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OBTAINING OXYTETRACYCLINE CONJUGATES WITH PROTEIN CARRIERS

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Abstract

Oxytetracycline is widely used in veterinary medicine for the treatment of animals, as well as growth promoters. They can have adverse effects on human health through animal products if the rules for the use of antibiotics are not followed. The World Health Organization has established maximum residue limits of antibiotics in milk and meat, which require accurate, rapid and inexpensive methods to determine. Immunochromatographic assay (ICA) is ideal for this purpose due to its speed of analysis, high sensitivity and ease of use. This paper describes the results of study on the obtaining an oxytetracycline (OTC) conjugate with bovine serum albumin (BSA) and/or ovalbumin (OVA). OTC has been chemically purified from the antibiotic hydrochloride salt (OTC HC), which is widely used in animal husbandry and is more affordable than its chemically pure analogue. Spectrophotometric analysis of the prepared conjugates and immunization of laboratory animals showed the suitability of OTC, chemically purified from OTC HC, for crosslinking into BSA and/or OVA molecules and obtaining hapten-specific antibodies. The results obtained could be used for the manufacture of ICA components - labeled OTC-specific antibodies and test line antigen.

Key words: oxytetracycline; bovine serum albumin; ovalbumin; conjugate; antibiotic; high performance liquid chromatography; spectrophotometry.

Basic position and Introduction

Oxytetracycline (OTC) is one of the first tetracycline antibiotics described in the late 1940s. This drug is characterized by a wide spectrum of action against gram-negative and gram-positive microorganisms [1]. Tetracyclines, including chlortetracycline, oxytetracycline, and doxytetracycline are widely used in veterinary medicine due to their low cost compared to other antibiotics [2]. They are also used to stimulate the growth of fattening cattle and poultry [3]. For example, in Kazakhstan, such antibiotics as «Oxytetracycline hydrochloride for injections» (BioPharmGarant LLC, Russia), Oksirala 20% (Agio Pharmaceuticals Ltd., India), Ashoksi 10% (Ashish Life Science Pvt Ltd, India) are registered in the State Register of Veterinary Drugs and Feed Additives [4]. Residues of tetracyclines get into animal products in cases of non-compliance with the instructions for the use of antibiotics or the time of exposure of animals before slaughter or milk production [4]. The constant consumption of such products is fraught with serious health consequences and could lead to allergic reactions and dysbacteriosis, and -may also cause nausea, vomiting, anaphylactic shock, and even death [5]. Violation of the use of antibiotics can lead to the emergence of antibiotic resistance genes in microorganisms, the transmission of such strains from animals to humans. [6]. In order to avoid harmful effects on human health, many countries have set maximum residue limits (MRL) of antibiotics in

milk and meat. The World Health Organization (WHO) has established that the content of tetracyclines in food should be no more than 0.01 mg/kg [7]. Currently, there are several methods for determining antibiotic residues, each with its own advantages and disadvantages. For example, microbiological -tests are available for use in poorly equipped laboratories, but are characterized by low sensitivity and specificity [8,9]. Instrumental methods such as gas chromatography, high performance liquid chromatography (HPLC), chromatography–mass spectrometry is sensitive and highly specific, but require expensive equipment and trained personnel. In addition, they take a lot of time and are not suitable for routine analysis [10]. Recently, enzyme-linked immunosorbent assay (ELISA) has become increasingly popular for screening food products for contamination with antibacterial drugs. European Union (EU) Directive 2002/657 recommends this test for the determination of veterinary drug residues in livestock products in the EU. However, this test is not used in practice, since the equipment of domestic laboratories for veterinary and sanitary examination in food markets leaves much to be desired. Therefore, for practice, simple-to-perform, but sufficiently sensitive and specific tests are needed to determine the presence or absence of antibiotics in animal products in a few minutes. These tests include immunological methods based on the principle of thin

layer chromatography, namely, immunochromatographic analysis (ICA) [11]. Antibiotics, including OTCs are haptens, and therefore competitive ICA is a suitable method for determining a contaminant in food products. The affordability of ICA tests for the detection of antibiotics is determined by the cost of its individual components, including the hapten conjugate with protein carriers. The use of chemically pure antibiotics in the preparation of the conjugate leads to a

