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Spectrophotometry

Practical in Medical Biochemistry

General Medicine

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Introduction to spectrophotometry

Spectrophotometry is one of the most widely used instrumental techniques in analytical practice. It is an optical method based on estimation of absorption of light either in the **UV range** having wavelengths **200 – 400 nm**, or in the **visible light (VIS) range** of wavelength **400 – 800 nm** by molecules of the analyte in solutions.

The principle of spectrophotometry is interaction of electrons placed in the bonding or non-bonding orbitals with the photons of UV-VIS radiation. The energy of radiation excites the electrons in orbitals to a higher excited level; it is associated with absorption of certain quantum of radiation, which has definite energy contents, hence also a definite wavelength.

The absorption of light by the analyte can be quantitatively expressed as **transmittance**, defined as the intensity of light passing the sample (I_S) in per cent of the intensity of incident light (I_0):

$$\mathbf{T = I_S/I_0} \qquad (\text{or } T = 100\% \times I_S/I_0)$$

In reality, however, the instruments do not really measure the intensity of incident light. Instead, the 100% transmittance is obtained by detecting intensity of light (I_B) that passes through a sample with a zero concentration of the analyte (blank sample):

$$\mathbf{T = I_S/I_B} \qquad (\text{or } T = 100\% \times I_S/I_B)$$

Transmittance is not very convenient as it has a non-linear relationship to the concentration of the light-absorbing analyte. It is better to use a negative log transformed value, known as **absorbance**:

$$\mathbf{A = -\log T}$$

Absorbance tells how much of light is consumed by the measured solution. If the absorption of light is zero (i.e., transmittance is 100%, or 1) , the absorbance $A = -\log 1 = 0$. In the real photometric measurements the zero absorbance is usually set at the beginning with the blank sample.

Then the relationship of absorbance on concentration is quantitatively described by the **Lambert-Beer law**:

$$\mathbf{A = \varepsilon . c . l} \qquad [\lambda]$$

A absorbance
ε molar absorption decadic coefficient, at the wavelength λ, unit is $l . mol^{-1} . cm^{-1}$
c concentration of the analyte in mol/l
l path length of the light (i.e. width of the cuvette containing the measured solution, through which the ray of UV-VIS radiation passes).

Obviously, spectrophotometry is only able to estimate a particular analyte if at least part of the analyte molecule absorbs radiation. It can be in the visible range – then we perceive the substance as colored – or in the ultraviolet. The configurations of atoms or functional groups that are responsible for absorption of light are called chromophores. Good example are **coordination complexes of transition metals** that often display vivid colors because of the ability of bound ligands to split energy levels in the *d* orbital of the central metal. In organic compounds the typical chromophores consist of **systems of conjugated double bonds**. In general, if a substance contains chromophores we can directly use photometry to estimate absorption of radiation by the substance and in this way determine its concentration.

In medicine, we typically determine the concentrations of a wide range of substances in various biological materials (serum, urine, cerebrospinal fluid etc.), which represent complex systems containing a large number of substances of various chemical nature. Each of the substances is characterized by its individual optical properties. For some substances their optical properties enable

a direct photometry. However, properties of most substances do not allow their direct photometric determination. It is therefore necessary to convert a specific analyte into a product suitable for photometry. Various chemical reactions are used for this, referred to as **indication reactions**.

Evaluation of spectrophotometric measurement

Spectrophotometric estimation of concentration of given substance requires a blank (reference) sample and usually one or more standards. The blank contains all components used in the assay except for the substance that is measured; it is used to set the absorbance to zero. The standard is a solution of the measured substance of known concentration.

