ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Obtaining of Specific Antibodies to Trenbolone Acetate Conjugated by Macromolecular Carriers.

R. Ryschanova[1]*, P. Ibragimov[1], M. Kuibagarov[2], Zh. Bermukhametov[1], P. Shevchenko[1]

[1]Veterinary School, Kostanay State A.Baytursynov University, A.Baytursynov Street 47, 110000 Kostanay, Kazakhstan.
[2]Kazakh Agrotechnical University named under S.Seifullin, Pobeda Street 62, 010000 Astana, Kazakhstan.

ABSTRACT

Were synthesized conjugates of trenbolone (TR) with macromolecular protein carriers: thyroglobulin (THY), the protein carrier keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), egg white albumin (OVA). Were developed schemes of immunization of rabbits and mice of balb/c line by the synthesized immunogenes - conjugates of trenbolone. Were received specific immune sera against trenbolone conjugated with different carriers. The resulting preparations of conjugates of trenbolone were tested with linked immunosorbent assey (ELIZA) and in the reaction of the immune diffusion (RID).

Keywords: trenbolone, conjugate, antiserum.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

One of the most powerful synthetic anabolic steroids that were developed to stimulate growth of animals is trenbolone. Trenbolone is a synthetic androgen that acts in times more active than the natural hormone testosterone. This fact, as well as the low cost of its synthesis defined the intensive implementation of this drug in the practice of livestock [1]. The risk associated with the presence of residues of anabolic agents in livestock production led to the prohibition of the use of these drugs as growth promoters in animals in the European Union since 1981 (Directive 2003/74/EU)[2]. In some countries (USA, Canada, Australia, New Zealand, Argentina, etc.), some natural and synthetic hormonal growth promoters of farm animals are officially allowed: testosterone, estradiol, progesterone, trenbolone, zeranol, melengestrol acetate [3]. To date for indication of hormones in livestock production is widely used linked immunosorbent assey (ELIZA), based on the use of immune serum containing antibodies specific to antigenic determinates hormone. One of the main problems in obtaining antibodies against steroid hormones is their relative molecular mass. This is the main reason for the lack of immune response to the intake of hapten into the animal organism, and requires a preliminary preparation of antigen – conjugation [4].

Objective

To synthesize highly active preparations of conjugates of hapten`s determinants with trenbolone for obtaining specific immune sera.

MATERIALS AND METHODS

The work was carried out in immunobiological laboratory of Innovative scientific and educational center of Kostanay State University named after Akhmet Baitursynov, the project grant funding No. 2410/GF4, 2015-2017.

The study used chemical and biological products made by Sigma-Aldrich Chemicals (USA, Germany), Paneko (Russia) and laboratory animals: outbred mice, mice of balb/c line, rabbits.

In studies were used serological, immunochemical, biotechnological, and physico-chemical methods.

When receiving the conjugate TR-THY was used the described earlier method by Jane Fitzpatrick et al [5] with modification.

Since the hormone trenbolone by itself is not able to form chemical bonds and form an immunogenic complex with the carriers of different nature, a preliminary preparation is necessary.

The derivatization of trenbolone with 17-hemisuccinate (17-Hemisuccinate) (TR-HS).

In the first stage was carried out the preparation of the reaction mixture with trenbolone by the method of W. R. Jondorf used in 1980 [6]. Was taken 100 mg of trenbolone and dissolved in 3 ml of dichloromethane, in 2 ml of dry pyridine, then was added 187 mg of glutaric anhydride and the resultant mixture was inoperable at room temperature for 48 hours. The reaction mixture was evaporated to dryness in a rotary vacuum evaporator with a heat gun. The freeze-dried residue was dissolved in 10 ml of chloroform. To remove an excess of glutaric anhydride, the freeze-dried residue was twice washed with water. The chloroform was removed from the vacuum. The resulting residue was stored in a dark place at room temperature.

The synthesis of the drug conjugate of trenbolone with thyroglobulin (TR-THY).

