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Short communication

Determination of hexoestrol residues in animal tissues based on enzyme-linked immunosorbent assay and comparison with liquid chromatography–tandem mass spectrometry

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Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of the hexoestrol (HES). Polyclonal rabbit antisera, raised against protein conjugate hexoestrol-mono-carboxyl-propyl-ethyl-bovine-serum-albumin (HES-MCPE-BSA), were utilized in immobilized antibody-based and competitive immunoassays. Assay conditions, including concentrations of antisera and Horseradish peroxidase (HRP)–HES were optimized. The effect of incubation time, surfactant concentration, ionic strength and pH of the medium were also investigated. The typical calibration curve gave an average IC_{50} value of 2.4 ng/ml, calibration range from 0.2 ng/ml to 30.5 ng/ml and a detection limit of 0.07 ng/ml. The specificity of the assay was tested against HES structurally related compounds, and the assay proved highly selective for HES. Assay performance was validated by using spiked pork and liver tissues samples. Moreover, it was compared with liquid chromatography–tandem mass spectrometry. The ion pair for quantification of HES was 269.4/134, and linear equation of HES was $Y = 0.2148X - 0.0374$ ($r = 0.9993$). The two analytical methods can be applied to monitor HES and other steroid residues in edible foods.

Keywords: Hexoestrol; ELISA; Immunoassay; Liquid chromatography–tandem mass spectrometry

1. Introduction

Hexoestrol (HES: 3,4-bis (*p*-hydroxyphenyl) hexane; CAS RN: 84-16-2), together with dienestrol and diethylstilbestrol belong to the group of stilbene estrogens. When used illegally in cattle feed, HES improved growth rate and increased feed conversion [1–5]. However, in most countries, the use of HES has been banned currently due to its teratogenic [6], mutagenic [7,22] and carcinogenic properties [8–11]. So, it is quite necessary to control its abuse.

The traditional method for the analysis of HES and other estrogens in the present is gas chromatography (GC) and liquid chromatography [12] with mass spectrometry [13–15,21]. Large-scale surveillance programs require a rapid analysis of synthetic nonsteroidal estrogen; therefore an enzyme-linked immunosorbent assay (ELISA) appeared suitable. Such assays have been developed in our laboratories since 2002. Here we report the synthesis of haptens and their protein conjugates, the development and characterization of antisera and the optimization and validation of physical and chemical conditions of the analytical medium. Finally, the optimized ELISA was applied to determine HES in spiked pork and liver tissues samples, and to compare with liquid chromatography and tandem mass spectrometry, in which internal standard was used.

2. Materials and methods

2.1. Reagents

Hexoestrol, diethylstilbestrol, dienestrol, 17β-estradiol, *O*-phenylenediamine (OPD), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were obtained from

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Sigma (St. Louis, USA). Internal standard, D_8-diethylstilbestrol (ring-3,3′, 5,5′-diethyl-1, 1,1′, 1′-D_8, 98%) was obtained from Cambridge Isotope Laboratories (50 Frontage Road, MA, USA). Zeranol, 19-nortestosterone, testosterone and cortisol were kindly gifted by Chinese Academy of Inspection and Quarantine. Bovine serum albumin (BSA, electrophoretic grade) and ovalbumin (OVA, electrophoretic grade) were purchased from Boao (Shanghai, China). γ-Bromobutyric Acid Ethyl Ester was brought from Yuyu Chemical Plant (Changzhou, china, import in bulk). Semi-preparative Silica gel GF254 plates were from Yoko Developmental Company (Wuhan, China). Protein G Sepharose (Mab Trap G) was from Pharmacia (Uppsala, Sweden). Horseradish peroxidase (HRP) was purchased from Kangcheng (Shanghai, China) as well as Tween-20. Isobutyl chloroformate was obtained from Feixiang Chemical Plant (Shanghai, China).Methanol, acetonitrile, hexane, chloroform, 1-propanol, and ethyl acetate for HPLC were all commercially available from Scharlau Chemic SA (Barcelona, Spain). Formic acid (99%), acetic acid (99%) and trifuoroacetic acid (99%) were from Acros Organics (New Jersey, USA). Anhydrous sodium sulfate and ammonium formic were all analytical grade (Beijing, China). Ultra pure water was made by the Milli-Q Ultra pure System (Millipore, Bedford, MA, USA). All standards were stored at −20 °C. Sep-Pak silica and amino-propyl solid phase extraction cartridges containing 500 mg materials (3 ml) were purchased from Waters Co. (Milford, MA, USA). To avoid contamination, all the glassware was baked for 4 h at 400 °C prior to use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination.