When the absorbances of the sample (A_{sa}) and standards (A_{st}) are measured, the value of concentration of the measured substance can be obtained in several ways:

1. Calculation from the Lambert-Beer law

Possible if the molar absorption coefficient is known. No standard is required in this case. The most commonly used unit for the molar absorption coefficient is $l \cdot mol^{-1} \cdot cm^{-1}$. This unit is convenient because usually cuvettes with the light path 1 cm are used. Then a simple calculation according to the formula

$$A = \varepsilon \cdot c$$

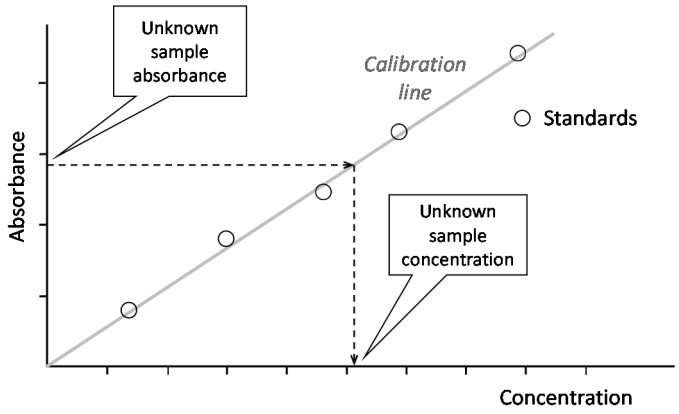
gives molar concentration of the solution (mol/l).

Another used unit is $cm^2 \cdot mol^{-1}$. This unit is 1000-times smaller than the $l \cdot mol^{-1} \cdot cm^{-1}$.

2. Calibration graph method

For construction of calibration graph we use the measured absorbances of the standards, i.e. solutions of known concentrations of the estimated substance.

On the x axis we plot the standard concentration (independent variable) and on the y axis the standard absorbance (dependent variable). The graphical expression of the relationship is a straight line with slope 'k' that passes through the origin.



The constructed graph is then used to convert absorbance values of the unknown sample(s) to concentrations.

3. Calibration factor method

Calibration factor (f) is the reciprocal value of the straight line slope (k).

$$f = \frac{1}{k}$$

The factor can be calculated from the values obtained for the calibration line: by dividing the value of concentration c_α with the corresponding measured value of absorbance A_α.

$$f_\alpha = \frac{c_\alpha}{A_\alpha} \quad \text{where} \quad \alpha = 1...n$$

From the values $f_1 - f_n$ the arithmetic mean is calculated that gives the value of the calibration factor f. The absorbance of the sample whose concentration is unknown is then multiplied with the factor. This method is equivalent to the calibration graph without constructing it. In practice it is possible to store the factor in the spectrophotometer memory and read directly concentrations of the analyzed samples. This method is therefore suitable for routine processing of high number of unknown samples.

4. One standard method

In addition to the sample of unknown concentration (c_{sa}), one standard sample (c_{st}) whose concentration is known is processed as well. The standard concentration is chosen so that it would be in the middle of the calibration line, or at the upper limit of the physiological range for the given analyte. The unknown sample concentration is calculated from the absorbances of both samples and the known concentration of the standard sample.

The absorbance is directly proportional to the concentration of the absorbing substance:

$$\frac{c_{sa}}{c_{st}} = \frac{A_{sa}}{A_{st}} \quad \text{then} \quad c_{sa} = \frac{c_{st}}{A_{st}} \cdot A_{sa}$$

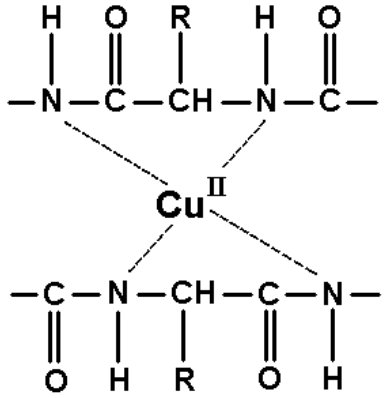
A_{st}	standard absorbance	c_{st}	standard concentration
A_{sa}	sample absorbance	c_{sa}	sample concentration

The one standard method is used the most often in the clinical chemistry because of its simplicity. The automatic analyzers calculate the factor from the measured standard and use it to convert the absorbances of unknown samples to concentrations.

