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One-step indirect competitive ELISA development for dexamethasone determination in chicken

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ABSTRACT

Dexamethasone (DEX) is a potent glucocorticoid that was widely employed in livestock production. In the present study, a one-step indirect competitive enzyme-linked immunosorbentassay (icELISA) was developed for the determination of DEX residue in animal-origin food. With this aim, hapten DEX-3c was firstly synthesized, conjugated to carrier protein, and then used as immunogen in rabbits. Polyclonal antibody was subsequently obtained and characterized. The half-maximal inhibition concentrations (IC50) and the limit of detection (LOD, calculated as IC20) of the icELISA for DEX were approximately 0.061 and 0.015 ng/mL, respectively. The antibody exhibited significantly cross-reactivity with some of DEX structural analogues. The average recoveries and coefficients of variation (CVs) of DEX from fortified samples by icELISA were in a range of 83.3-114.2% and 5.6-9.5%, respectively. The LOD of this assay for DEX in chicken muscle and liver extracts was 0.3 μg/kg and 0.5 μg/kg, respectively.

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1. Introduction

Dexamethasone (9α-Fluoro-11β, 17α, 21-trihydroxy-16α-methylpregn-1, 4-diene-3, 20-dione, DEX) is a potent and long-action glucocorticoid (GC) developed in late 1940s to early 1950s (1). DEX was used not only to treat inflammatory disease of human and animal for its anti-inflammation, anti-endotoxin, low dose and slight sodium retention, but also to promote animal growth for its improvement of feed conversion rate (2). So far, China became the world's largest DEX market for the disease treatment. DEX residue will move up the food chain to human body, and enrich in it. Redundant DEX in the human body can cause obesity, hypertension, high blood sugar, osteoporosis and other diseases. For this reason, the maximum residue limits (MRLs) of DEX in animal-origin foods were established in many countries including China, USA, Japan and the European Commission (3,4). The MRLs of DEX in pork (horse/cattle) muscle and pork (horse/cattle) liver was 0.75 ng/g and 2 ng/g, respectively, established by the Ministry of Agriculture of the People's Republic of China No. 235, promulgated on December 24, 2002. In 2012, "fast-growing chicken" incident was exposed in China for the usage of DEX as additive to promote chicken growth; White feather chicken can mature for the slaughter in 45 days (5). From that time, DEX residue in animal-origin food was scrutinized strictly in China, especially chicken muscle and liver.

There were several analytical methods have been reported for the determination of DEX in biological samples including liquid chromatography tandem

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mass spectrometry (LC–MS/MS) (6,7,8), high-performance liquid chromatography (HPLC) and so on (9). Though these instrumental methods are usually validated for the simultaneous detection of glucocorticoids in biological samples, they are limited by expensive equipment, skilled personnel, and complex sample treatments. Immunoassay has been proven to be a simple, high throughput, and cost-efficient tool in detecting trace constituents in a complex matrix, which was widely used for screening purposes. Positive samples from immunoassay are usually subjected to more rigorous confirmatory assay. Currently, there were already a few reported two-step immunoassays for the detection of DEX or other glucocorticoids in equine blood, bovine liver, milk, plasma and saliva (10,11,12). However, no screening assay for DEX has been reported for chicken muscle and liver. In this work, our aim was to develop a broad-specificity one-step icELISA for the determination of DEX and other glucocorticoids in chicken muscle and liver, which was great concerned for "fast-growing chicken" incident.

Therefore, this job involves (a) the preparation of DEX hapten and immunogen and produces a broad-specificity polyclonal antibody (pAb); (b) to evaluate this pAb and establish a one-step icELISA for the determination of DEX residue in chicken muscle and chicken liver; and (c) to compare icELISA with the confirmatory LC-MS/MS methods.

