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Designing an ELISA test system for identifying trenbolone hormone based on specific polyclonal and monoclonal antibodies

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Abstract

As a result of the research, methods of obtaining conjugate preparations of trenbolone with antigenic properties have been developed. Optimal schemes of animal immunization have been developed, which allow obtaining preparations of specific antibodies. Hybrid strains producing antibodies to the trenbolone have been obtained, their immunochemical properties have been studied, and their preparative quantity has been accumulated. The protocol for the enzyme-linked immunosorbent assay (ELISA) with the use of conjugation preparations with high molecular carriers, enzymes, and preparations of specific antibodies has been determined. Sensitivity and specificity of the developed ELISA test system have been checked. The protocol of preparing material samples for ELISA testing has been defined.

The research has been performed within the framework of the scientific project of grant financing by Ministry of Education and Science of the Republic of Kazakhstan "Development of the ELISA test system for trenbolone determination in animal products".

Keywords: trenbolone, antibodies, conjugates, immune-enzyme assay, livestock products, test system.

INTRODUCTION

The importance of quality and food safety increases every year, as ensuring safety of food raw materials and food products is one of the main facts that determine human health and gene pool preservation [1]. Important issue of product safety, on the one hand, is the prevention of fouling of raw materials for obtaining food products, which is ensured, in particular, by a system of monitoring the state of the environment, and on the other hand, by careful hygienic monitoring of production and finished products. Providing the structure, safety and quality of food products is an important strategic task of the state at the modern stage of development of the Republic of Kazakhstan [2, 3, 4]. Membership in the World Trade Organization (WTO) brings significant benefits for the economy of Kazakhstan, however there are problematic issues. One of the most important issues is ensuring food safety in regard to distribution in the global market of products containing xenobiotics, solving the problem of quality and competitiveness of domestic products on the world market [5].

In this respect, development of a technology that allows producing domestic highly sensitive and specific immune enzyme testing system for detecting residual quantities of steroid hormone trenbolone in products and raw materials of animal origin will allow equipping laboratories with available and inexpensive ELISA tests, which will allow monitoring hormonal stimulants' content in food products of animal origin [6, 7, 8, 9].

MATERIALS AND METHODS

The work has been performed on the basis of the immunological research laboratory of the Scientific-innovative Center of the Kostanay State University n.a. Akhmet Baytursynov.

To complete the task of the research, laboratory animals were used – rabbits, white mice and mice of BALB/s line.

During the research, serological, immune-chemical, biotechnological, physico-chemical and other methods were used.

MAIN REACTANTS AND EQUIPMENT

The chemical reagents used were the following: trenbolone (Sigma, China), bovine serum albumin (BSA), ovalbumin (OVA), thyroglobulin (THY), shell-fish haemocyanin (KLH), IgG-Free (Protease-Free) manufactured by Jackson Immuno Research (USA), orthophenylenediamine (OPD), tetramethylbenzidine (TMB), AntiRabbit, KBB, horseradish peroxidase (HRP), ammonium sulphate, Bradford solution, twin-20, anti-trenbolone antibody, OPD, distilled water, glacial acetic acid, carbodiimide (EDC) (Sigma-Aidrich, USA), glutaraldehyde (Fluka, Switzerland), line of myeloma cells X63.Ag.8.6.5.3 (Germany), medium RPMI 1640 (Sigma, USA), cow fetus serum of companies HyClon (USA), sodium pyruvate, 7.5% sodium bicarbonate, Hepes (Sigma, USA), L-glutamine (Sigma, USA), 2-mercaptoethanol, folic acid, complete and incomplete Freund adjuvants, polyethylene glycol (PEG) (Sigma, USA), dimethyl sulfoxide (DMSO) (PanEko, Russia), and preparations for enzyme immune research, immune diffusion reaction, and electrophoresis.

RESULTS AND DISCUSSION

The proposed test system for detecting trenbolone in animal products is based on the competitive variant of ELISA. This variant is based on the competition between the sought antigen and the marked standard one for the centers of binding with the antibody adsorbed on the solid phase.