Materials and methods

Laboratory animals. The Soviet chinchilla rabbits (3 heads, males, 6 months old, body weight 3300-3500 g) and outbred mice (3 heads, males, 2 months old, body weight 20-25g). Experiments with animals were approved by the Animal Ethics Committee, Faculty of Veterinary and Animal Husbandry Technology, S. Seifullin Kazakh Agrotechnical University, and were carried out in accordance with the Rules for the maintenance and care of laboratory rodents and rabbits (Interstate standard, GOST 33216-2014), as well as International Guiding Principles for Biomedical Research Involving Animals.

Reagents used to prepare antibiotic-protein conjugates. OTC HC for veterinary medicine (CJSC RPE Agrofarm, Russia), bovine serum albumin (BSA) (Jackson Immuno Research, USA) and/or ovalbumin (OVA) (Sigma-Aldrich, USA).

Chemical purification of the antibiotic. Three g of OTC HC was poured into a 0.5 L three-necked flask equipped with an addition funnel, a gas outlet tube, and a ground glass stopper.

significant increase in the cost of analysis. In this regard, antibiotics for animals available on the veterinary medicine market are of particular interest.

The aim of our study was the use of veterinary oxytetracycline hydrochloride (OCT HC) for the preparation of the antibiotic conjugate with bovine serum albumin (BSA) and/or ovalbumin (OVA) as well as specific antibodies, which are the main ICA reagents.

The antibiotic was dissolved in 50 ml of bidistilled water (pH=7.01) at a temperature of 30-35°C. Then, 100 ml of 7% sodium bicarbonate solution was added dropwise over 1 hour. Carbon dioxide released during the reaction of hydrochloric acid with sodium bicarbonate entered the calcium hydroxide solution. The mixture was stirred for 8 hours at room temperature at low speed of the magnetic rotor. After separation, the antibiotic was filtered on a Schott funnel. Subsequently, the filtrate was washed with distilled water and dried under vacuum.

Determination of purity and quantitative content of purified OTC using HPLC. Tetrahydrofuran, methanol, acetonitrile in ratios of 50:150:800 was used as the mobile phase - eluent, respectively. 20 mg of OTC substance was dissolved in 25 ml of 0.01 M hydrochloric acid. Endcapped octadecylsilyl silica gel for chromatography with a pore diameter of 5 µm was used as a sorbent for the chromatographic column. The following parameters were used: chromatographic column temperature,

50°C; eluent solvent pumping rate, 1.3 ml/min; amount of sample used, 10 µl. The result of the analysis was recorded at an optical density of 254 nm.

Conjugation of OTC with protein carriers was carried out using the methods described by T. Wongtangprasert et al. (2014) [12], Nail L. et al. (2014) [13], and Birader K. et al. (2021) [14].

Optical spectrophotometry was used to identify the hapten-protein conjugate. Briefly, 100 µl of test substance solution was poured into a clean cuvette. Then, it was placed in a holder, and a reference spectrum was recorded with the radiation source (dark spectrum) turned on and off. Then, the spectrum of light that passed through the sample was recorded in 10 repetitions in accordance with the manufacturer's instructions attached to the device. The absorption of light by the studied samples at each wavelength was calculated in the Spectra Suite program using the standard formula and obtained in the form of a graph-spectrum.

Studying the immunogenicity of the OTC-protein conjugate. On day 0 of immunization, mice were intraperitoneally injected with OTC-BSA at a dose of 25 µg in 0.1 ml of complete Freund's adjuvant (CFA). On the 7th, 15th, 22nd and 32nd days of immunization, the same dose of antigen was administered in incomplete Freund's adjuvant (IFA). Antiserum was separated from blood taken from the tail vein on the 38th day of immunization and stored at -20°C until use.