2. Materials and methods

2.1. Reagents and Materials

DEX, triamcinolone, prednisolone, betamethasone and hydrocortisone were all purchased from the China Institute of Veterinary Drug Control (Beijing, China). Bovine serum albumin (BSA), oval albumin (OVA), N-hydroxy succinimide (NHS) and Freund's adjuvant were all from Sigma (St. Louis, MO, USA). Goat anti-rabbit IgG-HRP (GaRIgG-HRP) was purchased from Jackson Immuno-research (West Grove, PA, USA). Ethyl acetate (EtAc), N, N-dimethyl formamide (DMF) and hexane were from Sinopharm Group Co.Ltd. (Beijing, China). HPLC grade acetonitrile and formic acid were purchased from Dima Technology Inc. (Muskegon, MI, USA). All chemicals used were of analytical grade, unless otherwise specified. Standard solutions: stock solutions of DEX and cross-reactants were prepared in DMF at the concentration of 2 mg/mL. The standard solutions of 0, 0.01, 0.02, 0.06, 0.18, 0.54 and 1.62 ng/mL were prepared by serial dilution in PBS solution.

2.2. Apparatus

Immunoassay absorbance was read by a multiscan MK3 microplate reader (Thermo Fisher Scientific, Vantaa, Finland). MALDI-TOF-MS was purchased from Kratos Analytical Co. (UK). Water was purified using a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). HQ-60-II Vortex mixer was from Beijing North TZ-Biotech Develop. Co. (Beijing, China). N-EVAP 112 nitrogen evaporator was purchased from Organomation Associates Inc. (Berlin, MA, USA).

2.3. Hapten synthesis

DEX derivative used in this work as hapten was prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, in the hydrogen of 21-position hydroxyl. The procedure for the synthesis of hapten was depicted in Figure 1. DEX (500 mg) was dissolved in pyridine (5 mL) and succinic anhydride (110 mg) was added in a 25 ml round-bottom flask. The mixture was stirred for 3 h at 80 ^{o}C, and then the organic phase was evaporated to dryness at reduced pressure. Ice-water was added to the residue and a white solid was precipitated and filtered. The isolated solid was washed thrice with water and dried in a vacuum oven at 50 ^{o}C for 8 h to give 350 mg product. The structure of the hapten was confirmed by MS. Dexamethasone21-hemisuccinate (DHS): M: M-H=491.2, M+: M+K=531.7.

2.4. Preparation of Protein-Hapten Conjugates

The hapten was conjugated with carrier protein BSA (or OVA) by the NHS active ester method for immunogen (or coating antigen). That is, the carboxylic acid hapten was coupled covalently with the amino group of carrier protein. The procedure for coupling haptens to BSA for DHS-BSA was described below. The hapten (0.045 mmol) was added to 1.5 mL of dry DMF and then 3-fold molar excess of 1-[3-(Dimethylamino) propyl]-3-ethylcarbodimide hydrochloride (EDC) and 0.22 mmol of NHS were added successively. The mixture was stirred at room temperature for 3 h. Fifty milligram of BSA (67 mg of OVA for DHS-OVA) was dissolved in 3.5 mL of carbonate buffer (0.1 M, pH 9.6) and stirred at 400 rpm for 10 min for dissolution. The active ester was added dropwise to BSA solution in ice-bath with stirring for 24 h at 4 ^{o}C. The conjugation was dialyzed against PBS (1 L) for 3 days at room temperature with three buffer changes per day. Finally, the conjugates were frozen in 0.5 mL aliquots. The