Stock solutions were prepared for standard substances at 1000 mg/l in methanol. Spiking and calibration mixtures at various concentration levels were obtained by combining stock solutions and internal standard with mobile phase, and stored at 4 °C. The concentration of internal standard in all the calibration mixtures and final sample solutions was 20 μg/l.

2.2. Instruments

Vertical saturate tank, plates (10 cm × 20 cm) and ZF-90 dark box UV transilluminater (Gucun Apparatus Plant, Shanghai, China) were used. The LC–MS spectrometer used for analyzing the protein HES-conjugates was a WATERS Platform ZMD 4000 (Waters Company, Milford, MA). AB104-N electronic chemical balance was from Metller Toledo Group (Shanghai, China). UV-2100 UV scanner was provided by Ruili Company (Beijing, China). Microtiter plates (Maxisorb) were purchased from Nunc (Roskilde, Denmark). MuLtiska Mks microplate reader was from Labsystem (Helsinki, Finland).

2.3. Synthesis and characterization of hexoestrol-MEBE derivative

The schematic principles of synthesis hexoestrol-MCPE is shown in Fig. 1 as described by Winkler et al. with slight modifications [16,17]. Briefly, 1.1 g of HES was dissolved in 25 ml of anhydrous acetone, and then 3.8 g of K_2CO_3–Al_2O_3 carrier reagent and 0.3 ml of γ-bromobutyric acid ethyl ester

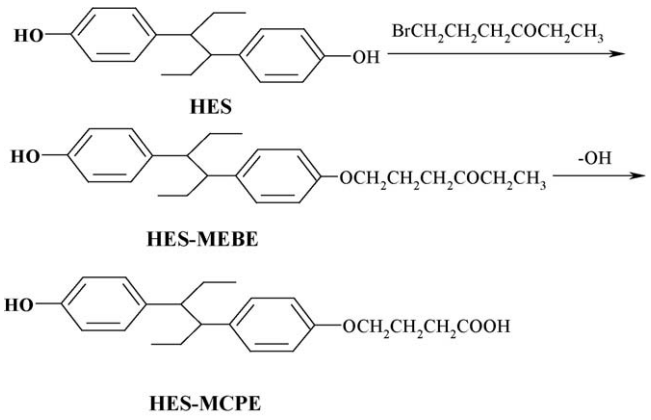

Fig. 1. Principles of connecting of space-arm to HES.

were added under the protection of nitrogen stream. This solution was refluxed in dark at 65 °C for 10 h. The catalytic agent was then removed by filtering; remained solution was evaporated and finally was streaked onto a preparative silica gel GF plate. The thin-layer chromatography conditions using a chloroform–methanol mixture (95:5 v/v) allowed the separation of a hexoestrol-mono-ether-butyrate-ethyl (HES-MEBE) derivative from hexoestrol with different R_f values. The band corresponding to the R_f of the HES-MEBE ($R_f = 0.4$) was scraped off and extracted with methanol. The organic phase was then concentrated by rotary evaporation and the obtained residue was further purified by HPLC using a Lichrospher C_{18} column (2.1 cm × 0.25 cm) at a flow rate of 0.3 ml/min with water as mobile phase. The elution volume corresponding to that of HES-MEBE was collected and evaporated to dryness under vacuum. The residue obtained was characterized by mass spectrum.

2.4. Preparation of protein-hapten conjugate

Hapten used in this study was conjugated to protein via its carboxylic group by the *N*-hydroxysuccinimide active ester method, according to Langone and Van Vunakis [18]. The hapten HES-MEBE was conjugated to BSA for forming immunogen, to OVA for coating conjugate preparation and to HRP for enzyme tracer. Approximately, 15 mg of hapten were dissolved in the appropriate volume of *N*,*N*′-dimethylformamide (DMF) for a final 100 mM concentration of the hapten and then activated by incubation at room temperature with 100 mM *N*-hydroxysuccinimide and dicyclohexylcarbodiimide for 5 h. The mixture was centrifuged, and the supernatant was collected. With gentle stirring, the active hapten was slowly added to 10 mg protein (BSA for immunogen, OVA for coating conjugate and HRP for enzyme tracer) dissolved in 1 ml of 50 mM sodium carbonate buffer, pH 9.6. The initial hapten-protein molar ratio was 100:1 for the three proteins. The mixture was stirred at room temperature for 2 h. Finally, the conjugates were separated from uncoupled hapten by gel filtration on Sephadex G-25 using PBS (pH 7.2) as eluant. Conjugate formation was confirmed by spectrophotometer. UV–vis spectra showed qualitative differences between carrier proteins and conjugates in the region of maximum absorbance of hapten (230 nm, for HES). The efficiency of