Immunization of rabbits was carried out by 4-fold subcutaneous injection of OTC-BSA into the back

area at a dose of 500 µg in 1.0 ml of adjuvant at several points with an interval of 10 days. On the 0th day of injection CFA was used, and on subsequent days IFA was used. Ear vein blood samples were taken 5 days after the last immunization. The separated immune sera were stored at -20°C until use.

Indirect ELISA (i-ELISA). The wells of a 96-well immunoassay plate (Suzhou CellPro Biotechnology Co., Suzhou, China) sensitized with OTC-OVA (1 µg/ml) in bicarbonate buffer, pH 9.6. The plate was incubated at +4°C overnight. The plate was washed 3 times with 300 µl of Tween-20 phosphate-buffered saline per well to remove unbound antigen. Then, two-fold dilutions of mouse and/or rabbit serum were prepared in 8 wells, starting from a dilution of 1:100 (0.1 ml), and incubated at +37°C for 60±5 minutes. Blood serum samples of animals taken before immunization were used as a control. After incubation, the plate was washed as described above to remove non-specifically bound antibodies, and anti-mouse (Jackson ImmunoResearch Inc., Pennsylvania, USA) and/or anti-rabbit (Jackson ImmunoResearch Inc., Pennsylvania, USA) conjugates were added to the wells in a volume of 0.1 ml and incubated at +37°C for 1 hour. The washing procedure was repeated to remove unbound reaction products. 0.1 ml of a stabilized solution of 3,3',5,5'-tetramethylbenzidine hydrochloride with hydrogen peroxide (CJSC "NVO Immunotech", Moscow, Russia) was added to the wells and the plate was incubated for 10-15 minutes at room temperature. The reaction was stopped by adding a solution of 0.5 M sulfuric

acid to the plate wells. The results of the ELISA were taken into account using a spectrophotometer (Hangzhou Allsheng Instruments Co., Hangzhou, China) with a vertical light flux at a wavelength of 450 nm. The reaction

was considered positive if the optical density (OD) of the immune serum exceeded the OD value of the control serum at a dilution of 1:200 by at least two times

Results

The yield of chemically purified antibiotic from OTC HC for veterinary medicine (CJSC RPE Agrofarm, Russia) was more than 90%. The results of the study of the purity and quantitative content of the antibiotic using HPLC are shown in Fig.1.