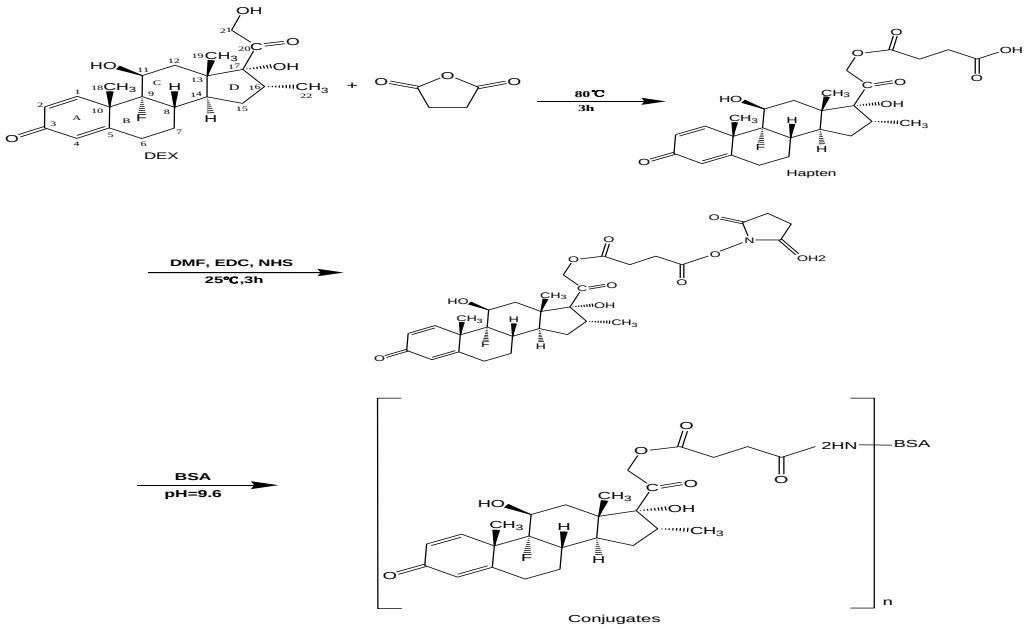


Figure 1. Reactions used to synthesize the hapten (DHS) and conjugates (DHS-BSA).

extent of coupling of hapten to BSA/OVA was determined by MALDI-MS-TOF.

2.5. Production of pAb Against DEX

Three female New Zealand white rabbits, weighing 2.0-2.5 kg, were injected subcutaneously with 1 mg of DHS-BSA conjugates at 2 weeks intervals, respectively. The first immunization was given with 1 mg DHS-BSA in 0.5 mL of 10 mM PBS emulsified with 2-fold volume of complete Freund's adjuvant. Complete adjuvant was followed by incomplete adjuvant in the subsequent boost. At seventh day after each boost immunization, the titers of antisera were tested. It was emphasized that antisera inhibition rate was preliminary evaluated by 0.5 ng/mL DEX at the same time. When a satisfactory titer and inhibition rate was obtained, the last injection was then given. The whole blood of rabbits was collected after the final injection, and antisera were purified by saturated (NH4)2SO4.

2.6. One-step icELISA procedure

Flat bottom polystyrene ELISA plates (Corning Coaster High Binding No. 42592, Cambridge, MA) were coated with 100 µL/well of DHS-OVA diluted (1: 20,000) in 50 mM carbonate-bicarbonate buffer (pH 9.6) by incubation overnight at 4 ºC. The following day, coating antigen was discarded and washed only one time with 10 mM PBS (1.5 mM KH2PO4, 7 mM Na2HPO4, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween-20 (PBST, pH 7.4) to remove excess coating antigen. And then the plate was blocked with 1% BSA and 3% sucrose in PBS (150 µL/well) and incubated for 2 h at room temperature. After then, 50 µL/well of standards or sample extractions, 50 µL/well GaRIgG-HRP in PBST (1: 1,000) and 50 µL/well antibodies in PBST with 0.5% BSA (1: 10,000) were added sequentially and incubated at room temperature for 30 min. The microplate was washed four times followed by the addition of 100 µL of TMB substrate solution into each well. The reaction was stopped with 1 M sulfuric acid solution after an incubation of 10-15 min at room temperature. The absorbance at 450 nm was read immediately, and the data were fitted to a four-parameter logistic equation using Origin (version 8.0, Microcal, studio city, CA) software packages

$$y = \{(A - D)/ [1 + (x/C) B]\} + D$$

where A is the asymptotic maximum (maximum absorbance in the absence of analyte, Amax), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration giving 50% inhibition of Amax, IC50), and D is the asymptotic minimum (background signal). Determination of spiked samples was performed by interpolating their mean absorbance values in the standard curve run in the same microplate.