The use of the competitive variant of ELISA for identification of trenbolone (antigen) is explained by the fact that this hormone is a low-molecular compound, haptene, which is unable to cause immune response. In this regard, the test system is based on the antibodies obtained to the conjugated haptene.

The first step of obtaining conjugated preparations of trenbolone was preliminary derivatization of haptene to cross-linking with high molecular proteins. The reaction components were activated with glutaric anhydride. Carriers were commercially available protein preparations - bovine serum albumin with the molecular weight of 690 kDa, ovalbumin (OVA) - 45 kDa, limpet hemocyanin (KLH) – 350 - 400 kDa,

thyroglobulin (THY) - 660 kDa. When choosing the carrier protein, its heterogeneity to antibodies of the immunized animal was taken into account. Mainly, the immune response was aimed at the carrier protein, and not at the haptene molecule, and this was taken into account when serum immunized animals were tested. The testing should show the antibodies content exactly to haptene, and not to the conjugate in general. The use of substances with the molecular weight less than 5-10 kDa incapable of inducing immune response results in the need for their covalent binding with the carrier protein. In response to immunization, antibodies are also formed to the determinants of the carrier, so we had to choose such a carrier that would not be used as an antigen in further studies. Since the size, the charge and the polar properties of haptenes determine induction of humoral response; these properties may greatly depend on the homogeneity and strength of binding between haptene and the carrier protein, and the nature of the media itself. For the purpose of choosing the most successful design of the immunogen, 4 conjugates with various media and with various ratios of haptene and the carrier were prepared and tested.

For obtaining trenbolone conjugates, 2 methods with general preparation of the antigen for cross-linking by the method of mixed anhydrides, which consisted of simple equimolar mixing haptene and protein with isobutyl chloroformate and medium – tributylamine, were tested.

The resulting preparations of conjugates were tested to determine the optimal working concentration by the ELISA method vs. commercially available antibodies. Thus, the concentration of 10 μg/ml was determined. During testing, synthesized conjugates also showed high antigenic properties in titrations: trenbolone with bovine serum albumin 1:51,200, with thyroglobulin 1:51,200 - 1:102,400, with limpet hemocyanin - 1:51,200, and with ovalbumin 1:25,600.

For obtaining antibodies specific to the trenbolone, laboratory animals (female rabbits weighing 2.5 to 3 kg) were immunized with synthesized conjugates. For these purposes, six various schemes of immunization with various antigen concentration, duration, intervals have been developed. The immune response was stimulated with complete and incomplete Freund's adjuvant.

The choice of the animal for immunization is also an important component of obtaining specific antibodies. In our case, female rabbits were used for the purity of the experiment, as trenbolone had high affinity to androgen receptors. Rabbits may also be kept in cages, they breed well in captivity, they are hardy, and live long, and their care, immunization and sampling blood are easy. The serum obtained from rabbits is stable during storage, and is easily cleaned from extraneous proteins.

Native serum was tested by the ELISA method against the heterologous antigen and the homologous carrier for the purpose of identifying the degree of specificity to trenbolone. The best results were shown by the serum obtained from animals immunized according to the schemes No. 2 (TR-THY), No. 3 (TR-BSA, TR-KLH), No. 1 (TR-OVA).

To clean serum from unwanted antibodies, and to obtain the purest preparations of immune globulins without reducing their immune activity, classic Castellani's method, sulfate-ammonium salting, and purification on immune-affine columns were used. The degree of immunoglobulins' purification was monitored using electrophoresis.

The stage of obtaining individual proteins is the most labor-consuming, since proteins possess conformational lability, and when working with them, denaturing effects are to be avoided. Most proteins denature and precipitate in case of short heating of the solution to 50-70°C, or in case of solution acidification up to pH-5. However, this precipitation is irreversible. Therefore, we used the method of proteins purification based on the differences of their solubility in various concentrations of salt in the solution. Thus, during precipitation with saturated ammonium sulfate, class G immunoglobulins precipitate, which almost do not contain admixtures of other proteins. With that, the greatest completeness of the precipitation occurs at pH equal to 7.2, i.e. close to the isoelectric point of the least charged IgG molecules.