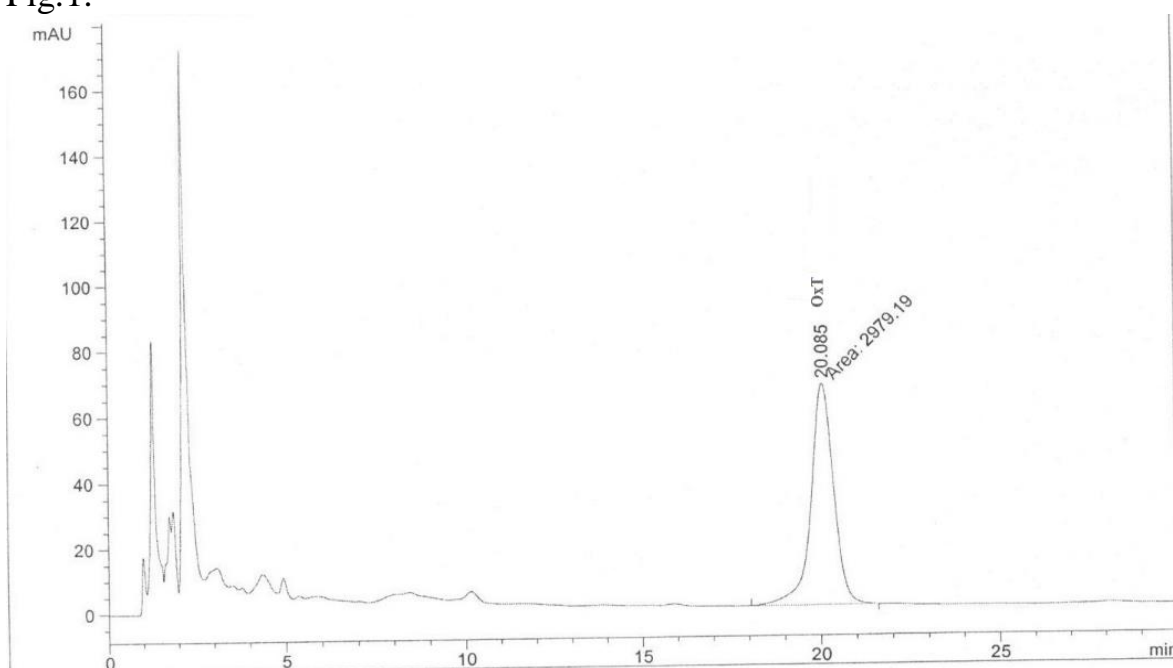


Figure 1 – HPLC chromatogram of purified OTC

As can be seen from Figure 1, the HPLC chromatogram of purified OTC contains several non-volume peaks identified as residual impurities and/or oxidation products in air during separation. At the same time, the content of the target component in the obtained substance was 96% in terms

of dry matter using the ChemStation software.

The identification of the obtained compound was carried out using IR spectroscopy by determining the main signals of functional groups in pressed tablets of potassium bromide (Fig. 2).

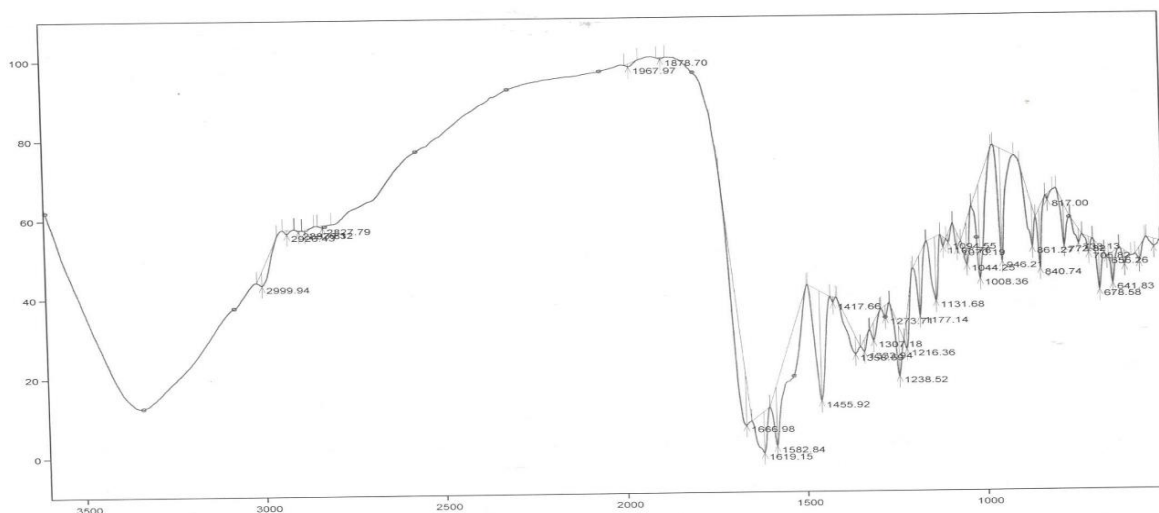


Figure 2 - IR spectrum of OTC

Analysis of Figure 2 shows that the spectrum of purified OTC contains signals at 3300-3600 cm^{-1} , which are characteristic of hydroxyl groups (OH-groups) and are contained in the molecule of the test substance in the amount of two units. Stretching vibrations of 3200 cm^{-1} and 1238 cm^{-1} correspond to the phenolic fragment of the molecule, 1666 cm^{-1} - to the C=O carbonyl group associated with the cycle and 1619 cm^{-1} , which indicates the presence of an amino group (NH₂-group). Signals from 2999 to 2827

indicate the presence of aliphatic bonds in the molecule.

