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RESEARCH ARTICLE

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Synthesis of artificial antigens of diethylstilbestrol and preparation of its antibody

Abstract Diethylstilbestrol-hemi-succinate (DES-HS) and diethylstilbestrol-monocarboxylic-propyl-ether (DES-MCPE) were synthesized in different ways. The two kinds of haptens were conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) to form the immuno-antigens and the coating-antigen, respectively. The antibody with high titer was obtained after immunising rabbits. The results showed that the antibody had a high affinity to diethylstilbestrol tested by enzyme-linked immunosorbent assay (ELISA) and Western blot. This study lays a basis for further research on the immune test kit for the DES remnant.

Keywords diethylstilbestrol, hapten, artificial antigen, antibody

1 Introduction

Diethylstilbestrol was first synthesized in early 1938 by Leon Golberg, as a kind of synthetic steroidal estrogen ethinyl estradiol (Dodds et al., 1938). It was approved by the FDA in 1941 for four indications: gonorrheal vaginitis, atrophic vaginitis, menopausal symptoms, and postpartum lactation suppression to prevent breast engorgement (Meyers, 1983). It was also used as a growth hormone in the beef and poultry industry. More than 30 years of research have confirmed that DES is a teratogen, an agent that can cause malformation of an embryo or fetus. However, it is reported that DES is still used in China, especially in the rearing of Chinese mitten crab from Yangcheng Lake. In 2002, the Ministry of Agriculture of the People's Republic of China regulated DES to prohibit its derivatives when used in all food animals. Therefore, it is necessary to research a sensitive, efficient and stable method for the detection of diethylstilbestrol residues. Gas chromatography (GC), high performance liquid chromatography (HPLC) with UV detection or HPLC mass spectrometry (HPLC-MS) have been well established for residue analysis of diethylstilbestrol. However, these methods are time-consuming and require sophisticated equipment available only in laboratories. Immunoassay is demonstrated as a simple, rapid, and cost-effective alternative compared with traditional methods above when high throughput and on-site screening tests are required. Furthermore, substances with a molecular weight of less than 1000 are not ordinarily antigenic, including DES with a small molecule that can elicit an immune response only when attached to a large carrier such as a protein. At present, the detection of diethylstilbestrol mainly relies on imported Kits. Many domestic scientific research institutions are actively developing the DES immunoassay reagent box. A variety of antigen synthesis methods for DES have been reported. Huang et al. (1990) used a chlorine sodium acetate and diethylstilbestrol direct reaction to prepare the polyclonal antibodies against diethylstilbestrol. Lei et al. (2004) used succinic anhydride to synthesize DES hemi-succinate. Actually, the antibody specificity was limited although the method to synthesize artificial antigens of diethylstilbestrol was used. In these researches, a single synthetic route for haptens and complete antigen was generally applied. In our study, we improved the general applicability of this method by synthesizing haptens for diethylstilbestrol. The innovative succinate and 4-bromine-ethyl butyrate by derivatives of diethylstilbestrol were synthesized and finally we prepared two kinds of complete antigen by active ester method and mixed-anhydride method.

Therefore, the purpose of this study was to transform chemicals to DES, prepare antibodies using antigenic hapten-carrier conjugates (complete antigens), compare specificities of antibody haptens of diverse structures and explore the relation between molar ratio and the titer of antibody.