2.7. UPLC-MS/MS analysis

Chromatographic separation was performed on an ACQUITY UPLC system (Waters, Miford, MA), equipped with a vacuum degasser and an autosampler, using a Waters BEHC18 column (50 mm × 2.1 mm, I.D., 1.7 µm particle size) at 25 °C. The mobile phase was composed of acetonitrile and water with 0.1% formic acid (1:1, v/v). The injection volume was 20 µL and the flow rate was 0.2 mL/min.

The MS/MS system consisted of a Quattro LC triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source. The MS/MS detection was obtained using multiple reaction monitoring (MRM) mode. The parameters used for the mass spectrometer were as follows: ionization mode, electrospray positive; gas (N2) temperature, 300 °C; gas (N2) flow rate, 450 L/h; source temperature, 120 °C; capillary voltage, 3.0 kV; extracted ion, 393.2 [M + H]; and scan range, m/z 100-400. The selected MRM transitions for DEX were m/z 393.2-355.2 and 393.2-147.1 with a dwell time of 1.66 min. The transition chosen for quantification was 393.2-147.1. The collision energies for the transitions of 393.2-355.2 and 393.2-147.1 were 12 eV and 30 eV, respectively.

2.8. Sample Preparation and Extraction

A total of 25 chicken muscles and 25 chicken livers were purchased from various supermarkets in Beijing, China. DEX-free chicken muscle, chicken liver confirmed by LC-MS/MS was obtained from the National Reference Laboratory for Veterinary Drug Residues (Beijing, China). For recovery study, DEX stock solution (100 ng/mL, prepared in DMF) was added into homogenized samples to produce various spiked concentrations. Fortified samples were prepared freshly prior to analysis.

For icELISA. (1) Chicken muscle sample. Homogenized chicken muscle samples (1g) were weighted into a 50 mL polystyrene centrifuge tube, which were spiked with DEX at the concentration of 0.3, 0.6 and 1.2 µg/kg. Then, 9 mL extraction buffer (1.56 g of NaHPO4•2H2O in 1 L of distilled water) was added, and the mixture was vortexed violently for 1 min and centrifuged at 4000 g for 10 min. Fifty microliter of the supernatant was used for icELISA. Dilution factor: 10. (2) Chicken liver sample. Homogenized chicken liver samples (1g) were weighted into a 50 mL polystyrene centrifuge tube, which were spiked with DEX at the concentration of 0.5, 1.0 and 2.0 µg/kg. Then, 1 mL of liver extraction buffer (10 g NaCl and 13 g K2HPO4•3H2O in 0.3 L of distilled water) was added and vortexed to adequately disperse tissue. Then, 5 mL of hexane-EtAc (25:75, v/v) was added and vortexed violently for 1 min and centrifuged at 4000 g for 10 min. The supernatant (1 mL) was evaporated to dryness under nitrogen at 50-60 °C. The residue was sequentially added 2 mL of hexane and 1 mL of PBS (0.02M), and then vortexed violently for 1 min and centrifuged at 4000 g for 5 min. The upper organic phase and middle impurity layer was discarded and the remaining solution was analyzed. Dilution factor：5. Blank samples were prepared in the same way above but without spiking step.