Chemically purified OTC as well as veterinary medicine OTC HC have been used to prepare antibiotic conjugates with BSA and/or OVA. Methods by L. Nail et al. (2014) and K. Birader et al. (2021) used for this purpose did not give the desired results due to the denaturation of proteins with the formation of a precipitate (Fig.3-a). An antibiotic-carrier conjugate was obtained by T. Wongtangprasert et al. (2014) only when purified OTC was used (Fig. 3-b).



a



b

Figure 3 - Appearance of OTC-BSA conjugates:
a - OTC HC for veterinary medicine; b - chemically purified OTC

Spectrophotometric analysis was used to establish the crosslinking of the purified OTC into the carrier molecules (BSA, OVA). For this purpose, OTC, carrier proteins and the prepared conjugate were studied using UV optical photometry in the wavelength range of 200-1100 nm (Fig. 4, 5, 6).

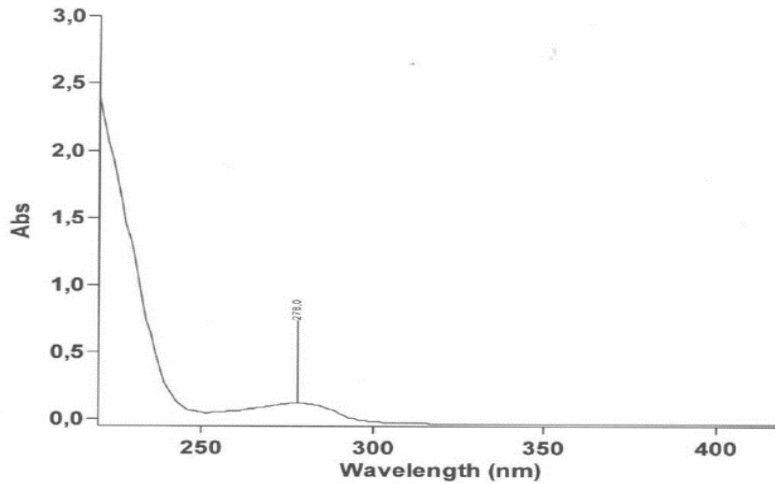


Figure 4 - UV spectrum of purified OTC

Figure 4 shows that the UV spectra of the chemically purified antibiotic contain one peak at a wavelength of 278 nm.

Spectrophotometric analysis of carrier proteins showed the presence of one peak in both the UV spectrum of BSA and OVA at 277 nm and 271 nm, respectively (Fig. 5).