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2 Materials and methods

2.1 Reagents and materials

Ovalbumin (OVA, MW45000), bovine serum albumin (BSA, MW67000) (enzyme immunoassay grade), 3,3',5,5'-Tetramethyl-benzidine (TMB), N-hydroxysuccinimide (NHS), N,N'-dicyclohedxylcarbodiimid (DCC), goat anti-rabbit IgG-horseradish peroxidase (IgG-HRP) and complete and incomplete Freund's adjuvants were all obtained from Sigma. Dimethyl sulfoxide (DMSO), N,N'-dimethylformamide (DMF), isobutyl chloroformate, Tri-n-butylamine, Na_2HPO_4 and NaH_2PO_4 were purchased from Shanghai Chemical Reagents Company (China). Pyridine and tetrahydrofuran were purchased from Tianjin Chemical Reagents Company (China). Silica gel for Thin-layer chromatography was from Qingdao Haiyang Chemical Reagents Company. Polystyrene 96-well micro-well plates were from Nunc (Roskilde, Denmark). Nitrocellulose filter membrane was from MILLIPORE (America). X-ray films were from Huineng Plastics Industry & Trade Co., Ltd (Tianjin, China). Hybridization bags were from Gibco (America). Super ECL Plus was from Santa CRUZ Company (America).

2.2 Instrumentation

^1H nuclear magnetic resonance (NMR) spectra were obtained by operating an AV-400 spectrometer (Bruker, America) at 400 MHz for solutions in DMSO-D, and the chemical shifts were expressed in parts per million (*ä* scale) using tetramethylsilane as an internal standard. The ESI-MS spectra were obtained on the Thermo-Finnegan LCQ Advantage HPLC with mass spectrometry (HPLC-MS) instrument. Infrared spectra were obtained using a Bruker Vector 22 spectrometer (Thermo, America). Ultraviolet-visible (UV-vis) spectra were recorded on a spectrophotometer (Unic Instruments Company, Shanghai, China). Thin-layer chromatography (TLC) was performed on silica gel G detected by an ultraviolet-visible (UV) detector. For column chromatography, silica gel 200–300 mesh was used. Immunoassay absorbance was read with a Multiscan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) in a dual wavelength mode (450–650 nm). Rotary evaporator was from Shenke Medical Instruments Company (Shanghai, China). Centrifuge was from ANKE (China). Electrophoresis apparatus (DYCZ-28A) was purchased from Liuyi Instruments Company (Beijing, China). Transmembrane equipment (TE70X) was purchased from Hoefer (America). UVP Biospectrum AC System was from UVP Company (America).

2.3 Synthesis of haptens to diethylstilbestrol

The design of specific haptens is necessary to obtain a mimic compound in structure and electronic and hydrophobic properties. Also, the haptens might present suitable functional groups (-COOH; $-NH_2$, -OH, -SH, etc.) to link the molecule to a carrier protein. Taking this into account, two strategies were followed to synthesize new haptens for diethylstilbestrol (DES-HS and DES-MCPE).

2.3.1 Diethylstilbestrol hemi-succinate (DES-HS)

Fifty mg (0.5 mmol) succinic anhydride was added to the solution of pyridine (5 mL) containing 134 mg (0.5 mmol) diethylstilbestrol. Then, the air was excluded with nitrogen. The mixture was stirred while the temperature was gradually increased to 60°C and maintained for 30 h. The solution was put into the mixed solution containing 30 mL ice-distilled water and 5 mL hydrochloride after the reaction solution was cooled down to room temperature, and placed overnight in order to counteract excess pyridine and remove the stub of succinic anhydride. The filtrate was sedimentated through a Buchner funnel, washed with distilled water to a pH value of about 5.5, and then vacuum dried to obtain crude products. The crude products were subjected to column chromatography [silica gel, chloroform/methanol/acetic acid (98.5∶1.5∶0.5)] so as to be refined into a colorless oil by collecting the components at a mobility ratio of 0.145 (Wainer et al., 1972; Dong et al., 1999; Gosling, 2000).

