ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

Selected Immunochemical Methods

Practical Lesson on Medical Biochemistry

General Medicine

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(translated by Jan Pláteník)

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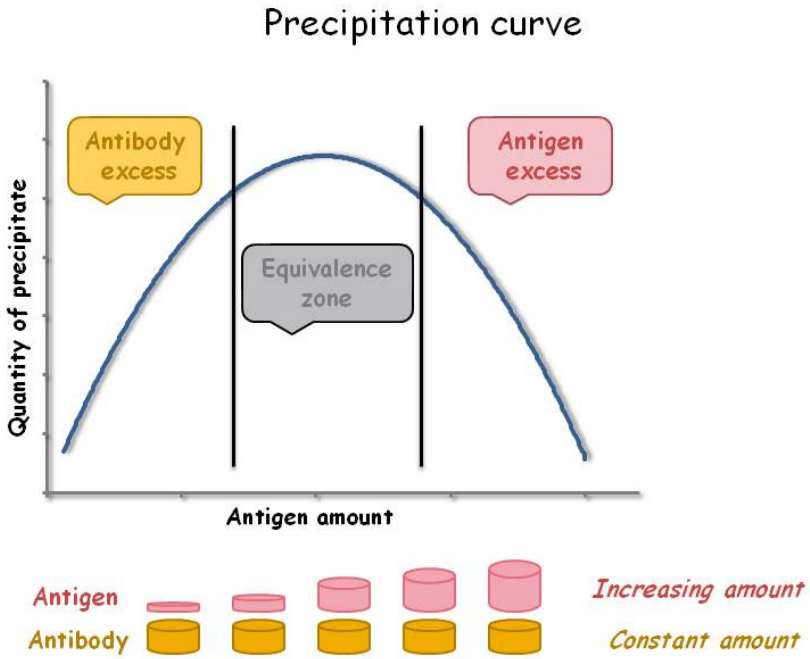
Task 1: Immunoprecipitation curve of human albumin and estimation of albumin concentration by means of immunoturbidimetry

Immunoprecipitation curve shows amount of the formed immunoprecipitate at ***different amounts of soluble antigen and constant amount of the corresponding antibody***. It is obtained if to a series of test tubes with the same amount of antibody in each tube we add increasing amounts of antigen (Fig. 1).

Fig. 1



After incubation, during which antigen reacts with the antibody, the amount of immunoprecipitate is measured in each tube. The immunoprecipitate consists of a space lattice formed by interconnected epitopes of antigens and the corresponding paratopes of antibodies. When the aggregate exceeds certain size, it loses its solubility and precipitates in the solution.

The prerequisite for immunoprecipitation is a ***reaction of antigen possessing several epitopes*** (polyvalent antigen) with antibodies that recognize these epitopes. If this requirement is not met, the immunoprecipitation does not occur. That is why haptens, which are equipped only with one epitope, cannot precipitate. Likewise, monoclonal antibodies that are directed against a single epitope are not suitable for immunoprecipitation.

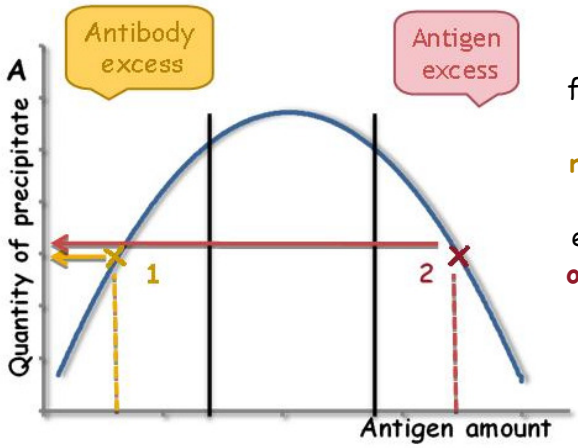
In our experiment human albumin and ram antibody against the human albumin were used for construction of the immunoprecipitation curve. The albumin is an important protein present in plasma at high concentration.

The used concentrations of albumin cover all parts of the precipitation curve: the area of antibody excess, the zone of equivalence and the area of antigen excess. Cloudiness resulting from the reaction of albumin with the corresponding antibody can be estimated by means of immunoturbidimetry. A common spectrophotometer can measure ***intensity of light that passes through the cloudy solution in forward direction***.

For the estimation of albumin concentration in an unknown sample the linear, ascending part of the precipitation curve (the area of antibody excess) should be used. In this area the antigen concentration is directly proportional to the amount of precipitate. It is important to stress that if the

immunoprecipitation is to be used for measurement of concentration of some substance, the reaction mixture must contain the ***antibody in excess***. If this condition is not kept, for high antigen concentrations we would get false low values. The sample must always be prediluted to reach a condition where the antibody is in excess, and multiply the result with the dilution factor used. Fig. 2 shows an example.