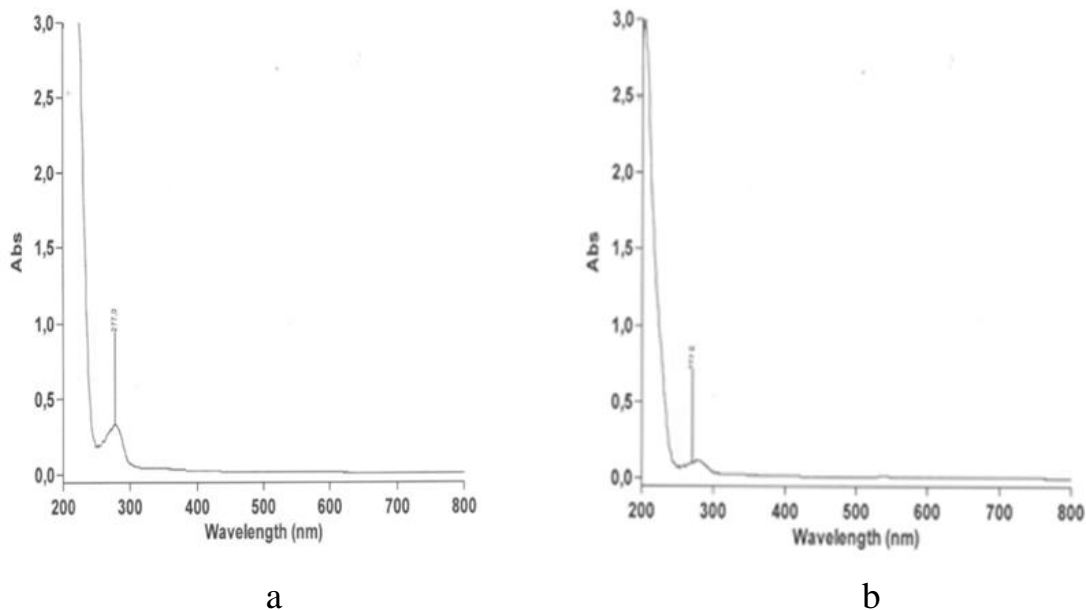


Figure 5 - UV spectra of proteins: a - BSA, b – OVA

Spectrograms of antibiotic conjugates with carrier proteins are shown in Fig.6.

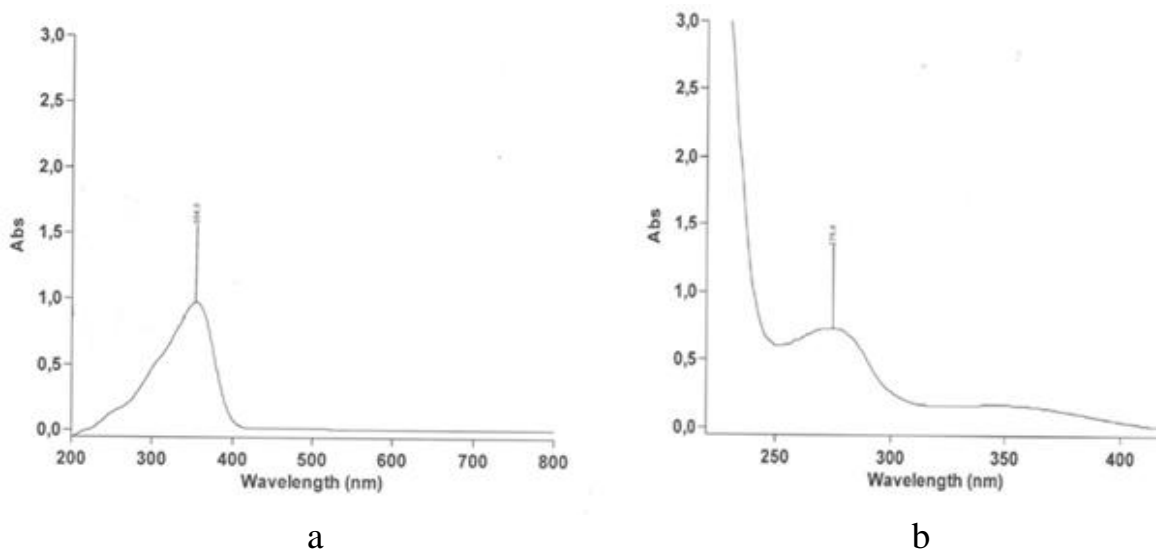


Figure 6 - UV spectra of conjugates: a - OTC-BSA, b - OTC-OVA

In the spectra of optical photometry, there are continuous signals with characteristic peaks of the initial substance, which testify the homogeneity of the final products obtained (Fig. 6). In the spectral regions, the presence of signals characteristic of the substrates of protein polymers is demonstrated, while the presence of the initial compounds of an antibiotic nature is not noticed, which is due to the

complete absorption of low molecular weight compounds by macromolecules. The obtained UV spectra prove the suitability of OTC, purified from the hydrochloride salt of the antibiotic, for the preparation of a conjugate with BSA and/or OVA.

Figure 7 shows the results of studying the immunogenicity of OTC crosslinked into carrier protein molecules in mice and rabbits.