2.3.2 Diethylstilbestrol-mono-carboxylic propyl ether (DES-MCPE)

Load reagent was prepared using 10 g anhydrous potassium carbonate (K_2CO_3), neutral alumina 15 g plus (Al_2O_3) together with 30 mL ultra pure water, stirred at 60–70ºC for 1 h, crushed after activation for 3 h at 130–140ºC and stored in a desiccator. 500 mg load reagent in 25 mL tetrahydrofuran was added to a stirred mixture of 134 mg diethylstilbestrol (0.5 mmol) in nitrogen atmosphere. After heating reflux stirring for 1 h in 60°C water bath, 0.6 mmol (86 μL) 4-bromine-ethyl butyrate dissolved by 2 mL

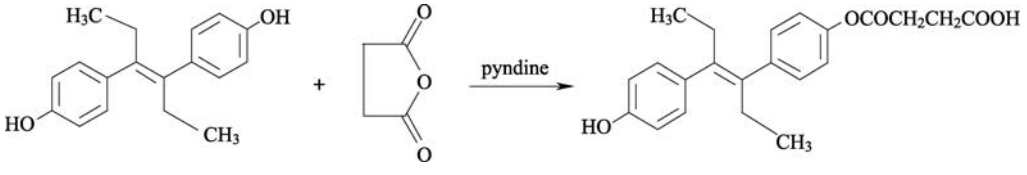

Fig. 1 The synthetic route for haptens (DES-HS)

anhydrous tetrahydrofuran was added dropwise to the stirred mixture and the reaction was maintained for 4 h in nitrogen atmosphere. The reaction solution was placed in a refrigerator at −20°C overnight, removed from the refrigerator and filtered the next day, adjusted to pH 11 using 0.1 mol·L^{-1} NaOH. After wiping off tetrahydrofuran with a rotary evaporator under reduced pressure, the solution was stirred for 1 h at room temperature, filtered, adjusted to pH 4–5 using 1 mol·L^{-1} HCl and extracted with ethyl acetate (30 mL×3). The organic extract phase was washed with water and dehydrated with anhydrous sodium sulfate. Ethyl acetate in the extract was removed using a rotary evaporator under reduced pressure. The crude product was subjected to column chromatography [silica gel, chloroform/methanol/acetic acid (98.5∶1.5∶0.5)] to refine the product to a colorless oil by collecting the components at a mobility ratio of 0.185 (Degand et al., 1989; Zhan et al., 1994).

2.3.3 Identification of haptens

The molecular structure of the hapten was identified by electrospray ionization (ESI) mass spectrometry, ^{1}H nuclear magnetic resonance (NMR) spectra and Fourier transform infrared spectra.

2.4 Preparation of complete antigens

Diethylstilbestrol, when used as hapten, is not immunogenic. However, on conjugating with carrier molecules, it elicits an antibody response. Carbodiimides and isobutyl chloroformate are heterobifunctional coupling reagents utilized for coupling reaction through carboxyl and amino groups. The procedures were generally used for coupling carboxyl and proteins with haptens resulting in conjugates that generate immunodominant antibody responses directly against the neodeterminants on the carrier protein.

2.4.1 Hapten-protein conjugates prepared by a active ester method

0.1 mmol DES-HS was added to the solution of 0.5 mL DMF containing 15 mg NHS and 40 mg DCC and stirred at 4°C overnight. After being centrifuged (10000 r·min^{-1}, 5 min) to remove the precipitated urea, the solution (active ester) was added slowly to BSA solution (40 mg BSA in 10 mL of 0.05 mol·L^{-1} PBS at pH 8.0) and stirred in an ice bath for 6 h. Then, the reaction solution was dialyzed against distilled water (500 mL×3), followed by PBS (pH = 7.4, 0.01 mol·L^{-1}), by changing the dialysis solution every 6 h till UV scanning the dialyzed solution outside the dialysis bag without UV absorption of hapten and storing the artificial immunogen at −20ºC individually (Zhu et al., 2002, 2003). The synthetic routes for hapten-carrier conjugates using the active ester method are illustrated in Fig. 3.