Electrophoretic separation of proteins is based on specific pH value and on the ionic strength of the solution, in which proteins move in an electric field at a rate proportional to their total charge. The main method is electrophoresis in dissociating DDS-Na medium, according to the procedure described by Laemmli [10]. The electrophoretic method was used to determine the specificity of the obtained immunoglobulins, and to determine the presence of admixtures. During proteins' separation, the molecular weight of the obtained immunoglobulins was determined, which amounted to 160,000 Da, which corresponded to the molecular weight of IgG.

Avidity is determined by many complex factors, in particular, by heterogeneity of antibodies in this serum, directed to a specific antigenic determinant, and by the heterogeneity of the determinants themselves. The following circumstance should also be taken into account. Polyvalency of most antigens results in a kind of "reinforcing" effect: the strength of the bond between two molecules of the antigen via molecules of antibodies is always higher – usually many times higher than the strength of the arithmetic sum of the bonds of antigens via each antibody.

It is the avidity that characterizes the immune response in vivo, and is a measure of functional affinity of an antiserum to the natural polyvalent antigen. High avidity has an advantage over the low one for many reactions.

The ability of the obtained antibodies to form the antigen-antibody complex with source trenbolone not conjugated with high-molecular carriers was determined by competitive ELISA followed by building calibration graphs of optical density dependence on the concentration of trenbolone. As a result, the best result was shown by antibodies to trenbolone-thyroglobulin. This was due to the fact that the antigen was bonded with the most specific antibodies [11].

The monoclonal antibody technology was used for obtaining monoclonal antibodies [12]. Mice of line Balb/c were subjected to immunization with conjugated antigens of trenbolone (TR-OVA) (TR-THY). Immunization of linear mice according to the two-month scheme ensured obtaining sufficiently stimulated lymphocytes. Titers of antibodies in blood serum of immunized mice in case of indirect solid-phase immunoenzymometric analysis were 1:12,800 to 1:25,660, which indicated high production of clones of B – lymphocytes producing antibodies of given specificity. Lymphocytes of mice that produced antibodies against conjugated antigen TR-THY in the highest titers were used for hybridization with myeloma line X63-Ag8.653.

The obtained serums were tested in ELISA analysis, where TR-BSA conjugate was adsorbed antigen. Figure 1 shows the results of testing studied serums taken from groups of animals immunized with TR-THY and TR-OVA conjugates, compared to the serum from an intact animal. Figure 1 shows the average values of optical density of three groups of mice, three animals in each group. The titer of the antiserum in each of the three groups of animals immunized with TR-THY conjugate was higher than in animals immunized with TR-OVA conjugate. The splenocytes' donor for hybridization was a mouse immunized with the TR-THY conjugate, with the highest titer of the antiserum. Optical density in the ELISA analysis at the wavelength of 450 nm was 1.6 points.

As a result of the hybridization of splenocytes of the mice immunized with TR-THY conjugate with the cells of the X63-

Ag8.653 myeloma line, hybridomes producing monoclonal antibodies to the trenbolone were obtained.

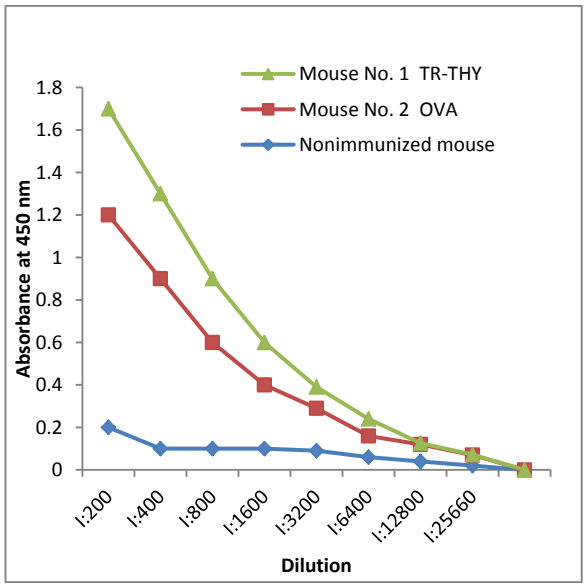

Figure 1. The results of analyzing immune serums from groups of mice immunized with various conjugates, compared to normal mouse serum

Hybrid cells that stably produce antibodies to the conjugated antigen TR-THY were cloned three times. During the first cloning, production of antibodies of hybrid cells' subclones was 16%, during the second cloning - 50%. After cloning, hybrid cells did not change their activity for 15 passages. Productivity of the hybrid was determined within 8 days, which in the cells' culture was 0.05 µg, in ascite - 8 to 16 mg.