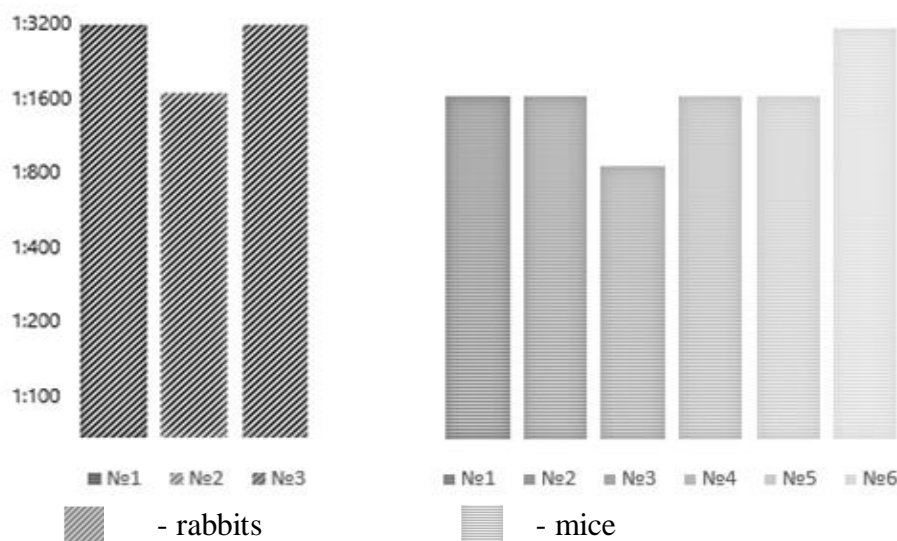


Figure 7 - Titers of anti-OTC antibodies by i-ELISA

Studies of antisera samples showed that BSA-coupled OTC was recognized by the immune system of laboratory animals as a separate epitope of the integral antigen

and stimulated B-lymphocytes to synthesize antibiotic-specific antibodies. This is evidenced by the titers of antibodies against OTC by i-ELISA in samples of both mouse and rabbit antisera. It follows from the diagram that antibodies specific to the antibiotic were detected within the range of blood serum dilutions from 1:1600 to 1:3200, which is evidence of the immunogenicity of the antibiotic purified from its hydrochloride salt.

Discussion

The veterinary practice of the country needs simple, but sufficiently sensitive and specific tests to determine the presence of antibiotics in food products in a few minutes, which is very important for the reliable protection of public health. Such tests can be developed on the basis of competitive ICA, the main reagents of which are antibodies specific to various antibiotics and their conjugates with high molecular weight carriers. The difficulty in creating ICA tests for determining the MRL of antibiotics lies in the preparation of stable conjugates of hapten with carriers and the production of highly specific antibodies, since antibiotics, including OTC, are small substances with a molecular weight in the range of 460–434 Da [15]. In addition, imported ICA tests available on the market of veterinary drugs remain expensive for small producers of meat and milk in Kazakhstan, which produce more than 90% of milk and about 70% of meat in slaughter weight. One of the main components of ICA is an antibiotic-carrier conjugate, which is used in the diagnosticum design not only as a test

line reagent, but also as an immunogen for obtaining specific antibodies. The use of chemically pure antibiotics for these purposes makes a significant contribution to the rise in the cost of the test system. In this study we propose a method for purifying OTC from its hydrochloride salt, which is widely used in veterinary practice and is affordable. Purified OTC conjugated with OVA served as an antigen in i-ELISA, while an antibiotic cross-linked into a BSA molecule was used as an immunogen. The results of spectrophotometric analysis showed the formation of a whole molecule consisting of a chemically purified antibiotic and OVA and/or BSA. The last conjugate showed its immunogenicity for the body of laboratory animals, which made it possible to obtain mouse and rabbit antisera with titers against the antibiotic hapten in the range of 1:1600 - 1:3200.

Thus, the developed method for obtaining chemically pure OTC from its commercial hydrochloride salt could reduce the cost of ICA tests designed to detect antibiotic residues in food.