2.4.2 Hapten-protein conjugates prepared by mixed-anhydride method

A modified method of Krejcarek and Tucker (1977) was used. 0.1 mmol DES-MCPE and 15 μL tri-n-butylamine were dissolved in 1 mL anhydrous DMF. The reaction mixture was slowly added to 0.08 mmol isobutyl chloroformate (15 μL) in 0.5 mL DMF on the stirring stand with an ice bath for 1 h. The solution was then heated to room temperature for 1 h. 40 mg BSA was dissolved in 4 mL PBS (0.05 mol·L^{-1}, at pH 8.7), the above-mentioned reaction solution was slowly added to BSA solution, stirred overnight at 15ºC and then dialyzed against distilled water (500 mL×3), followed by 0.01 mol·L^{-1} PBS (pH = 7.4), by changing the dialyzed solution every 6 h till UV scanning of the solution outside the dialysis bag without UV absorption of haptens and storing the artificial immunogen at −20°C individually (Erlanger et al.,

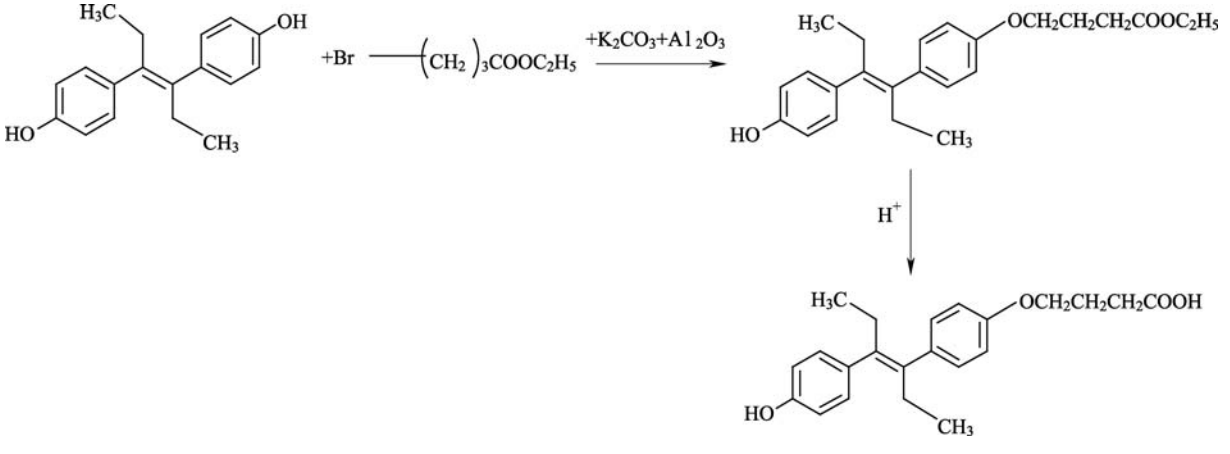

Fig. 2 The synthetic routes for Haptens (DES-MCPE)

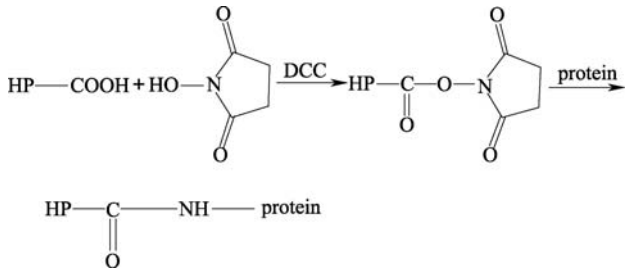

Fig. 3 The synthetic routes for complete antigens
Note: HP represents the DES-MCME or DES-MCPE.

1958). The synthetic routes for hapten-carrier conjugates of the mixed-anhydride method are illustrated in Fig. 4.

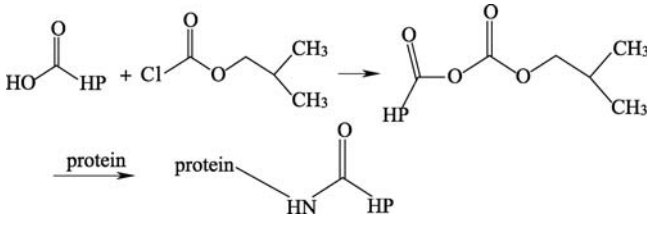

Fig. 4 The synthetic routes for complete antigens
Note: HP represents the DES-MCME or DES-MCPE.

