ENZYME LINKED IMMUNOSORBENT ASSAY

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Annotation: *The current state of enzyme immunoassay of medicinal substances is considered. The main methods for obtaining and purifying specific antibodies, immunogens, enzyme conjugates are described. Examples of definitions are given medicinal substances by enzyme immunoassay, lists the possible causes of errors in the determination of medicinal substances by enzyme immunoassay methods analysis.*

Key words: *enzyme immunoassay, enzyme, conjugate, medicinal substances.*

Introduction. Instrumental methods of analysis (chromatographic, electrochemical sky, spectrometric), used for detection and quantification division of medicinal substances in biological logical liquids, as a rule, require preliminary sample preparation.

The use of specific mutual antigen-antibody interactions allowidentify and quantify new definition of a large range of target analytes. Since the reaction conducts directly in the bioliquid, then not additional application required isolation and purification methods. Methods of immunochemical analysis make it possible to simultaneously analyze a large number of samples, which is convenient for the purposes of express analysis.

Enzyme immunoassay is widely used to determine medicinal substances and their metabolites. This article discusses the history of development, the possibility of using enzyme immunoassay.

History of development enzyme immune analysis

In 1959 R.S. Yalow and S.A. Berson proposed an immunological method for the quantitative determination of insulin in human serum. The method is based on competition between unlabeled insulin and 131I labeled insulin for the number of binding sites on insulin antibodies. The amount of insulin in plasma is inversely proportional to the amount of labeled insulin bound to the antibodies. The ease of

measuring low levels of radioactivity, the high specificity of the binding of the analyte by antibodies were the basis of a new method called immunological.

Along with the advantages (universality, selectivity and specificity, sensitivity and ease of implementation), radioimmunoassay (RIA) also has disadvantages. The main disadvantages include: short half-life of isotopes, danger to health when working with isotope labels, radiation damages the structure of labeled molecules, the difficulty of automating RIA, etc.

In 1971, E. Engvall, R. Pelmann, V.K. van Veemen and A.N. Shuurs proposed another type of sensitive and versatile labels for immunoassays - enzymes.

Enzyme labels are the most sensitive and versatile, as they are protein molecules with a powerful catalytic effect. Most enzyme labels are capable of converting 10 substrate molecules per 1 enzyme molecule into products in 1 min. The catalytic activity of an enzyme depends significantly on its three-dimensional structure (configuration). Another advantage enzyme labels due to the presence their molecules contain numerous functional groups (amino groups, carboxyl, sulfhydryl, tyrosine residues) through which ligand molecules can be attached.

The activity of the label in the fraction is measured after the separation of labeled and unlabeled antigens bound to antibodies free antigens. This method is referred to as immunoassay with separation of components and is called heterogeneous enzyme-linked immunosorbent assay (ELISA – Enzyme Linked Immunosorbent assay).

Designed by K.E. Rubenshtein et al. in 1972 y. analysis does not require separation of antibody-bound and free antigens. This method measures the specific activity of an enzyme when antibodies bind to labeled antigens. This method is called homogeneous immunoassay, since such an assay does not require heterogeneous phases to separate bound and free antigens. A more appropriate term for such a method is “separation-free immunoassay”.

Enzyme immunoassay without separation of components using lysozyme as an enzyme label was proposed for the determination of morphine. The method was called homogeneous ELISA, since in this method there is no need to separate the forms of the enzyme-labeled antigen (the free form of the antigen and associated with antibodies).

The antigen (morphine) in the sample competes with the enzyme-bound antigen to form a complex with the antibodies. The resulting Ab-Ag-E complex has
has low enzymatic activity. In the presence of an antigen in the sample, the Ab-Ag complex, part of the Ag-E remains in an unbound state and catalyzes the transformation of substrates into products. In this variant of ELISA, enzymatic activity is directly proportional to the amount of free antigen in the sample.

ELISA with separation of components (heterogeneous analysis) is characterized in that the enzyme-labeled antigen (Ag-E) competes with the antigen of the analyzed sample for a limited number of antibodies located (immobilized) on a solid carrier.

After incubation (specified temperature and duration) separate the complex Ag-E-Ab from free Ag-E and analyze the fraction associated with antibodies.

ELISA with separation of components has high sensitivity. For example, ELISA with component separation allows you to determine ferritin with sensitivity up to 2x10-19 M.

There are two types of enzyme immunoassay heterogeneous methods - competitive and non-competitive (sequential) analysis.