the coupling procedure assessed by the ratios of absorbancy at 230 and 280 nm on the excluded fraction relative to the HES reference standard gave a yield of 17, 10 and 3 haptens per mol of BSA, OVA and HRP, respectively. The immunogenic conjugate was stored at −20 °C and coating conjugation at 4 °C.

2.5. Production of antisera against HES-MCPE-BSA

A 0.5 ml (150 μg) of HES-MCPE-BSA derivative was emulsified with equal volume of CFA and injected subcutaneously into multiple sites along the back of New Zealand white male rabbits individually. The booster doses were made in IFA at 4-week intervals with the doses of 300 μg. Blood from rabbits were collected after 7 days of each booster injection for the titer monitoring by indirect ELISA with the HES-MCPE-OVA as coating antigen. Antisera R1, R2 and R3 with adequate titer, affinity and specificity were obtained 3 months after the first immunization. Polyclonal IgGs were purified from antisera by affinity chromatography on a Mab Trap G.

2.6. Sample preparation

Ten grams of each sample was weighed, and each sample was put into a 100 ml glass conical flask and spiked with 10 ng internal standard. Ten milliliters 0.2 mol/l acetate buffer (pH 5.2) was added and the samples were homogenized with an ultra turrax machine for about 1 min. The pH of each mixture was readjusted to 5.2 and 100 μl β-glucuronidase/arylsulfatase from Helix Pomatia (Roche Diagnostics GmbH, Mannhein, Germany) was added and incubated overnight at 54 °C. Then the sample was cooled to room temperature and 35 ml methanol was added to homogenize the sample. Each mixture was centrifuged at 2000 × *g* for 10 min at 0 °C. The supernatant was decanted into a separatory funnel and extracted with 20 ml *n*-hexane twice to remove the parts of fat. The upper layer was discarded (*n*-hexane) and 5 ml 1-propanol was added to prevent foaming during evaporation. Methanol was evaporated at 50 °C with a rotary evaporator. One hundred milliliters of water was added and the aqueous solution was subjected to solid phase extraction (SPE).

An HLB cartridge was conditioned sequentially with 6 ml methanol containing 50 mmol/l triethylamine, 6 ml methanol, and 6 ml water. The aqueous extract was applied to the cartridge at a flow rate of 3–4 ml/min. The glass reservoir and cartridge were rinsed with 2× 4 ml water. The cartridge was dried with high purity nitrogen. The crude analytes were eluted with 10 ml methanol containing 50 mmol/l triethylamine. The eluate was dried under a gentle nitrogen stream. The residue was dissolved by ultrasonication for 30 s with 0.5 ml chloroform, and 5 ml *n*-hexane was added. The solution then was normally passed through a Sep-Pak Silica solid phase extraction cartridge conditioning with 6 ml *n*-hexane without any pressure. Five milliliters of *n*-hexane was used to wash the interference. The analytes were eluted sequentially with 6 ml water-saturated ethyl acetate. The eluate was dried under a gentle nitrogen stream, and the residue was redissolved with 2 ml methanol–ethyl acetate (40:60, v/v). The methanol–ethyl acetate solution was applied to amino-propyl solid phase extraction cartridges conditioned with 4 ml methanol–ethyl acetate (40:60, v/v) and 4 ml water-saturated ethyl acetate. The eluate was collected and another 2 ml methanol–ethyl acetate (40:60, v/v) was used to rinse the analytes. The eluate was dried under a gentle nitrogen stream. The residue was reconstituted with 0.5 ml mobile phase and mixed in a vortex stirrer.

2.7. LC/MS/MS analysis

Identification and quantification of analytes were carried out using an Alliance 2695 (Waters Co., USA) liquid chromatograph equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer. The CAPCELL PAK Phenyl column (250 mm × 2.0 mm i.d., 5 μm) was used for LC separation. The column oven was 40 °C, the flow rate was 0.2 ml/min, and the injection volume was 15 μl acetonitrile and water were used as mobile phases. The initial composition was 35% of organic phase and 65% of water. After 3 min, the organic phase was programmed to linearly increase to 100% in 27 min and to hold for 5 min and then decrease to the initial composition in 1 min. The column was then equilibrated for 20 min.