Task 1: Spectrophotometric estimation of total serum protein with the biuret method

Principle:

The possibilities of determining the concentration of a certain substance by spectrophotometry will be demonstrated on the example of the determination of total protein in serum, for which the biuret reaction is used as an indication reaction. It is based on the formation of a violet complex of Cu^{2+} ions with nitrogens of the peptidic bonds in an alkaline environment.



The intensity of the complex color is directly proportional to the concentration of proteins and is assessed photometrically. The reaction depends on the presence of peptidic bonds, not on the properties of amino acid side chains; hence, all proteins react – the reaction does not distinguish specific proteins in the analyzed sample.

In general, any substance that has at least two adjacent groups -CO-NH_2 (amide) or at least two peptide bonds -CO-NH-, will give the biuret reaction. Thus, the simplest reacting compounds are oxamide H_2N-CO-CO-NH_2, or biuret H_2N-CO-NH-CO-NH_2 (bis-urea, dimer of urea H_2N-CO-NH_2) that gave the reaction its name.

The determination of total protein is one of the basic biochemical examinations in serum/plasma. Examination of total protein concentration provides us with indicative information about protein biosynthesis, utilization and excretion. Quantitative changes in the composition of serum proteins can result from various diseases.

Reagents:

A commercial kit Celková bílkovina liquid 500 S made by Erba -Lachema Diagnostika s.r.o., is used for analysis.

1. Biuret reagent:

 Copper sulfate	12.0 mmol/l
Sodium-potassium tartrate	31.9 mmol/l
 Sodium hydroxide	0.6 mol/l
Potassium iodide	30.1 mmol/l

2. Stock standard protein solution: 100 g/l

3. Serum sample of unknown concentration

Procedure:

Preparation of solutions for calibration curve

The stock protein solution is to be diluted to 5 standard solutions that have concentrations as directed by the table below.

1. Calculate the volumes of the stock protein solution and the purified water that are needed to prepare 0.1 ml each of the diluted standard solutions. Fill the calculated volumes to the table:

Test tube No.	Purified water	Stock protein solution	Protein concentration in the standard solution
1	–	100 µl	100 g/l
2			80 g/l
3			60 g/l
4			40 g/l
5			20 g/l

2. Prepare and label 5 test tubes (1–5). To these tubes measure the calculated volumes of the stock protein solution and purified water according to the table. Mix contents of the tubes by vortexing. Use automatic pipettes – results of the quantitative analysis depend on the pipetting precision.

Biuret reaction

3. Prepare and label another set of 7 test tubes (1–7). Pipette according to the table:

Measure in ml	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**
	Standard 1 20 g/l	Standard 2 40 g/l	Standard 3 60 g/l	Standard 4 80 g/l	Standard 5 100 g/l	Unknown	Blank
Biuret reagent	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Standard solution 1–5	0.02	0.02	0.02	0.02	0.02	–	–
Serum (unknown)	–	–	–	–	–	0.02	–
Purified water	–	–	–	–	–	–	0.02

4. Mix the tubes and incubate 10 minutes at the ambient temperature. Protect from direct light.
5. Together with your teacher measure absorption spectrum of the solution in tube No. 1 against the content of tube No. 7 (blank) in the visible range (380–740 nm). Find the wavelength of the absorption maximum for the colored complex of Cu^{2+} with peptidic bonds. Set this wavelength at your spectrophotometer.
6. Insert a cuvette filled with the content of tube No. 7 (blank) and set the zero absorbance. Then measure absorbances of all standards (tubes No. 1-5) and the unknown (No. 6).