Fig. 2 Evaluation of immunoturbidimetry



The same values of absorbance can be found for **sample 1** with concentration of the estimated substance that lies **in region of antibody excess**, as well as for **sample 2** with a high amount of the estimated substance, which lies **in region of antigen excess**. In case of sample 2 an incorrect, false low value would be obtained under this condition.

Immunoturbidimetric methods are widely used for estimation of various analytes in biological fluids (e.g., various proteins in blood plasma).

Task 2: Evaluation of single radial immunodiffusion for estimation of IgG and IgM

Antigen (in this case human IgG or IgM), which is applied into a round start, radially diffuses into agarose gel that contains a specific (animal) antibody. Reaction of antigen with the specific antibody produces a precipitation ring whose area is proportional to the amount of antigen.

Reference values: IgG: 8 – 18 g/l IgM: 0.6 – 1.8 g/l

Task 3: Estimation of antibodies in unknown samples by means of ELISA test

1. Principle of method:

The enzyme immunoanalysis (EIA) methods represent a large group of immunoanalytical techniques that employ an ***enzyme*** as a ***label***. Technique called ***ELISA (enzyme-linked immunosorbent assay)*** is one of the EIA. A typical feature of ELISA is that either antigen or antibody is firmly anchored to a solid phase, usually a surface of the wells in microtitration strips. The ELISA methods are divided to ***competitive*** and ***non-competitive***. They can be used ***for estimation of antigens or antibodies***.

Our model ELISA kit is an example of an ***enzyme immunoanalysis*** designed for estimation of specific antibodies, such as the antibodies against various infectious agents (bacteria, viruses, etc.), autoantibodies directed against the body's own structures or IgE antibodies against particular allergens.

In this kit a specific antigen (Ag), which can be e.g. some virus or allergen, is attached on a surface of the wells in microtitration strips. In the unknown samples that will be added to the wells, presence of antibodies against this antigen will be examined. If antibodies against the antigen are present in the sample, they will bind to it and form immunocomplexes (Fig. 3, the first step).

After washing out the unbound components of the sample the bound immunocomplexes are detected with a conjugate of animal antibody against human antibody (antiimunoglobulin – anti-Ig) with enzyme peroxidase. An immunocomplex originates, consisting of the antigen anchored to the well surface, the human specific antibody and the conjugate (Fig. 3, the second step). Any unbound conjugates must be removed by washing.

The amount of attached labelled antibodies is visualized with an enzymatic reaction, catalyzed by peroxidase. After removal of the unbound molecules of conjugate we add a substrate that is converted to a colored product by the action of peroxidase. The enzymatic reaction is stopped by addition of an acid (stopping solution). Presence or absence of antibodies in tested samples is evaluated according to the color intensity (Fig. 3, the third step). A quantitative or qualitative way of evaluation can be employed.

Reaction conditions of non-competitive ELISA

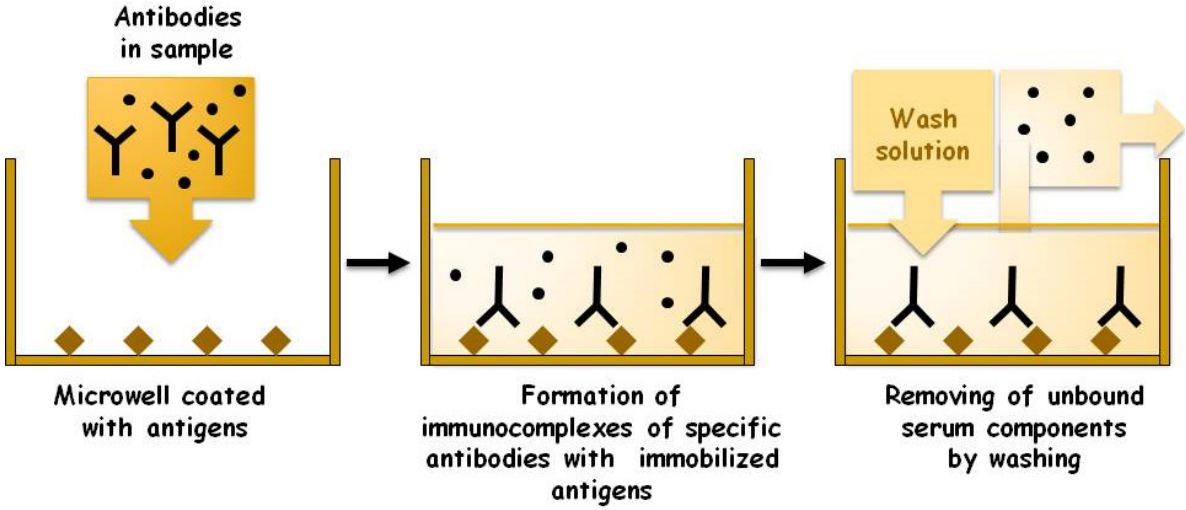
The non-competitive ELISA for estimation of antibodies requires the amount of antigen attached in the wells to be in excess over the expected amount of antibodies. In case of insufficient amount of the antigen some of the antibodies in tested samples could not bind and would be removed in the washing step. The resulting concentration of antibodies in the sample would be false low. Likewise, the amount of added conjugate must be in excess. A lack of conjugate would again lead to a false low result, because the requirement that all the immunocomplexes made of the antigen and the antibody get the conjugate bound, would not be met.