2.4.3 Preparation of coating antigen

The preparation of coating antigen was conducted using the method in accordance with that in section 2.4.1, wherein the BSA was replaced by OVA.

2.5 Identification of the conjugates and molar ratio in the conjugates

The concentration of conjugates were determined by Braford method (Wang and Fan, 2002). The UV spectra and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were used to identify qualitative differences between carrier proteins and conjugates. Molar ratios of the haptens to carrier proteins in the conjugates were confirmed by UV-vis spectral method (Hermanson, 1996) and estimated using the spectral data of haptens, proteins and corresponding conjugates.

2.6 Immunization schedule and antiserum preparation

Each immunizing conjugate ($1\,mg\cdot mL^{-1}$ PBS) was suspended in 1 mL Freund's complete adjuvant and injected into two male New Zealand white rabbits in the subcutaneous intramuscular tissue. The animals were boosted at 14-day intervals with the same immunogen suspended in 1 mL Freund's incomplete adjuvant. Ten days after each boost, the blood was collected by bleeding the ear vein of the rabbits and allowed to coagulate overnight at 4°C. Then the serum was separated by centrifugation and the aliquots of sera were stored at 4°C in 1‰ sodium merthiolate (Liu and Gou, 1989).

2.7 Titers of the anti-sera and antibody

The serum antibody responses elicited with the diethylstilbestrol-protein conjugates produced were determined in an indirect ELISA (Elliott et al., 1994). The anti-serum or its purified antibody was diluted serially with $0.05\,mol\cdot L^{-1}$ PBS. The titer dilution of the anti-sera and antibody was considered suitable while OD = 450nm value was around 1.0. The optimum concentration of the coating antigen and the antibody was confirmed according to the phalanx titration principle using competitive indirect ELISA. Then, Western blot method was used to verify the accuracy of the results of ELISA. SDS-PAGE was used to separate coating antigens (DES-HS-OVA and DES-MCPE-OVA). The proteins were then transferred to a membrane (nitrocellulose), detected using antibodies specific to the target proteins (Renart et al., 1979; Towbin et al., 1979).

3 Results

3.1 Identification of molecular structure of the hapten by ESI-MS, ^{1}H-NMR and IR spectra

3.1.1 Identification of molecular structure of DES-HS

Identification result of DES-MCPE by MS (ESI): Found: m/z 367 $[M-H]^-$, 734.7 $[2M-H]^+$, calcd for $C_{22}H_{26}O_5$ Mr = 368. Results available: the molecular weight of synthetic products in line with the DES-HS.

Identification result of DES-MCPE by IR spectra (KBr): 2958, 2853 (m, C-H), 1711 (s, C = O), 1592, 1511 (s, C = C), 1227 (s, Ar-O-C). Results available: the compound has the characteristic peak of DES-HS.

Identification result of DES-HS by ^{1}H-NMR (300 MHz, DMSO-d6, ppm): 9.269 (s, 1H, OH), 6.993 (d, J = 8.1 Hz, 4H, Ar-H), 6.769 (J = 8.6 Hz, 4H, Ar-H), 2.075 (q, J = 7.5 Hz, CH_2CH_3). Results available: the compound contains 24 hydrogen atoms. These data suggested that the reaction product was just the anticipated compound.

3.1.2 Identification of molecular structure of DES-MCPE

Identification result of DES-MCPE by MS (ESI): Found: m/z 353.23 $[M-H]^-$, 706.94 $[2M-H]^-$, calcd for $C_{22}H_{28}O_4$ Mr = 354. Results available: the molecular weight of synthetic products in line with the DES-MCPE.

Identification result of DES-MCPE by IR spectra (KBr): 2966, 2868 (m, C-H), 1703 (s, C = O) 1609, 1510 (s, C = C), 1321 (m, Ar-H), 1235 (s, Ar-O-C). Results available: the compound has the characteristic peak of DES-MCPE.