For UPLC-MS/MS. Five gram homogenated samples were weighted into a 50-mL polystyrene centrifuge tube, which 20 µL of internal standard 6-alpha- methylprednisolone (0.5 µg/mL) and 10 mL of NaOH solution (0.3 M) were added. And then 20 mL of EtAc was added for the extraction of analyte from samples, which was vortexed violently for 1 min and shook for 20 min, followed by centrifugation at 3000 rpm for 5 min. The upper organic phase was transferred into a pear shape flask through a barrel-type funnel containing 25 g of anhydrous sodium sulfate. The sample extraction was repeated with 20 mL of EtAc. The merged supernatant was evaporated to near dryness with a rotary evaporator at 45 °C in water bath, followed by nitrogen evaporation at 60 °C to complete dryness. The residue was reconstituted with 1 mL of mobile phase. This solution was passed through a filter (0.2 µm) prior to LC-MS/MS analysis.

3. Results

3.1. Synthesis and preparation of immunogen

In this work, the spacer arm was attached through the hydroxyl group by O-alkylation and DHS was prepared. The syntheses of the haptens were carried out as outlined in Fig. 2. The molecular weight of hapten (DHS) was 492.53. MALDI-TOF-MS was used for the measurement of BSA and DHS-BSA. As shown in Fig. 3, molecular weight of BSA and DHS-BSA was 67485.901 and 73828.009, respectively. Conjugation ratio: (MWDHS-BSA-MWBSA)/MWDHS=12.88.

3.2. Binding characterization of antibody

Under the optimal condition, the standard inhibition curve of icELISA for DEX was shown in Fig

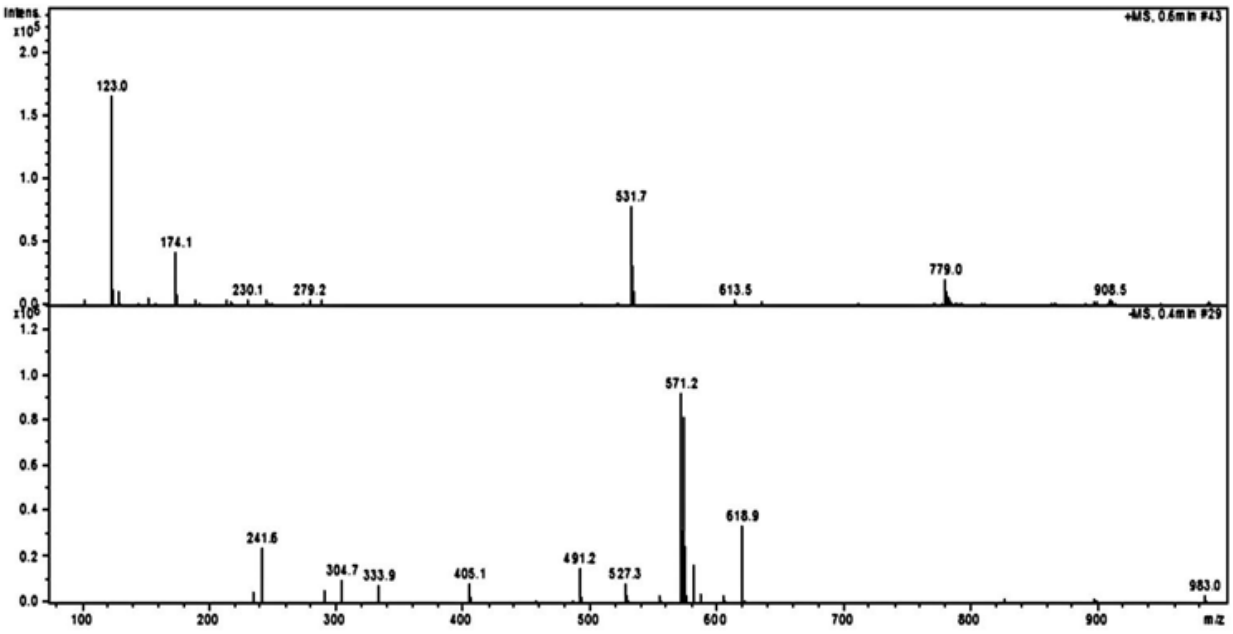


Figure 2. MS spectrum for DHS.