Explanation of some terms

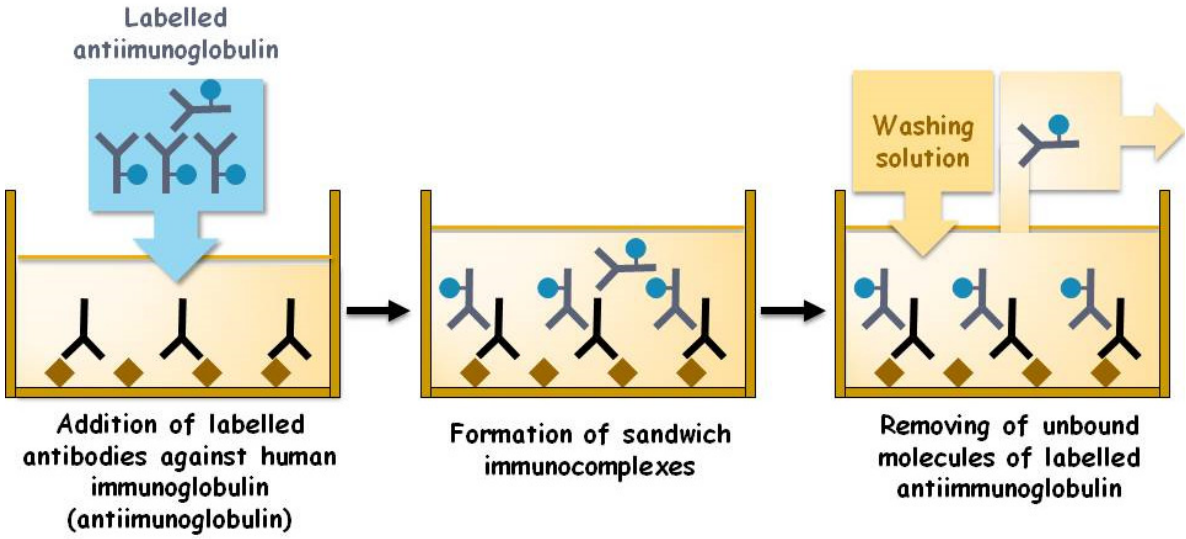
1. **Washing:** between particular steps of the ELISA procedure the unbound components of the serum (the first step) or the unbound enzyme-labelled antibody (the second step) must be removed. It is done by filling the wells with washing solution followed by its removal. This treatment is repeated several times. If the enzyme-labelled antibodies were not washed out, the resulting color in the wells would be the same regardless of the amount of specific antibodies in the samples.
2. **Conjugate:** denotes a junction of two or more substances by means of chemical or another reaction. In the EIA methods usually an animal antibody against a human immunoglobulin (anti-Ig) or antigen is conjugated with a label, e.g. an enzyme, which enables detection of the immunocomplexes. In our ELISA method the conjugate is anti-Ig with peroxidase (anti-Ig/Px).
3. **Substrate solution:** depends on the enzyme present in the conjugate. For peroxidase a benzidine derivative tetramethylbenzidine is used, whose reduced form is colorless. By oxidation with hydrogen peroxide in the presence of peroxidase it is converted to a colored product (Fig. 4). If the peroxidase conjugate is absent from the wells, the color conversion of tetramethylbenzidine does not occur and the contents of the wells remains colorless.
4. **Stopping solution:** is a weak solution of acid that terminates the enzymatic reaction. In order to produce a mutually comparable results, the reaction is stopped after certain period of time. Simultaneously the color changes from blue to yellow, which is measurable with a spectrophotometer at wavelength 450 nm. In case of positive samples the yellow coloration is visible even by a bare eye.

