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ELISA: METHODS, PRINCIPLES AND APPLICATION

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Abstract
Enzyme-linked immunosorbent assay (ELISA) is widely used as diagnostic tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample.
This article discusses the history, types, principle of the method, and importance of enzyme immunoassay in diagnostics.

Keywords: Enzyme immunoassay, enzyme, conjugate, medicinal substances.

INTRODUCTION

Although the basic principle of ELISA and radioimmunoassay (RIA) techniques dates back to 1941, RIA method was first used by Yalow and Berson in 1960s to measure the endogenous plasma insulin level. In fact, ELISA method was invented simultaneously by two research teams at the same time. However, ELISA method was pioneered largely by the Swiss scientists Engvall, and Perlmann who died in 2005. These two researchers developed the ELISA method in 1971 by modifying the RIA method. In other words, they devised the immunological ELISA method by conjugating the tagged antigen and antibody radioisotopes in RIA with enzymes rather than radioactive iodine 125. They employed this new method to determine the levels of IgG in rabbit serum. In the same year, a different research team succeeded in quantifying human chorionic gonadotropin amounts in the urine by using horseradish peroxidase (EC 1.11.17) enzyme with the EIA method [1,3]. The researchers applied for a patent both in the USA and Europe.
Following the invention of ELISA, a number of researchers used it: Carlson and colleagues in 1972, Holmgren and Svennerholm in diagnostic microbiology in 1973, Ljungstrom and colleagues to identify the presence of trichinosis in parasitology in 1974, and Voller et al. to diagnose malaria in 1975. Bishai and Galli, Leinikki et al. and Ukkonen et al. made use of the ELISA method to identify infections caused by influenza, parainfluenza and mumps viruses in 1978, 1979, and 1981, respectively [2]. In 1980, Siegle et al. modified the ELISA test and incorporated microtitration plates to identify the concentrations of various hormones, peptides, and proteins. The method

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which has found different fields of application and grown beyond infancy over time has become a routinely used method in research and diagnosis laboratories around the world [1,4].

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" [2,5].

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.

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 [6,7].

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 ligand.

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 [3].

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,

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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) [7,8]. 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 [2,4,5].

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

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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) [3,8].

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 [1,2].

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 [9].

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 azide, 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.

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methods (thin-layer chromatography, gas chromatography, high-performance gas chromatography, chromato-mass spectrometry) [2,4].

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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