Conclusion

- A method for obtaining chemically pure OTC from OTC HC for veterinary use has been developed, and OTC-BSA and/or OTC-OVA conjugates have been prepared;
- The homogeneity of the conjugates has been proven by HPLC and spectrophotometric analysis;

- OTC-BSA, as an immunogenic preparation, may be used to obtain oxytetracycline-specific antibodies;
- The obtained results will be used in our further study in order to design a domestic competitive ICA test for the detection of OTC in livestock products.

Information on financing

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Аннотация

Окситетрациклин широко используется в ветеринарии для лечения животных, а также в качестве стимуляторов роста. При несоблюдении правил использования антибиотиков, они могут оказывать неблагоприятные последствия на здоровье людей при употреблении продуктов животноводства. Всемирная организация здравоохранения установила предельно-допустимые концентрации антибиотиков в молоке и мясе для определения которых требуются точные, быстрые и недорогие методы. Для этой цели идеально подходит иммунохроматографический анализ (ИХА), благодаря скорости анализа, высокой чувствительности и простоте в применении. В настоящей работе описаны результаты исследований по получению конъюгата окситетрациклина (ОТЦ) с бычьим сывороточным альбумином (БСА) и/или овальбумином (ОВА). ОТЦ был химически очищен из гидрохлоридной соли антибиотика (ОТЦ ГХ), который широко используется в животноводстве и по цене более доступен, чем его химически чистый аналог. Спектрофотометрический анализ приготовленных конъюгатов и иммунизация ими лабораторных животных показали пригодность ОТЦ, химически очищенного из ОТЦ ГХ, для сшивки в молекулы БСА и/или ОВА и получения гаптен-специфичных антител. Полученные результаты могут быть использованы при изготовлении компонентов ИХА - меченых ОТЦ-специфичных антител и антигена тестовой линии.

Ключевые слова: окситетрациклин; бычий сывороточный альбумин; овальбумин; конъюгат; антибиотик; высокоэффективная жидкостная хроматография; спектрофотометрия.

Түйін

Окситетрациклин ветеринарияда малды емдеу үшін, сондай-ақ өсу стимуляторлары ретінде кеңінен қолданылады. Егер мал шаруашылығында антибиотиктерді қолдану ережелері сақталмаса, олар тағам өнімдері арқылы адамдардың денсаулығына кері әсерін тигізуі мүмкін. Дүниежүзілік денсаулық сақтау ұйымы сүт пен етте антибиотиктердің шекті рұқсат етілген - мөлшерлерін белгіледі, ал оларды анықтау үшін дәл, жылдам және арзан әдістер қажет. Осы мақсатта нәтиже жылдамдығына, жоғары сезімталдығына және қолданудың қарапайымдылығына байланысты иммунохроматографиялық талдау (ИХТ) өте қолайлы әдіс. Бұл жұмыста окситетрациклиннің (ОТЦ) бұқа сарысуы альбуминімен (БСА) және/немесе овальбуминмен (ОВА) конъюгатын дайындау бойынша зерттеу нәтижелері баяндалған. ОТЦ мал шаруашылығында кеңінен қолданылатын және оның химиялық таза аналогына қарағанда қол

жетімді болып табылатын антибиотиктің гидрохлоридті тұзынан (ОТЦ ГХ) химиялық әдіспен тазартылған. Дайындалған конъюгаттарды спектрофотометриялық зерттеу және олармен зертханалық жануарларды иммунизациялау ОТЦ ГХ-нен химиялық тазартылған ОТЦ-нің БСА және/немесе ОВА молекулаларымен қосыла алуына және гаптенге тән антиденелерді түзе алуына жарамдылығын көрсетті. Алынған нәтижелер ИХТ компоненттерін, атап айтсақ тест жүйесінің ОТЦ-мен таңбаланған арнайы антиденелерін және сынық сызығының антигенін өндіруде пайдаланылуы мүмкін.

Кілт сөздер: окситетрациклин; бұқа сарысуы альбумині; овальбумин; конъюгат; антибиотик; өнімділігі жоғары сұйық хроматография; спектрофотометрия.