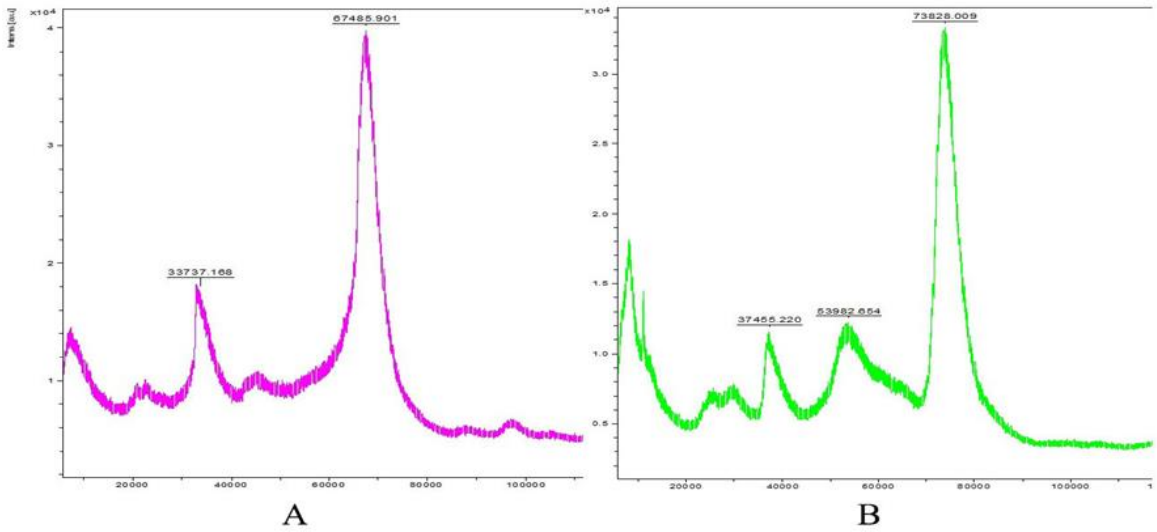


Figure 3. MALDI-TOF-MS spectrum. (A) BSA; (B) DHS-BSA.

4 The 50% inhibitory concentration (IC50) of DEX obtained from the four-parameter equation and LOD (limit of detection, IC20) of icELISA were 0.061 and 0.015 ng/mL, respectively, indicating that the prepared antibody had high sensitivity to DEX. The specificity of the antibody was estimated by the cross-reactivity with DEX analogues (i.e., glucocorticoids). Some of glucocorticoids showed high cross-reactivity with the prepared antibody (Table 1).