In a glass test tube was added 5 mg of TR-HS, and it was dissolved in 500 µl of dioxane, was added dry N-hydroxysuccinimide (NHS) to obtain a final molarity of 0.1 M. To the resulting solution was added 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) (EDC), which was dissolved in 250 µl of distilled water and it was incubated for 10 min with stirring. In the solution was added 10 mg of thyroglobulin (THY), in 700 µl of 0.05 M phosphate buffer with pH 7.8 and it was incubated for 75 minutes. Then it was dialyzed for 48 hours against four changes of phosphate-saline buffer (PBS) at +4° C.

Receiving of a drug conjugate of trenbolone with protein carrier keyhole limpet hemocyanin (TR-KLH) was carried out in a similar way as in the synthesis of the conjugate TR-TG.

Obtaining of a conjugate of TR-OVA and TR-BSA was performed by the method described by Jane Fitzpatrick et al [5] and other researchers [7] with some changes and preliminary preparation of trenbolone. It was taken 10 mg of TR-HS, then it was dissolved in 200 µl of dimethylformamide (DMF), was added 8 µl of tributylamine and 2.5 µl of butylchloroformate and the resultant mixture was stirred on ice for 12 minutes. Solution containing 20 mg of carrier BSA or OVA in 1 ml of water, and 2.5 ml of dimethylformamide, and 25 µl of 1 M NaOH was cooled to 0° C and it was added to an activated hapten. The resulting mixture was incubated at +4°C for 20 hours. To remove non-conjugated hapten a mixture was dialyzed over night against 5 liters of PBS.

Immunization of rabbits.

To obtain polyclonal antibodies were selected according to the principle of analogues 4 groups of rabbits (3 females in each group, with weight of 3-4 kg) that were injected with previously obtained preparations of the conjugates TR-THY, TR-KLH, TR-OVA, TR-BSA. Two schemes of immunization were developed: a short-term immunization was 14 days and a long-term was 49 days. Preparations of conjugates were injected at a dose of 0.5 ml for each rabbit with the antigen concentration of 0.2 mg/ml, subcutaneously in the back at several points with the subsequent reimmunization. As an immunostimulant was used a complete and incomplete Freund adjuvant in a 1:1 ratio. On the 4th day according to the short circuit and on the 7th day according to the long-term immunization. After the last injection of an antigen was performed a blood sampling for testing of sera by enzyme immunoassay (ELIZA) and the reaction of the immune diffusion (RID). In the process of immunization from the marginal vein of the ear of rabbits was taken a small blood sample to assess the specificity of the antibody. For a negative control before the immunization was taken the blood of rabbits. To study the immunochemical properties of specific antibodies the blood was taken in a volume of 15-20 ml directly from the heart by cardiac puncture.

Immunization of white mice of balb/c line.

Immunization of mice was carried out by short-circuits (3-fold immunization with an interval of 7 days) with a concentration of 0.1 mg/ml of antigen-conjugate. Drugs were administered intraperitoneally in a dose of 0.1 ml of antigen with 0.1 ml of incomplete Freund's adjuvant. In 5 days after the last injection the antigen was carried out the taking of blood for testing serum by enzyme immunoassay.

Firm-phased indirect-linked immunosorbent assay (ELISA).

Cells of 96-well plate were sensitized with a conjugate of trenbolone with heterologous carrier at a concentration of 5 µg/ml at a temperature of $+4^0$ C over the night. To remove non-associated antigen the plate was washed 3 times with phosphate-saline buffer (PBS) with 0.5 % tween-20 (TV). After washing was added the immune serum and it was incubated at a temperature of $+37^0$ C for 60 minutes. After incubation to remove the not binding antibody, the plate was washed in the described manner. Then in the wells of the plate were added antitypical antibodies, labeled with horseradish peroxidase (HRP) (antiserum conjugate) in a volume of 100 µl and it was incubated at $+37^0$ C for 60 minutes. Was repeated a procedure of washing for removal not binding reaction products and was added 100 µl of tetramethylbenzidine (TMB) and incubated for 15 minutes in a dark place at room temperature. The reaction was terminated by adding into the wells of a 5% solution of sulfuric acid. The results of the ELISA were calculated using spectrophotometer (BIO RAD, USA) with a vertical stream of light at a wavelength of 450 nm. [8].

Reaction of the immune diffusion by method of Ouchterlony O. (RID).

