creatine + ATP$$

$$ATP + glucose \xrightarrow{Hexokinase} glucose - 6 - P + ADP$$

$$glucose - 6 - P + NADP^{+} \xrightarrow{G6PDH} gluconate - 6P + NADPH + H^{+}$$

G6PDH = Glucose-6-phosphate dehydrogenase

By multiplying the values of the activity by the factor 2, it will give the activity of CK-MB in serum.

Reagents

1. Reagent R1 buffer: (imidasole 100 mM (pH=6.7), glucose 20 mM, Mg-Acetate 10 mM)

2. Reagent R2 (enzyme/antibody reagent): 5mM ADP, 5mM AMP, 10μM diadenosine-P-5P, 2mM NADP, 30 mM creatine phosphate, 2500 U/L hexokinase, 1500 U/L G-6-PDH, 20 mM N-acetylcysteine and antibody to CK-MB

Preparation of the working reagent: Dissolve contents of enzyme reagent R2 with the corresponding volume of buffer R1. Gently swirl to completely dissolving of the reagents. Do not shake!

Procedure

Pipette into the test tube as follows:

Reagents, μl	Sample
Working reagent	1000
Serum	40

Incubate the tubes for 7 minutes at room temperature and read the absorbence of sample at λ = 340 nm, zeroing the spectrophotometer with air or distilled water (A_1). Wait 5 min and read again the sample absorbence at λ = 340 nm, zeroing the spectrophotometer with air or distilled water (A_2). Calculate $\Delta A = A_2 - A_1$

Calculation

Calculate ΔA and then the activity of CK-MB is calculated with the formula:

$$CK - MB\ activity\ =\ \Delta A\ x\ 1651\ (U/L)$$

Myocardial damage is indicated when the following 3 factors are present:

- CK men > 80 U/L,
- CK women > 70 U/L and
- CK-MB > 10 U/L and the
- CK-MB activity between 6% - 25% of the total CK-activity.

Pathological modifications

- *Important increases:* in myocardial infarct, dermatomyosites, progressive muscle dystrophy, rabdomyolysis, myoglobinuria.
- *Moderate increases:* after intramuscular shuts, hypothyroidism.

- *After certain drugs:* statins can cause elevated levels of CK-MB; often at a low level indicating mild muscle injury

Possible error factors in the enzymatic quantification of necrosis:

- The grade of local perfusion: the non-perfused central zone of necrosis does not liberate the enzyme, so that the massive and compact necroses are underestimated.
- The speed of enzyme degradation, in the necrotic tissue and in circulation.
- The renal elimination speed of the enzyme.
- The apparition of the myocardial reperfusion: the reperfusion flux sends in circulation a high enzyme quantity and mimics an extended necrosis, but reduces, on the other side, by oxygenation, masses of necrotic tissue.

Between the conditions that can affect the CK-MB specificity for the acute myocardial infarct and that can influence the diagnosis, there are:

- The moderate increase of CK-MB, sometime observed after a severe physical effort, especially at performing athletes.
- Possible limit increases of CK-MB in unstable angina, in the absence of a certain cardiomyocyte lyses.

Lab Notes

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