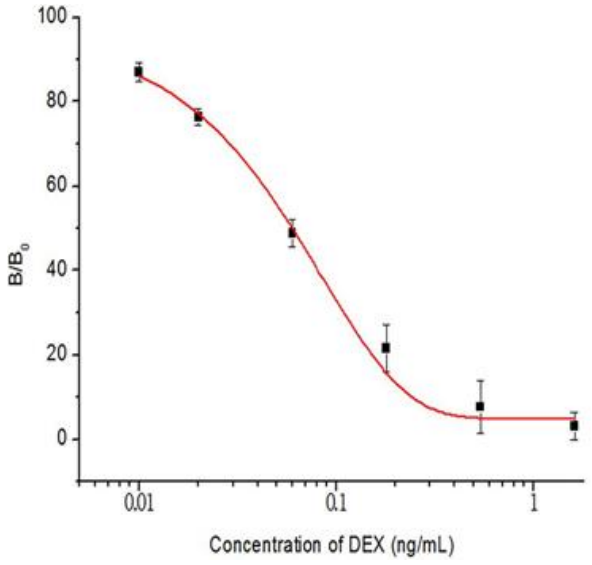


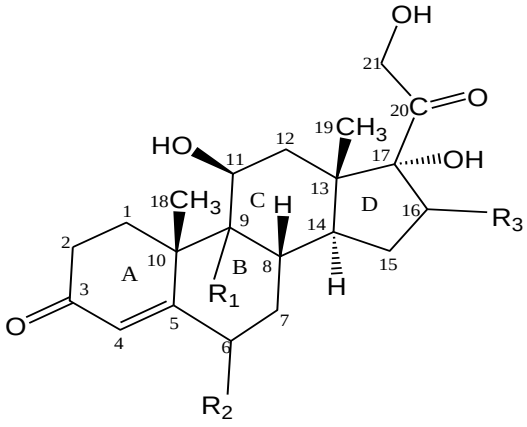
Figure 4. Standard curve of dexamethasone by indirect ELISA.

3.3. Analysis of Spiked Samples

The sensitivity of this icELISA for real samples was evaluated by blank samples, including 20 blank chicken muscles and 20 blank chicken livers. The LOD (mean value + 3SD) of this assay for DEX in chicken muscle and liver extracts was 0.3 µg/kg and 0.5 µg/kg, respectively. The blank chicken muscle samples spiked with DEX standard solution at three different concentration levels (0.3, 0.6 and 1.2 ng/g) and chicken liver samples fortified with 0.5, 1.0 and 2.0 ng/g were analyzed by icELISA and UPLC-MS/MS. The average recoveries and CVs by icELISA were in a range of 83.3-114.2% and 5.6-9.5%, respectively (Table 2), which was acceptable. The average recoveries and CVs were in the range of 78.0-95.8% and 3.6-7.2% for UPLC-MS/MS, respectively.

Table 1. Cross-reactivity of Anti-DEX Antibody with Various glucocorticoids.

Compound	1-position	R_1	R_2	R_3	IC_{50} (ng/g)	Cross-reactivity (%)
Dexamethasone	═	F	H	α-CH_3	0.061	100.0
Triamcinolone	═	F	H	OH	0.060	101.6
Prednisolone	═	H	H	H	1.070	5.7
Betamethasone	═	F	H	β-CH_3	0.063	96.8
Hydrocortisone	—	H	H	H	4.067	1.5
Flumethasone	═	F	F	α-CH_3	0.264	23.1



Glucocorticoids

Table 2. Recoveries of DEX fortified in blank sample by icELISA and LC-MS/MS (n=5).

Sample	Fortified (ng/g)	icELISA			LC-MS/MS		
		Test (ng/g)	Recovery (%)	CV (%)	test (ng/g)	Recovery (%)	CV (%)
muscle	0.3	0.25±0.017	83.3	6.8	0.25±0.018	83.3	7.2
	0.6	0.57±0.028	95.0	4.9	0.52±0.022	86.7	4.2
	1.2	1.37±0.130	114.2	9.5	1.15±0.041	95.8	3.6
liver	0.5	0.46±0.036	92.0	7.9	0.39±0.025	78.0	6.4
	1.0	1.03±0.058	103.0	5.6	0.84±0.047	84.0	6.0
	2.0	2.24±0.167	112.0	7.5	1.83±0.121	91.5	6.6

3.4. Application to real samples

DEX residues in 25 chicken muscles and 25 chicken liver sample were collected from the local supermarkets. DEX was not detectable in chicken muscle, but a chicken liver sample was shown DEX-positive with an approximate concentration of 1.04 µg/kg. The positive sample determined by icELISA was analyzed by UPLC-MS/MS. Both methods showed consistent results for this positive sample. DEX residue in the positive sample was validated by UPLC-MS/MS analysis to be 0.97µg/kg, which is far below the MRLs of 2.0 ng/g in chicken liver. In the present work, the LOD of the developed icELISA method was lower than the MRLs for DEX residue in chicken muscle and liver at 0.75 µg/kg and 2.0 µg/kg, respectively, set by the European Commission.