On the surface of the defatted glass slides, placed at a strictly horizontal surface, was filled with molten 1% agarose to form a layer having a thickness of 1.5 mm. After solidification of the agar using a special stencil and the punch were cut holes with a diameter of 2-3 mm. In the central hole was added 0.005 ml of the investigated antigens (conjugates), and in the rest of 0.005 ml of specific antisera in accordance with figure 1. Petri dish [State standard - GOST 25336-82-E] with slides [GOST 92-84-75] were incubated in a humid chamber for at least a day at room temperature. The reaction results were taken into account by the presence of bands of precipitation between wells with test antigens and specific antisera [9].

Salting out method with ammonium sulfate.

To the examined serum was added 4 M of ammonium sulfate of an equal volume, then it was incubated for 3 hours at a temperature of +4° C. Then it was centrifuged for 30 min at 25,000 rpm at +4^0 C. The supernatant was removed, the residue was dissolved in distilled water and dialyzed to remove ammonium sulfate. Reprecipitation of the protein fractions was carried out twice.

Carrying out a column gelfiltration chromatography.

Sephacryl S-200 was washed in a distilled water to remove preservatives, small gel particles suspended in the supernatant liquid were removed by decanting. A column was filled (size 2x80см) with gel, pre-installing it vertically. After filling the column until forming a dense gel column with a height of 75 cm, and several volumes of buffer were passed through it for a balance. The outlet of the column was opened and the buffer solution was released until then, when its level in the upper part of the column could reach the level of the gel. Then the gel was carefully layered 4-5 cm^3 of the sample, previously dialyzed for 1 hour against buffer solution. After the inflicted drug has entered the gel, its residues were washed in three portions of buffer solution, by 4 - 5 ml. For elution through a chromatographic column was passed through a buffer solution in total amount of 700-800 ml at a speed of 40 - 60 ml/h and were gathered fractions in a volume of 5-7 ml. The obtained fractions were combined and concentrated up to 1 mg/ml by dialysis against 50% solution of polyethylene glycol with a molecular weight of 6000.

Purification of the immune serum from the antibodies to the carriers according to the method of Castellani.

The method is based on adsorption (depletion) of the immune serum with an excess of the corresponding protein. The immune serum was diluted from 1:2 to 1:256 in the phosphate buffered saline in the amount of 2 ml. To each tube was added 0.5 ml of a carrier (media) at a concentration of 1 mg/ml. The test tube with dilution which yields a precipitate was centrifuged at 3000 rpm for 10 minutes. Supernatants were selected and tested for antibody levels to the carrier (media).

RESULTS AND DISCUSSION

To practice the methods of obtaining the conjugate preparations with antigenic properties as high molecular weight proteins-carriers (that are having sufficient molecular weight) for conjugation with the molecules of haptens-trenbolone were used Thyroglobulin from bovine thyroid (THY), the protein carrier keyhole limpet hemocyanin (KLH), Bovine Serum Albumin (BSA), egg white albumin (OVA). The features of trenbolone formula (TR) do not allow the use of its pure product for direct conjugation with the protein carrier. In this regard, a work was carried out to obtain hemisuccinate trenbolone - TR-17-Hemisuccinate (TR-HS). The obtained preparation TRE-HS was used for conjugation with protein carriers.

To determine the number of hapten molecules bound to the protein was used the method of UV spectroscopy. In the result of the study it was determined that epitope density of the obtained protein conjugates made up of 14-15 trenbolone molecules in one mole of the carrier protein that allows to use these medicines to immunize laboratory animals and obtaining antibodies specific to the original drug. A conjugation of trenbolone with heterogeneous protein carriers for a basis took known methods for the synthesis of conjugate of hapten with the carriers with a replacement of some crosslinking agents. At the stage of derivatization of hapten as an activator of components of the reaction is used glutaric anhydride in contrary to succinic anhydride that is having a high reactivity. The problem is solved due to the fact that in this method is the obtaining of conjugate by reaction between EDC and NHS in the presence of DMF. Purification of conjugates of trenbolone from the non-associated components was carried out by vacuum evaporation and washing. For the process of conjugation of a modified hapten with proteins-carriers was used dry hapten.