Identification result of DES-MCPE by ^{1}H-NMR (300 MHz, DMSO-d6, ppm): δ 12.000 (s, 1H, COOH), 9.317 (s, 1H, OH), 6.980 (d, J = 8.1 Hz, Ar-H), 6.766 (dxd, J = 8.6Hz, Ar-H, 2.3 Hz, 4H), 3.392 (t, J = 5.7 Hz, 2H, OCH_2CH_2), 2.236 (t, 7.5 Hz, 2H, CH_2COOH), 2.063 (q, J = 7.5 Hz, 4H, CH_2CH_3), 1.631 (m, 2H, $CH_2CH_2CH_2$). Results available: the compound contains 26 hydrogen atoms. These data suggested that the reaction product was just the anticipated compound.

3.2 Identification of the conjugates and molar ratio in the conjugates

3.2.1 SDS-PAGE of conjugate and carrier protein

SDS-PAGE (Fig. 5) showed that the electrophoretic mobility of the conjugates of haptens of BSA was less because the mobility ratio and the molecular weight was inversely proportional, proving the molecular weight of artificial antigens was greater than that of the carrier proteins. Test results showed that the synthesis of artificial antigens was successful.

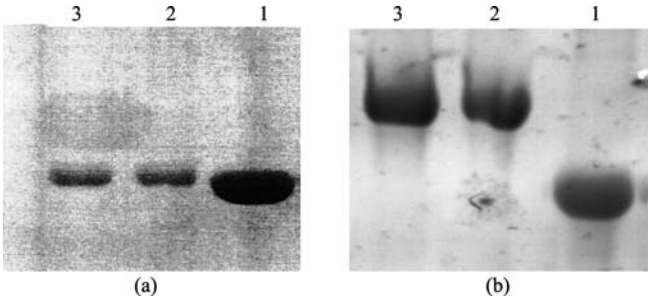

Fig. 5 SDS-PAGE of conjugates and BSA

Note: (a) represents hapten-protein conjugates prepared by the mixed-anhydride method. (b) represents hapten-protein conjugates prepared by the active ester method. 1, 2 and 3 represent BSA, DES-HS-BSA and DES-MCPE-BSA, respectively.

3.2.2 UV-vis spectrum of conjugates and carrier proteins

UV-vis spectrum (Fig. 6) showed that, on the spectra, there existed qualitative differences between carrier proteins and conjugates with a region of maximum absorbance of