Fig. 3 Procedure of the non-competitive ELISA method for estimation of antibodies

First step: binding of specific antibodies from sample on antigen anchored to well surface



Second step: addition of conjugate (enzyme-labelled antiimmunoglobulin) to immunocomplexes anchored to well surface and formation of "sandwich"



Third step: visualization of amount of bound conjugates by means of enzymatic reaction

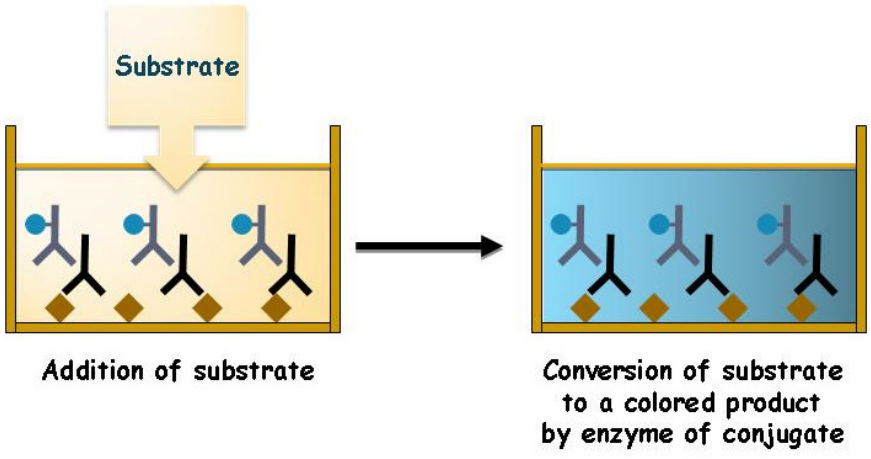
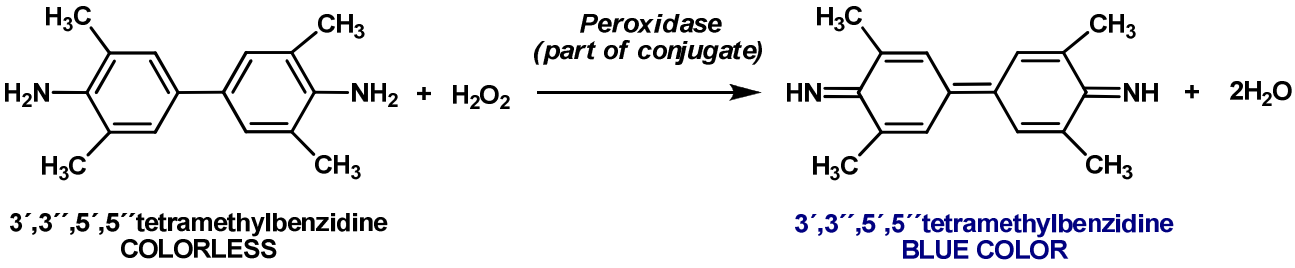


Fig. 4 Principle of the chemical reaction catalyzed by peroxidase of the conjugate



2. Evaluation

Performance check of ELISA method

In the ***blank*** wells ***no visible color should develop***, because instead of sample they only received the diluting solution, which does not contain any antibodies. The conjugate has nothing to bind and is removed during the washing steps. Weakly yellow color can be caused by remnants of the conjugate due to poor washing.

Evaluation of samples

If the tested samples contain antibodies against the antigen immobilized to the well surface, after the addition of the stopping solution the contents of the wells are colored yellow. The intensity of yellow coloration can be evaluated ***qualitatively or quantitatively***.

a. Qualitative evaluation

In this way of evaluation the samples are considered either positive or negative. It is necessary to know how to differentiate between the sample positivity and negativity. The necessary data for this are usually provided by the manufacturer of the kit. In our ELISA method we process a cut-off control sample whose absorbance represents the borderline value between the positive and negative samples. The specific procedure for the interpretation of the results is described in the instructions to the experiment.

Another option how to evaluate the extent of sample ***positivity*** is an assessment by means of dilution (titration) of the sample. In low dilution all the wells with positive samples are strongly colored. If diluted more, the strongly positive samples remain intensely colored, while the weakly positive samples display a color of reduced intensity. ***Negative*** samples do not contain any specific antibodies and the unbound conjugate is removed by washing. Therefore, the wells remain colorless. Likewise, antibodies against other antigens are not bound by the antigen in the wells and are also washed out in the subsequent step.

b. Quantitative evaluation

If a quantitative estimation of antibodies is required, standards in several dilutions must be processed together with the samples. The intensity of yellow color in the wells is (in certain concentration range) proportional to the amount of antibodies present in the sample.