The obtained trenbolone conjugates with different media were initially tested for activity against commercial antiserum. To study the antigenic properties of the synthesized preparations of conjugates was conducted the immunization of laboratory rabbits and white mice of balb/c line according to the developed schemes. It is important to note that the use of conjugated drugs for immunization of laboratory animals entails certain challenges when testing serum for the presence of specific immunoglobulins, since specific antibodies are produced to a greater extent on high molecular weight antigenic determinants of the carrier as

they have an overwhelming advantage. In order to confirm the presence of antibodies specific directly for the used hapten in the composition of the immune serum, it is necessary to use a conjugate in immunological reactions, consisting of the same hapten and heterologous macromolecular carrier. Given the above, in testing of blood serum obtained after immunization by conjugate TR-THY, TR-KLH, TR-OVA as an antigen we were used the conjugate TR-BSA and vice versa.

In the result of testing of the obtained preparations it was determined that all conjugates used for immunization have the ability to induce an immune response. However, the study of the immune serum of experimental groups of rabbits by enzyme immunoassay showed that in a long-term immunization a working antibody titer was higher than in a short-term immunization. Testing of the antiserum after long term immunization of rabbits by conjugates of trenbolone with carriers THY, KLH, OVA and BSA showed that relatively the greatest activity has the conjugate TR-THY, where the antibody titer in enzyme immunoassay was 1:25600-1:51200 (table 1), then a good titer of 1:25600 show the conjugates of the TR-KLH and TR-OVA.

Table 1 - Results of testing the trenbolone conjugates with heterologous carriers after long-term immunization of rabbits

Methods	Conjugates			
	TR-THY	TR-KLH	TR-OVA	TR-BSA
ELIZA	1:25600-1:51200	1:25600	1:12800-1:25600	1:6400-1:12800
RID	1:6	1:6	1:4-1:6	1:4

The relatively low specificity show antibodies with titer of 1:6400-1:12800, obtained by immunization of rabbits with conjugate TR-BSA. It should be noted that in the preparation of the conjugates TR-THY, TR-KLH and conjugates of trenbolone with OVA and BSA were used different methods, which likely affected the results. The high activity of the conjugate trenbolone with thyroglobulin (TR-THY) we associate with its high molecular mass (660 kDa) relative to other proteins-carriers. The dependence of immunogenicity of the molecular weight appears to be determined by the increase of residence time of the antigen in the body with increasing its molecular weight, in addition, in high molecular weight antigens significantly increases the ability to interact with macrophages. And also with the increasing of molecular weight in the antigen increases the total number of antigenic determinants and their diversity, which increases the efficiency of the interaction of antigens with both B lymphocytes and T lymphocytes [10]. In RID three conjugates TR-THY, TR-KLH and TR-OVA formed a line of precipitation in dilutions of 1:6 and TR-BSA in 1:4. From the animals with the highest titer of specific antibodies were selected samples of blood serum (in average - 10 ml).

Good results were obtained by immunization of the mice balb/c line, which were injected with immunological preparations that were made according to various methods (table 2). As a result of testing it was determined that all preparations of conjugates used for immunization have the ability to induce an immune response.

Table 2 – Test results of antiserum of the mice`s blood in enzyme immunoassay

Conjugates			
TR-THY	TR-KLH	TR-OVA	TR-BSA
1:6400	1:3200	1:1600	1:800

The antibody titers of mice specific for antigenic determinants of trenbolone amounted to 1:6400 (TR-THY), and 1:3200 (TR-KLH), 1:1600 (TR-OVA), 1:800 (TR-BSA).

Purification of immune sera and selection of preparations of immunoglobulins and study of their immunochemical properties.

To allocate fractions of immunoglobulins was used ammonium sulfate (50% concentration). Purification of the obtained fractions of antibodies was performed using column gelfiltration chromatography with the length of the columns 30-60 cm, diameter of 1.5 cm with sephacryl S-200. As a buffer systems was used a phosphate buffered saline (PBS) pH 7,2-7,4. The resulting immunoglobulin fractions were concentrated to 1 mg/ml by total protein by dialysis against polyethylene glycol with a molecular weight of 6000 KD (PEG-6000). As a further purify of antisera obtained from the use of trenbolone conjugates of antibodies to carriers

was used the method of Castellani. The method is based on adsorption (depletion) by the excess of the corresponding protein.