Results and evaluation:

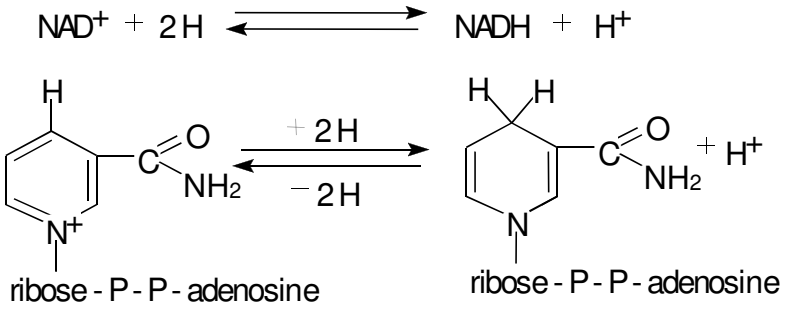
1. Make a sketch of the absorption spectrum of the colored complex of Cu^{2+} with peptidic bonds to your report, and mark the absorption maximum.
2. Record the values of absorbances for the solutions in tubes No. 1-6 to the table.
3. Use the results for plotting a calibration graph and calculate the average factor.
4. Determine the concentration of total protein in the unknown sample by three ways:
    - Graphically, by reading from the calibration graph
    - Calculation using the calibration factor method
    - Calculation using the one standard method

Task 2: Estimation of catalytic activity of lactate dehydrogenase in serum by means of Warburg optical test

Principle:

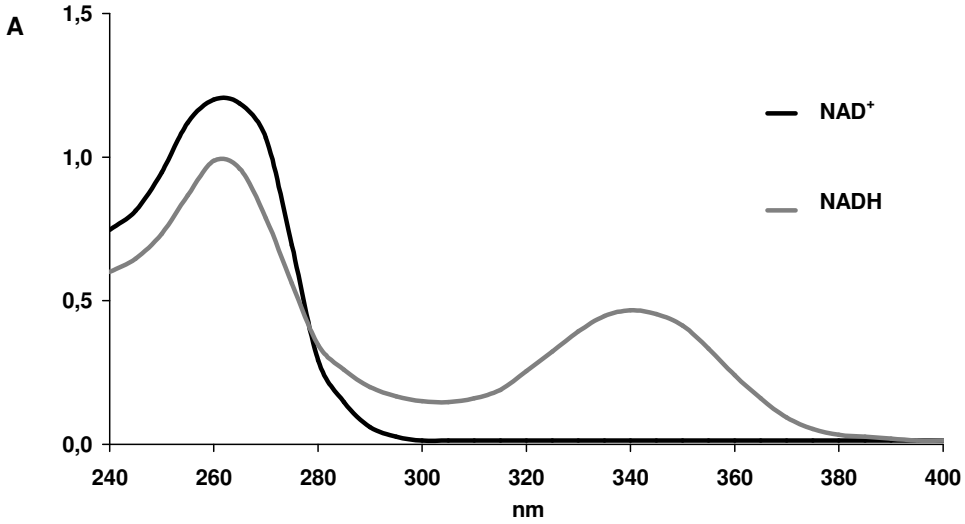
Nicotinamide adenine dinucleotide, NAD in short, is a coenzyme consisting of nicotinamide, adenine, two ribose molecules and two phosphates, connected together in the same way as nucleotides are (adenosine diphosphate, then ribose and then nicotinamide attached).

In the cell NAD participates **in redox reactions**, i.e. it is a coenzyme of **oxidoreductases**. The coenzyme occurs in two forms: **NAD^+** is the oxidized form that accepts electrons from other molecules and is itself reduced. In this way **$NADH + H^+$** originates which can provide electrons and itself get oxidized. This reversible electron transfer is the major function of NAD^+.



Reversible hydrogenation of nicotinamide adenine dinucleotide, which occurs on the pyridine ring in nicotinamide, leads to the reduced form and is associated with a distinct change in the **absorption spectrum**. The oxidized form (NAD^+) has an absorption maximum at wavelength 260 nm. Reduction cancels the aromatic character of the pyridine ring and its transition to a quinoid form ($NADH + H^+$) is associated with emergence of **another absorption maximum at 340 nm**. This maximum can be utilized for estimation of concentration of the reduced coenzyme in a reaction mixture. Historically, this approach has been called **Warburg optical test**.