The mass spectrometer was operated in electrospray ionization mode, and negative mode was used. The capillary voltage was held at 3.1 kV. The cone voltage was 80 V. The RF lens 1 and RF lens 2 were set at 40 and 0.5 V. The entrance and exit voltage were set to 10 and 20 V. Nitrogen was used as the nebulizing, desolvation and cone gas. The multiplier voltage was 650 V. The nebulizing gas was adjusted to the maximum, and the flow of the desolvation gas and cone gas were set to 450 and 0 l/h, respectively. The source temperature and desolvation gas temperature were held at 100 and 300 °C. The collision energy gain was 3.0. During tandem mass spectrometric analysis, UHP argon was used as the collision gas, and the pressure of the collision chamber was held at 2.8×10^{-3} mbar. The analyte retention time of hexoestrol and D_8-diethylstilbestrol was 16.5 and 16.3 min, respectively. The most sensitive mass transition was from *m/z* 269.4 to 134 for hexoestrol, from *m/z* 275.3 to 259.3 for D_8-diethylstilbestrol.

2.8. ELISA development

HES (125 ng/ml) standard solution was prepared from a 1 mg/ml stock in anhydrous acetone and was diluted to provide a series of standards containing 0, 0.05, 0.25, 1.25, 6.25, 31.25 ng/ml HES.

2.9. Antibody-coated format

Antiserum concentration and enzyme tracers dilutions with absorbencies from 0.3 to 1.2 of were chosen for further analysis. Antibody affinity was determined by measurement of the binding of serial concentrations (from 5×10^{-3} to 0.8 mg/l) of the enzyme tracer in PBST to a micro titer plate coated with different dilutions of serum (from 1:10,000 to 1:160,000). Next, plates were coated with 60 ng of the purified polyclonal IgGs diluted in coating buffer (100 mM sodium carbonate–biocarbonate, pH

9.6) overnight at 4 °C. After washing (PBS +0.05% Tween-20, pH 7.4 containing), the wells were blocked (2% OVA in PBS + 0.05% Tween-20, pH 7.4) for 2 h at 37 °C. Then wells were washed three times and 100 μl aliquots of the mixture of HES standards or urea samples and the HES-HRP as tracer were added duplicate to the assay wells. After gentle incubation (1 h, RT) and three washing cycles, the enzymatic activity of bound HRP was revealed with the addition of peroxide substrate solution (100 μl/well of 0.5 mg/ml OPD and 0.006% hydrogen peroxide in 0.15 M citrate buffer, pH 5.0) for 20 min at room temperature. The absorbance was measured at 492 nm with MuLtiska Mks microplate reader.

2.10. Optimizing of the competitive ELISA method

The assay optimizing was performed using HES as analyte. A set of experimental parameters (incubation time, surfactant concentration, ionic strength and pH of the medium) were assayed to achieve maximum sensitivity, and the minimal sensitivity is a minimal detectable dose based on the mean values added three times standard deviation from blank samples. The plates were coated with diluted antiserum in carbonate buffer, and incubated overnight at 4 °C. Several HES standard curves were run in triplicate on the same plate for each selected parameter.

Optimal concentrations for antiserum dilution and enzyme tracers were determined by checkerboard titration. The antisera dilution was 1: 80,000 and the HRP–HES concentration used was 0.1 mg/l. Once the optimum concentrations of the specific compounds of the assay system were selected, the influence of several non-specific parameters on assay characteristics was examined.

2.11. Date analysis

Using Sigmaplot software package, sigmoidal competitive curves were fitted to a four-parameter logistic equation:

$$\frac{B}{B_0} = \frac{A-D}{[1+(x/C)^B]} + D$$

where A is the asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), B the curve slope at the inflexion point, C the χ value at the inflexion point (corresponding to analyte concentration giving 50% inhibition of A_{max}, IC_{50}) and D is the asymptotic minimum (background signal).