4. Discussion

DEX is a small (MW=392.5), simple organic molecule, consisting of three six-membered rings and one five-membered ring, and it is non-immunogenic by itself and lacking a functional group for coupling to proteins. Therefore, the synthesis of hapten should preserve the analyte structure and electronic distribution as far as possible, which is a critical factor in the preparation of high-affinity antibodies (13). It was known that chemical properties of the DEX molecule are depending mainly on 9-position F and 16-position CH3 (14). Hapten was conjugated to carrier proteins by the active ester method to elicit an immune response. To obtain evidence of successful conjugation, the results indicated that DEX was successfully conjugated to BSA. The conjugation of OVA and DHS was evaluated the same way mentioned above. After a high titer antiserum was obtained, a checkerboard method was further employed to screen the optimal concentrations of antibody and coating antigen via the inhibition of two different concentrations (0.05 and 0.5 ng/mL) of the analyte in phosphate buffer, using an icELISA for DEX. The inhibition ratio was defined as: I %= {1– (B/Bo)} ×100%, namely, a percentage of the difference between the absorbance of the analyte-free buffer (Bo) and the absorbance of the analyte-containing buffer (B), divided by the former (15). Numerous ELISAs for small molecules are performed in a two-step competitive protocol, which is generally time-consuming. The high sensitivity, speed and easy operation give the one-step ELISA promising application (16). In the present work, a rapid one-step icELISA was substituted for the traditional two-step assay to improve the detection process. Compared to the two-step method, the one-step process in our study needs less time and fewer steps, which is important for the screening assay. So, one-step assay was therefore chosen for the subsequent study. It was reported that the antibodies for glucocorticoids were raised generally from immunogen synthesized by the attachment of the carboxylacid group at C-3, C-4 and C-21, which linked to carrier protein (11, 17); high cross-reactivity was observed by anti-GC-21-HS-BSA; negligible cross-reactivity was exhibited by anti-GC-3(4)-HS-BSA and anti-GC-4-HS-BSA. It was deduced that the A-ring and B-ring on the structure of DEX (or GC) plays an important role in epitope determination, which can also explain the prepared antibody has high cross-reactivity with triamcinolone and betamethasone, though there was a large structure difference in R3 substituent of D ring between DEX, triamcinolone and betamethasone (shown in table 1). Antibodies with high cross-reactivity can be a base of immunoassays, which are capable of detecting a class of chemicals, and thus avoid the need for a series of different assays to recognize undesired presence of contaminants (18).

A very important evaluation for any analytical method is the assessment of its sample preparation procedure. It is a great challenge to reduce matrix effects in complex sample (e.g. chicken liver). DEX was a stable compound, which can easily keep a stable structure. In the present study, DEX was fortified in chicken muscle and liver samples at three concentrations. Chicken muscle samples were simply

extracted with phosphate buffer and no cleanup step was employed prior to analysis. For chicken liver, sample pretreatment involved an extraction with hexane-EtAc and a nitrogen blow. It was mentioned that the average recoveries of DEX by icELISA were higher than those by UPLC-MS/MS (Table 2), which can be contributed by the matrix effect of the test sample. The good recoveries suggest the pretreatment procedure for DEX in chicken muscle and liver was suitable method.

In the present work, the highly sensitive icELISA for the detection of DEX was developed and confirmed by LC–MS/MS. Based on this work, a commercially DEX ELISA kits was developed by Beijing WDWK Biotechnological Co., Ltd., which was applied for the monitoring of DEX in animal-origin food in China.

5. Conclusion

In this work, the developed icELISA could be an appropriate method for screening of DEX residue in chicken muscle and liver, and then we could confirm the residue by LC-MS/MS method. Based on this work, a commercially DEX ELISA kit was developed, which could be carried out in any routine laboratory without special facilities. This method is simple, high throughput, credible for the detection of DEX residue in chicken muscle and liver.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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