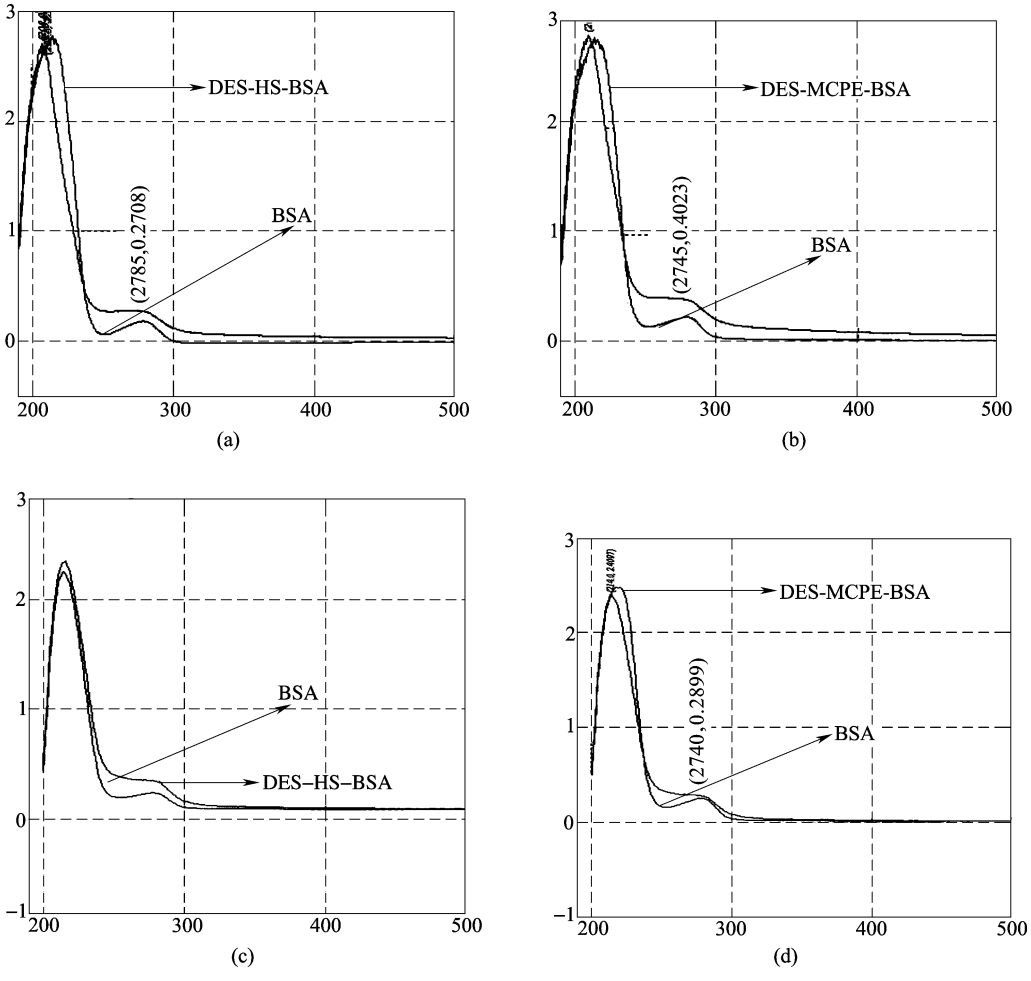

Fig. 6 UV-vis spectrum of carrier proteins and conjugates

Note: (a) and (b) are hapten-protein conjugates prepared by the active esters method. (c) and (d) are hapten-protein conjugates prepared by the mixed-anhydride method.

haptens. This indicated that carrier molecules had been successfully connected to a certain number of haptens. The molar ratio of haptens to proteins in conjugates was then estimated based on the spectral data of the haptens, proteins and corresponding conjugates.

3.2.3 The Molar ratio of haptens to proteins (Table 1)

Table 1 The Molar ratio of haptens to proteins

methods	DES-HS	DES-MCPE
active ester method	24:1	20:1
mixed anhydride method	12:1	9:1

3.3 Titers of the anti-sera and antibody using ELISA

The titers of the anti-sera from rabbits are listed in Table 1. The concentration of the coating conjugates was 10 $\mu g \cdot mL^{-1}$.

Table 2 Titers of the anti-sera

immunogen	DES-HS (24:1)	DES-HS (12:1)	DES-MCPE (20:1)	DES-MCPE (9:1)
titers	1:7200	1:6400	1:6400	1:6400

Note: Numbers in parentheses represent the Molar ratio of haptens to proteins.

3.4 Titers of the antibody using Western blot

Western blot (Fig. 7) showed that it is appropriate to measure the titers using ELISA.

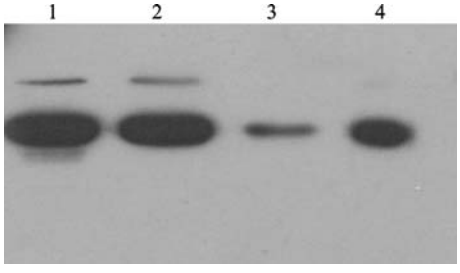

Fig. 7 Western blot of antibody
Note: 1 and 2 are antibodies of DES-HS-BSA and DES-MCPE-BSA prepared by the active ester method. 3 and 4 are antibodies of DES-HS-BSA and DES-MCPE-BSA prepared by the mixed-anhydride method.