2.12. Assessment of the specificity of the antisera

The specificity of the antisera was assessed by evaluating the extent of cross reactivity studies with structurally related compounds, such as diethylstilbestrol, dienestrol and 17β-estradiol. The cross reactivity values were calculated according to the following equation:

$$CR(\%) = \frac{IC_{50}(HES)}{IC_{50}(\text{cross-reacting compound})} \times 100$$

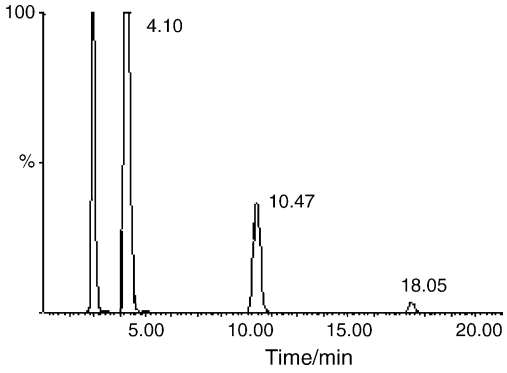

Fig. 2. HPLC illustration of solution after reaction.

2.13. Application of the ELISA and LC/MS/MS method on samples

Once the method was optimized, it was applied to determination the pork and liver samples according to the steps as described above.

3. Results and discussion

3.1. Analysis of HES and its derivatives

In the following HPLC, the retention time at 4.10, 10.47 and 18.05 min were corresponding to HES, HES-MEBE and HES-DEBE respectively which were shown in Fig. 2. The conversion rate of HES-MEBE was 23%. The characteristic ions (mass to charge ratio m/z) were 383.7 $[M-H]^-$, 297.6 $[M-(C_2H_5)_3]^-$, and 407.8 $[M+Na]^+$ for HES-MEBE, 521.8 $[M+Na]^+$, and 407.9 $[M+Na\text{-}(CH_2)_3COOC_2H_5+H]^+$ for HES-DEBE.

3.2. Antigenic of the BSA-HES conjugate

Antigenic of BSA-HES was evaluated by indirect non-competitive ELISA. The titer value for R1, R2 and R3 was 1: 640,000, 1:160,000 and 1:320,000, respectively which indicated that the BSA-HES conjugate is highly antigenic. Antiserum titer value, by definition, corresponds to the antiserum dilution resulting in uninhibited assay signal three times the background signal under given assay conditions [19]. Because R1 displayed the highest titer value our subsequent experiments were carried out with it.

3.3. ELISA optimization

3.3.1. Tween-20 effect

The influence of Tween-20 concentration on the analytical characteristics of the HES immunoassay was examined. Competitive curves with different Tween-20 concentrations, from 0.001% to 0.5%, were obtained, as shown in Fig. 3a. The general trend of A_{max} is to decrease as Tween-20 percentage increases, but the sensitivity fluctuates widely. When the concentration of Tween-20 was 0.05%, the A_{max} was 1.12 with lower IC_{50} (3.21 ng/ml) and gave the lowest background. For this reason, the addition of 0.05% Tween-20 is the best.