Sequential heterogeneous analysis consists of two stages. In the first step, antibodies adsorbed on solid carrier, interact with the antigen or some other analyzed protein. Unbound components are washed off and a solution containing enzyme-conjugated antibodies is added. Then the unbound components are washed (second stage) and the appropriate substrate is introduced into the system to carry out the enzymatic reaction. In a competitive heterogeneous assay, enzyme-labeled and unbound antigens compete with antibodies immobilized on a solid support.

After incubation, wash off the excess unbound components and into the system introduce the appropriate substrate. During immunoassay analysis, it is necessary to take into account the influence a number of factors: nature and method of preparation of the carrier, type and content of the conjugated enzyme, sequence of reactions, incubation time, the possibility of manifestation of "matrix effects".

To date, many ELISA technologies have been developed that combine the use of enzymes as labels and the possibility of their detection using appropriate enzyme systems. Modern works devoted to ELISA consider various aspects of the structure of immunogens (hapten design, choice of carrier protein), type of antibodies obtained (polyclonal, monoclonal) and ELISA format.

Antibody production and purification

One of the main stages of development any immunoassay technique - obtaining specific antibodies to the antigen being determined. To a large extent, the sensitivity and specificity of the analysis technique depends on the quality of antibodies. In immunological studies, it is often necessary to obtain purified antibody preparations, i.e. antigen-specific or non-specific immunoglobulins. Antibodies are obtained by immunizing animals (mice, guinea pigs, rabbits) with the appropriate antigen.

Isolation of non-specific immunoglobulins from serum is usually carried out by sequential protein fractionation, which includes the following steps: precipitation of gamma globulins in 30-50% ammonium sulfate solution, gel filtration to obtain molecules of the appropriate size, ion exchange chromatography to isolate molecules, carrying a net positive charge at neutral pH, affinity chromatography using natural immunoglobulin ligands.

Isolation of antigen-specific immunoglobulins is carried out by affinity chromatography. The antigen is "sewn" to the particles of sepharose and the "pure"

antibodies bound to it are eluted from the immunosorbent buffer solution (glycine-HCl) or sodium thiocyanate solution. Affinity chromatography is also used to obtain purified antigen preparations. One cycle of affinity chromatography allows you to purify proteins by 1000 times or more.

However, even this purification method does not completely eliminate the heterogeneity of the antibody preparation. The way out of this difficulty is to obtain antibodies with one specificity, reacting with a single antigenic determinant. Such antibodies are called monoclonal. They are obtained by cell engineering methods by hybridization of immunocompetent B lymphocytes and myeloma tumor cells capable of rapid reproduction, an unlimited number of divisions (unlike most non-tumor cells, in which the number of divisions is limited). Preparations of monoclonal antibodies are characterized by the constancy of the composition and physico-chemical properties, low probability of cross-reaction with "foreign" antigens. This is a high tech product. Its disadvantage is often a relatively low affinity for the substrate, low affinity.

Synthesis of immunogens

An immune response occurs in the body only with the introduction of a compound whose molecular weight exceeds 3000. Therefore, obtaining antibodies to low molecular weight antigens is complicated by the fact that they themselves do not induce the formation of antibodies. To convert small molecules into an immunogenic state, they can be aggregated into larger particles or attached to a carrier protein (i.e., synthesize an immunogen). The most common carrier protein is human or bovine albumin.

Obtaining enzyme conjugates

Most commonly used in ELISA enzymes: horseradish peroxidase, alkaline phosphatase, β-galactosidase, acetylcholinesterase, catalase, urease, glucose-6 phosphate dehydrogenase, malate dehydrogenase and etc. (Table 1).

Enzymes used in ELISA must meet a number of general requirements:

- high specificity and specific catalytic activity of the enzyme, which makes it possible to detect the enzyme label at low concentrations;
- the availability of enzymes, the possibility of obtaining sufficiently pure enzyme preparations, stability during storage and after modification;
- the enzyme must not be present in analyzed biofluid;
- simplicity and sensitivity of the method for determining the products of the enzymatic reaction.

Enzyme activity is detected by changes in optical density, fluorimetric and electrochemical methods.

The development of ELISA methods is associated with the need to obtain conjugates of marker enzymes with antigens or antibodies in which the antigen or antibody retains immunological activity and does not inactivate the enzyme. However,

all the main approaches used for the chemical conjugation of proteins and haptens lead to partial inactivation of enzymes and heterogeneity of conjugates, which affects the specificity and sensitivity of enzyme immunoassay. Genetic engineering methods can be used to obtain recombinant conjugates of proteins with antibodies. Such conjugates have a number of advantages: they are homogeneous in composition, have a 1:1 stoichiometry, retain the functional activity of both the marker protein and the antigen/antibody, as well as reproducibility and relative ease of preparation.