Absorption spectrum of NAD^+ and NADH



By following decrease in absorbance (due to oxidation of $NADH+H^+$ to NAD^+) or increase in absorbance (if NAD^+ is reduced to $NADH+H^+$) at 340 nm per unit of time ($\Delta A/At$, e.g. during three minutes) it is possible to measure the rate of enzymatically catalyzed reactions that employ NAD as coenzyme. The change of absorbance is directly related to number of converted molecules of the coenzyme. Conversion of one mole of coenzyme corresponds to conversion of one mole of the substrate.

Warburg optical test can serve e.g. for estimation of enzymatic activities that depend on NAD (if substrates are in excess), or for estimation of substrates that can be converted by NAD-dependent oxidoreductases (if the enzyme is in excess).

As an example of Warburg optical test in this practical lesson we will measure the activity of the glycolytic enzyme **lactate dehydrogenase** (LD, EC 1.1.1.27.).

A reaction mixture is prepared from lactate dehydrogenase, its coenzyme NAD^+ and corresponding substrate (lactate) and under optimal condition the reaction rate is followed by measuring changes in absorbance of NADH at the wavelength 340 nm.

$$CH_3\text{-}CHOH\text{-}COOH + NAD^+ \longleftrightarrow CH_3\text{-}CO\text{-}COOH + NADH + H^+$$

Lactate *Pyruvate*

If the reaction mixture containing the enzyme, lactate and NAD^+ is placed into a cuvette of a spectrophotometer equipped with UV-light source and ability of continuous recording, the enzyme activity will manifest as a gradual and steady increase in absorbance at 340 nm. The conditions are chosen so that both substrates are in excess and the rate limiting factor is the amount of the catalytic enzyme activity.

Then, if the molar absorption coefficient of NADH at this wavelength is known, dilution of the sample is taken into account and time interval 1 sec. is chosen, it is possible to express the activity of LD in sample (serum) in µkat/l[1]. If continuous recording is not available, it is possible to resort to a discontinuous measurement of 340 nm absorbance in 1 minute intervals ($\Delta A_{340\ nm}$), and convert to the interval 1 sec.

Reagents:

A commercial kit Lactate dehydrogenase-l Bio-La-Test manufactured by Erba-Lachema s.r.o., is used for analysis.

1. Reagent 1: N-methyl D-glucamine buffer 406 mmol/l 
2. Reagent 2: N-methyl D-glucamine buffer 406 mmol/l 
   L-lactate 62.5 mmol/l
3. Reagent 3: NAD^+ 50 mmol/l 
4. Serum

[1] The basic unit of enzyme activity is katal (kat), defined as the activity that convert 1 mole of substrate to product per one second. In case of µkat it is one micromole of substrate converted to product per one second.

Procedure:

a. Cuvettes and solutions are pre-warmed to 37 °C.

b. Prepare the blank sample (directly to a cuvette): 800 µl reagent 2 (buffer and L-lactate)
200 µl reagent 1 (buffer)
20 µl serum.

c. Mix.

d. According to the instructions for use of the spectrophotometer Lightwave II+ set the measurement of absorbance at the wavelength 340 nm.

e. Insert the blank sample to the instrument and set it to zero.

f. Into another cuvette measure: 800 µl reagent 2 (buffer and L-lactate)
200 µl reagent 3 (NAD^+)
20 µl serum.

g. Mix well and after exactly 1 minute read A for the wavelength 340 nm, and write the value to the table.

h. Repeat the measurement 5 times more in **exactly one minute intervals**, and write down the absorbances to the table.

i. From the measured absorbances A_{340} calculate the difference in absorbance per one minute (ΔA_{340}). Calculate the arithmetic mean of these differences. Use the average value of ΔA_{340} for calculation of the catalytic activity of LD related to 1 liter of undiluted serum and time interval 1 sec., utilizing the molar absorption coefficient of NADH for 340 nm:

$$\mathbf{LD\ (\mu kat/l) = \Delta A_{340} \times 136.7}$$

Explanation of calculation:

The calculation of enzyme activity is based on the **Lambert-Beer law:**

$$A = \varepsilon \cdot d \cdot c \qquad (1)$$

$$c = \frac{A}{\varepsilon \cdot d} \qquad \Delta c = \frac{\Delta A}{\varepsilon \cdot d} \qquad (2)$$

The estimation of enzymatic activity assumes the reaction follows the zero order kinetics, i.e. the course of the reaction is linear (consumption of substrate or accumulation of the product per unit of time is constant).