4 Conclusions and discussion

Diethylstilbestol-4-carboxymethyl ether was synthesized through the method of organic chemistry. It was successfully synthesized owing to the MS, IR, and H-NMR. Then the haptens were conjugated to BSA and OVA to form the immunogen and the coating antigen, respectively. The corresponding polyclonal antibodies were also obtained after the immunogen was injected to rabbits. Moreover, the specificity of both was satisfied. Two kinds of antibodies had the strong specificity of haptens with diverse structures. The Molar ratio of the hapten to carrier proteins prepared by the active ester method was higher than that by the mixed-anhydride method. In addition, there was a direct proportional relationship between the titers of anti-sera and the molar ratio within a certain range. In our research, DES was transformed into artificial antigens that were used to immunize rabbits, through which the anti-DES corresponding polyclonal was successfully obtained. This offered a good technique platform for developing DES residual immunoassay reagent box.

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References

Degand G, Schmitz P, Maghuin-Rogister G (1989). Enzyme immunoassay screening procedure for the synthetic anabolic estrogens and androgens diethylstilbestrol, nortestosterone, methyltestos-terone and trenbolone in bovine urine. Chromatogram, 489: 235–243

Dodds E C, Goldberg L, Lawson W, Robinson R (1938). Estrogenic activity of certain synthetic compounds. Nature, 141: 247–248

Dong M, Li G F, Li J (1999). Immunological detection of arbutin. Tsinghua Science and Technology, 4(3): 311–316

Elliott C T, Francis K S, Mccaughey W J (1994). Investigation of dissociation enhanced lanthanide fluoroimmunoassay as an alternative screening test for veterinary drug residues. Analyst, 12: 119–124

Erlanger E F, Borek F, Beiser S M (1958). Steroid-protein conjugates II. Preparation and characterization of conjugates of bovine serum albumin with progesterone, and deoxycorti-costerone, and estrone. Biological Chemistry, 234: 109–210

Gosling J P (2000). Immunoassays-A Practical Approach. Oxford: Oxford University Press, 174

Hermanson G T (1996). Bio-conjugate Techniques. San Diego: Academic Press

Huang S Y, Cheng Z Y (1990). Synthesis of diethylstilbestrol immunogen by mixed anhydride. Atomic Energy Agricultural, 11 (4): 183–184 (in Chinese)

Krejcarek G E, Tucker K L (1977). Covalent attachment of chelating groups to macromolecules. Biochem Biophy Res Comm, 77: 581–585

Lei L H, Qun Z N, Liu S (2004). Synthesis of diethylstilbestrol-hemisuccinate. The Progress of Animals Medicine, 25(2): 62–64 (in Chinese)

Liu Y B, Gou S J (1989). Animal Experimental Technique in Immunology. Changchun: Jilin Science and Technology Press (in Chinese)

Meyers R (1983). DES, The Bitter Pill. New York: Seaview/Putnam

Renart J, Reiser J, Stark G R (1979). A method for studying antibody specificity and antigen structure. Proc Natl Acad Sci, USA, 76(7): 3116–3120

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci, USA, 76(9): 4350–4354

Wainer B H, Fitch F W, Rothberg R M (1972). Morphine-3-succiny-lbovine serum albumin: an immunogenic hapten-protein conjugate. Science, 176(39): 1143–1145

Wang J Z, Fan M (2002). Technical Manuals of Protein. Beijing: Science Press (in Chinese)

Zhan Z Y, Lu W F, Shi Y Z (1994). Single-etherification reaction of hydroquinone and KB2BCOB3B-AlB2BOB3 Bload reagents. Applied Chemistry, 11(5): 14–17 (in Chinese)

Zhu G N, Wu G, Wu H M (2003). Synthesis and Identification of antigens for the organophosphorus insecticide Chlorpyrifos. Scientia Agricultura Sinica, 36: 657–662 (in Chinese)

Zhu G N, Wu Y L, Cheng J L (2002). Synthesis and identification of the antigens for carbofuran. Zhejiang University Science (Agriculture and Life Sciences), 28(1): 47–53 (in Chinese)