Table 1

Enzymes used in ELISA

Enzyme	Indicator system	Registration Method enzyme activity
horseradish peroxidase	H_2O_2/chromogen (o-phenylenediamine, 5-aminosalicylic acid)	photometric, fluorimetric, chemiluminescent, electrochemical
alkaline phosphatase	4-nitrophenyl phosphate	photometric, fluorimetric
β-galactosidase	2-nitro-β-D-galactoside	Photometric
Acetylcholinesterase	acetylcholine/5,5'-dithiobis (2-nitrobenzoic acid)	photometric
glucose-6-phosphate dehydrogenase	NADP+/NADPH	photometric, fluorimetric
glucose oxidase	H_2O_2/chromogen	Photometric

ELISA variants and examples definitions

The choice of ELISA technology depends on a specific applied problem to be solved by the analysis. Often, in a chemical-toxicological study, it is sufficient to establish only the fact of the presence or absence of substances in the samples.

Sometimes, however, it is important to determine the concentration of substances in samples with high accuracy.

Promising variants of ELISA used in practice are ELISA technologies (Enzyme Linked Immunosorbent Assay- heterogeneous enzyme-linked immunosorbent assay),

EMIT (Enzyme Multiplied Immunoassay Tests), CEDIA (Cloned Enzyme Donor Immunoassay - cloned enzyme-donor immunoassay), KIMS (Kinetic Interaction of Microparticles in Solution – kinetic interaction of microparticles in solution).

In the literature, there are numerous reports on the use of ELISA for the diagnosis and determination of narcotic and medicinal substances in various biological fluids. The minimum detectable concentrations for sulfanilamide preparations (sulfamerazine, sulfatiazole, sulfamethazine, sulfadiazine) were determined, lisinopril, enalapril, derivatives of barbituric acid, derivatives of benzodiazepines, morphine, amphetamines, cannabinoids. There is evidence of the use of ELISA for the analysis of post-mortem blood for the presence of cocaine and opiates.

Currently, ready-made commercial kits of reagents are being produced that allow the detection of medicinal substances with a guaranteed detection limit of 300 - 500 ng / ml from Syva (USA), F. Hoffmann-La Roche Ltd (France), IPAV RAS (CIS), Abbot (USA). Commercial diagnostic kits are predominantly based on the principles of solid phase ELISA. Most of the kits produced use polyclonal antibodies, since their production involves with less cost. The kits are most often implemented on microplates or in test tubes.

Possible sources of error ELISA

Errors that occur in the determination of medicinal substances by ELISA can be due to a number of reasons. The biological fluids used for analysis can affect the activity of the enzyme-marker due to the salt composition, which changes the pH value and ionic strength of the analyzed sample.

The result of the analysis may be affected an admixture of an endogenous enzyme or salt forms of metabolites that lose the ability to compete in an immunological reaction due to protein binding biofluids.

The possible entry into the reaction mixture of chemical inhibitors of proteins should be avoided. Many heavy metal salts, such as mercury-containing preservatives, are enzyme inhibitors. Anticoagulants, EDTA, and some drug metabolites also reduce enzyme activity.

So, for example, ELISA tests can give false negative results if preservatives are present in the sample (sodium acid, sodium benzoate, which are added to preserve the samples), since the preservatives used block the activity of the horseradish peroxidase enzyme.

A special specific problem of determining medicinal substances by immunochemical methods of analysis is cross-reactivity or cross-reaction (binding of structurally related substances). Cross reactivity for analytes should be confirmed. Most manufacturers investigate potential interfering substances and a list of them is included with the immunokits.

The blood of the deceased decomposes over time, producing biogenic amines that cross-react with antibodies in immunoassays, which also leads to false positive results.

To avoid false positive results, it is required that all specimens positive in immunochemical tests be confirmed by others. methods (thin-layer chromatography, gas chromatography, high-performance gas chromatography, chromato-mass spectrometry).

Conclusion. A number of advantages of enzyme immunoassay analysis (high sensitivity, specificity, small sample volumes, speed of analysis) make it possible to use it as a preliminary method for screening diagnostics of drugs and narcotic substances in biological media.

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