In the kinetic estimation of the enzymatic activity the change of substrate or NADH concentration ($mol \cdot l^{-1}$) is related to time interval 1 sec. and volume of undiluted serum 1 liter.

$$\text{Catalytic activity/1 liter of serum} = \frac{\Delta A}{\varepsilon \times d} \cdot \frac{1}{60} \cdot \frac{V}{v} \cdot 10^6 \quad \mu mol \cdot l^{-1} \cdot s^{-1} \qquad (3)$$

The change of substrate concentration is expressed in terms of the equation (2), 1/60 is conversion from minutes to seconds, and V/v stands for dilution of the serum. The unit of enzyme activity is katal (kat) = $mol \cdot s^{-1}$. In clinical chemistry the derived unit µkatal is preferred (1 kat = 10^6 µkat).

When measuring a specific enzyme, lactate dehydrogenase in our case, specific values are entered to the equation 3 (4):

$$LD = \frac{\Delta A_{340}}{\varepsilon \times d} \cdot \frac{1}{60} \cdot \frac{V}{v} \cdot 10^6 \quad \mu mol \cdot l^{-1} \cdot s^{-1} \qquad (4)$$

$$LD = \frac{\Delta A_{340} \times 1.02 \times 10^{-3} \times 10^6}{6.22 \times 10^3 \times 60 \times 0.02 \times 10^{-3}} = \Delta A_{340} \times 136.7\ \mu kat/l$$

Symbol		**Unit**	Specific value for LD and used method
V	Volume of reaction mixture in cuvette	l	**$1.02 \cdot 10^{-3}$**
v	Volume of added serum	l	**$0.02 \cdot 10^{-3}$**
ε	Molar absorption coefficient for NADH at 340 nm	$l \cdot mol^{-1} \cdot cm^{-1}$	**$6.22 \cdot 10^3$**
d	Light path (cuvette thickness)	cm	1
10^6	Conversion from mol to µmol		10^6
60	Conversion from min. to sec.		60

Note: the first three values (in bold) depend on the estimated enzyme and method, whereas the following three values (light path, conversion from mol to µmol, conversion from min. to sec.) are usually the same for any kinetic measurement of enzyme activity.

Task 3: Spectroscopic examination of cerebrospinal fluid

Principle:

Analysis of absorption spectrum can be used for identification of substances, because for the given substance the spectrum is often characteristic, displaying one or several absorption maxims.

One possible application is demonstration of subarachnoid bleeding by direct spectroscopy of cerebrospinal fluid (CSF).

In subarachnoid bleeding blood enters the CSF. Already 4–8 hours since the beginning of bleeding in CSF we can demonstrate hemoglobin, which has maximal absorption at wavelength 415 nm and further small peaks are apparent at 540 nm a 580 nm. In older bleeding we can find bilirubin in CSF as the product of catabolism of hem. Bilirubin has a broad maximum at around 450 nm.

Physiologically the CSF is colorless and its spectrophotometric curve is flat or slightly rising from 600 nm to 370 nm. In the visible range the absorbance does not exceed 0.02.

Procedure:

Measure spectra of three samples of CSF against purified water in the wavelength range from 350 to 600 nm.

Results and evaluation:

Draw the obtained absorption spectra of CSF samples, describe the positions of eventual absorption maxims and conclude whether the findings suggests fresh or old subarachnoid bleeding, or no bleeding at all.