For testing the optimum parameters of formulating a competitive ELISA based on the use of polyclonal and monoclonal antibodies for determining a standard (reaction monitoring) solution of trenbolone, we studied the influence of main physico-chemical factors: temperature, ionic strength and pH value, concentration ratio, buffer, duration of interaction, which determined the flow of the reaction.

Identification of trenbolone in this variant of ELISA was based on free trenbolone competition from the studied sample and the trenbolone marked with the enzyme. Thus, the amount of trenbolone in the studied sample was inversely proportional to the enzyme activity on the solid phase.

Setting of the reaction consisted of the following main stages:

- sensitization of specific polyclonal/monoclonal antibodies on a solid substrate, i.e. immobilizing antibodies in the holes of the tablet to conjugates of trenbolone with a heterologous protein carrier;
- introduction of studied samples;
- introduction of the antigen marked with the horseradish peroxidase enzyme;
- incubation;
- introduction of tetramethylbenzidine;
- stopping the reaction by introducing a stop reactant;
- controlling the reaction on a spectrophotometer with vertical flow of light at the wavelength of 450 nm.

Based on the data obtained in research, for further development of the trenbolone quantitative determination method in animal products, we chose the following optimal conditions of reactions: the working dilution of polyclonal/monoclonal antibodies - 0.005 mg/ml, incubation temperature of the tablet - 37ºC, incubation time - 2 hours, phosphate buffered saline with Tween-20 (0.05%) used for washing, standard concentration of trenbolone solutions - 0, 25, 50, 100, 200, 500 µg/kg. Incubation of samples with trenbolone conjugate with horseradish peroxidase lasted for 1 hour at room temperature (20-25°C). The studied samples were introduced for analysis in two repetitions.

The results were interpreted according to the calibration curve in a semi-logarithmic system of coordinates, which was built on the relative values of optical density calculated for the standard solutions, and on corresponding values of trenbolone concentration in µg/kg.

Efficiency of any method for identifying hazardous substances, including residues of hormones in animal products, largely depends on properly chosen method of preparing samples of materials for the study. We aimed at developing an optimum method of extracting the hormone from samples of meat, allowing detection of the substance in the minimum quantity. Solutions containing hormone trenbolone at the rate of 50 µg/l of active substance were artificially introduced into meat samples. Next, the effect of various chemicals on muscle tissue was studied. During the experiment, the same sample of meat experimentally contaminated with the hormone was subjected to various physico-chemical factors, and their actions were compared. Chemical compounds to be used as the extracts were chosen based on their polarity. In this study we used 3 methods of extraction of the hormone:

- method No. 1 - extraction with tret-butyl-methyl ether;
- method No. 2 - extraction with ethyl acetate;
- method No. 3 - extraction with acetone.

Based on the results obtained, the optimal method of muscle tissue sample preparation was determined for ELISA testing – method No. 1, extraction with tret-butyl-methyl ether. It consisted in freeing the sample of meat from fat and connecting tissues, weighing a 15 g portion and homogenizing with 15 ml of phosphate-saline buffer. The homogenized sample was transferred to a vial, and shaken "up and down". Then the homogenate was transferred into a centrifuge tube, tret-butyl-methyl ether was added, and was stirred in a shaker. After centrifugation, supernatant was taken into a glass vial, and n-hexane was added, thoroughly shaken, and settled. After removing the top hexane layer, the bottom layer was evaporated until dryness. The dry residue was diluted in methanol, then in a phosphate buffer. Further purification was performed in RIDA C18 columns. 50 µl per hole were used for the study.

Conclusion

Thus, the research resulted in developing a test system for detecting residual quantities of trenbolone based on the competitive ELISA variant. The optimal parameters and conditions for ELISA have been established, based on the polyclonal and monoclonal antibodies obtained to the conjugates of trenbolone with high molecular carriers.

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