The color intensity is estimated by measurement of absorbance using a special spectrophotometer with vertical light beam. The absorbance values are used for construction of calibration curve. The concentration of antibodies in the unknown samples is then read from the calibration curve.

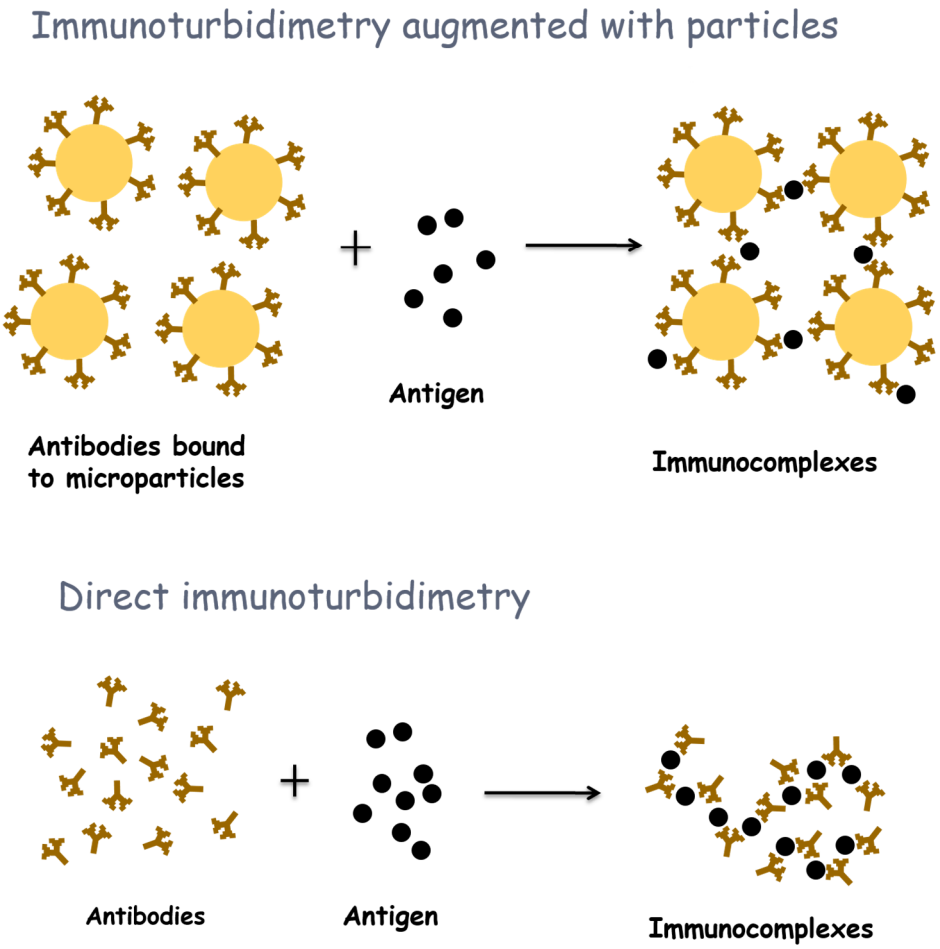
Task 4: Estimation of concentration of C-reactive protein in serum by means of turbidimetric POCT[1] test

Principle of method

C-reactive protein (CRP) is one of the acute phase proteins.[2]

CRP will be estimated by an immunoturbidimetric method that employs microparticles coated with antibodies against CRP (imunoturbidimetry augmented with particles). This modification increases sensitivity of the method, because immune complexes formed by CRP present in the examined sample with antibodies attached to the particles produce larger aggregates and so more intense turbidity than in case of usage of free antibodies (direct immunoturbidimetry – see Task 1) (Fig. 6).

Fig. 6 Principle of direct immunoturbidimetry and immunoturbidimetry augmented with particles



[1] POCT (point-of-care testing) means performing diagnostic tests directly at the place of patient care (e.g. in a medical office or bedside) with the aim to quickly obtain information critical for diagnosis, treatment or monitoring of vital functions. For POCT special diagnostic kits are developed that require only small and user-friendly instruments, or require no further equipment.

[2] Acute phase proteins will be discussed in detail in the summer semester, in practical lesson on blood plasma proteins.

Literature:

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Leaflet to kit ELISA-VIDITEST EDUCO-Diagnostic.

Leaflet to kit recomLine Campylobacter IgG/IgA ALL DIAG (český návod LABOSERV s.r.o.).