By titration were determined the optimal parameters for absorption of antibodies specific to the protein carrier - BSA. The preparations were divided into aliquots and stored before use at -20°C. To check the stability of the specificity was carried out the reaction of the immune diffusion (RID) against heterologous conjugate and a carrier. When setting a reaction was used 1% solution of agarose in phosphate-saline buffer with pH 7.2. Preparations were incubated in a humid chamber for at least a day at a room temperature. The reaction results were taken into account by the presence of bands of precipitation between wells with participant preparations. As a result it was determined that the immunoglobulins of antisera after the procedure ammonium sulphate salting out and chromatographic purification have not changed their qualities and react in RID with a heterologous conjugate through formation of precipitation line in dilutions 1:4 and 1:6. The results of enzyme immunoassay of purified immunoglobulins did not change significantly and remained at the same level (1:6400-1:51200).

CONCLUSION

Thus, the result of this work determined that the used methods of conjugation of trenbolone with heterologous carriers allow obtaining drugs protein of trenbolone conjugates TR-THY, TR-KLH, TR-OVA and TR-BSA with the necessary degree of antigenicity. Were developed optimal schemes of immunization of animals with synthetic immunogens, allowing obtaining a specific antiserum with a high titer. Was conducted a clearing of immune sera of the blood, were selected preparations of immunoglobulins. The results of this experiment proved the possibility of obtaining an immune response to the trenbolone when using the synthesized conjugates.

The obtained preparations can find application as immunogens to generate antibodies to the trenbolone, and also as the reagents for enzyme immunoassay and other immunoanalytical methods of detection of residual quantities of trenbolone in animal products.

GRATITUDE

The studies were performed due to the project No. 2410/GF4 of grant funding from the Ministry of education and science of the Republic of Kazakhstan.

REFERENCES

[1] Schiffer, B.; Daxenberger, A.; Meyer, K.; Meyer, H. H. D. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: Environmental studies.EnViron. Health Perspect. 2001, 109, 1145-1151.

[2] The EU directive 2003/74/EC of the European Parliament and of the Council of 22 September 2003 "On the prohibition of the use in livestock farming of certain substances having a hormonal or thyreostatics action and of beta-agonists".

[3] Federal state budgetary institution the all-Russian state center for quality and standardization of medicines for animals and feed of the FSBI "VGNKI". The centre of the world organization of animal health for food safety, diagnosis and control of animal diseases for Eastern Europe, Central Asia and the Caucasus. "Organization of work of veterinary laboratories and actively monitoring" the meeting of the Rosselkhoznadzor on June 27, 2013

[4] P. K. Nakane, A. Kawaoi. The Journal of Histochemistry and Cytochemistry, 1974, v. 22, N 12, p. 1084-1091

[5] Jane Fitzpatrick, Bernadette Manning and Richard O'Kennedy. Enzyme-Linked Immunosorbent Assay-Based Detection of Free Trenbolone in Bovine Bile. J.Agric. Food Chem., Vol. 52, No. 14, 2004, 4351-4354.

[6] Jondorf W.R. On the raising of antibody to the synthetic anabolic steroid trenbolone, its partial characterization and preliminary application for radioimmunoassay. *Experientia* 1980, *36*, 394-395.

[7] «Generation of anti-trenbolone monoclonal antibody and establishment of an indirect competitive enzyme-linked immunosorbent assay for detection of trenbolone in animal tissues, feed and urine»

Yuanyang Zhanga, Fangyang Hec, Yuping Wanc, Meng Mengb, Jing Xua, Jian Yia, Yabin Wanga, Caiwei Fengc, Shanliang Wangc, Rimo Xib. Talanta - Volume 83, Issue 3, 15 January 2011, Pages 732–737.

[8] Egorov A. M., Osipov A. P., Dzantiev B. B., Gavrilova E. M. Theory and practice of enzyme immunoassay. M. 1991.

[9] Ouchterlony O. Diffusion – in gel methods for immunological analysis// Prog. Allergy. – 1958. – V. 5. – P. 1-78.

[10] Butenko R. G., et al. The scientific journal “Cell engineering”. Biotechnology series No. 3/ M.: Vysshaya Shkola, 2001.