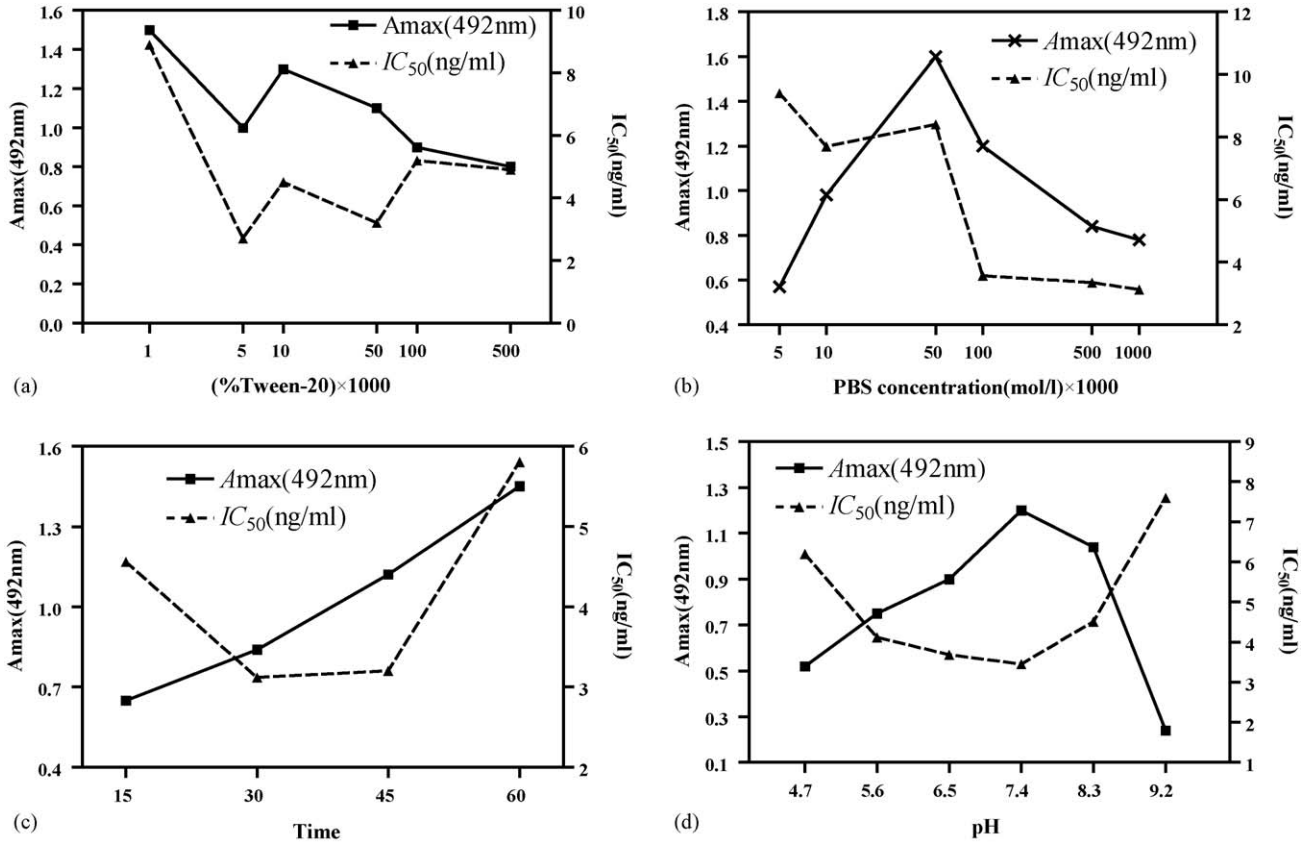

Fig. 3. (a) Influence of Tween-20 concentration on the maximum signal (A_{max}) and assay sensitivity (IC_{50}). Each point represents the mean of three replicates. (b) Influence of buffer concentration (PBS) on the maximum signal (A_{max}) and assay sensitivity (IC_{50}). Each point represents the mean of three replicates. (c) Variation of the immunoassay performance (A_{max}, IC_{50}) as a function of the competition time step. Each point represents the mean of three replicates. (d) Variation of maximum signal (A_{max}) and assay sensitivity (IC_{50}) at different pH values of the assay medium. Each point represents the mean of three replicates.

3.3.2. Ionic strength

Competitive curves were obtained using several different concentrations of PBS (from 0.005 to 1 mol/l) supplemented with 0.05% Tween-20 as assay buffer of the competitive step. As shown in Fig. 3b, the lower A_{max} value was obtained when the concentration of PBS was lower than 0.05 mol/l. In all cases, the IC_{50} value decreased as PBS concentration increased. Although the antibody obtained the highest signal when the concentration of PBS is 0.05 mol/l, the specificity is the lowest (highest IC_{50} value). While the concentration of PBS is 0.1 mol/l, the A_{max} is 1.21 with an IC_{50} value 3.5 ng/ml, which is suitable for the assay. For these reasons, the optimum concentration of PBS for HES is 0.1 mol/l.

3.3.3. Incubation time

The influence of the incubation time on the competitive step was investigated because it can affect seriously the ELISA response [20]. Standard curves at different (from 15 min to 1 h incubation times), were performed using the optimal immunoreagent concentrations as shown in Fig. 3c. In all cases, A_{max} increased and sensitivity decreased as incubation time expanded. The optimum time chosen was 45 min since there was equilibrium between A_{max} (1.12) and IC_{50} (3.2 ng/ml).

3.3.4. pH effect

In order to evaluate the effect of the pH of the medium on assay performance, PBS buffers with pH values between 4.7 and 9.2 with an increment of 0.9 pH units were prepared. These buffers were supplemented with 0.05% Tween-20. Using these buffer-detergent solutions as assay media, HES standard inhibition curves were measured in triplicates at each pH. Maximal signal intensity was shown at neutral pH (7.4). Fig. 3d indicated that the system better tolerates slightly acidic than alkaline media. Assay performance appears to be only moderately affected by changes in pH between 6.5 and 8, and has an optimum around 7.4.

3.4. Analytical parameters of the optimized ELISA

Under the optimized conditions (PBS (0.1 mol/l, pH 7.4) with 0.05% Tween-20 as assay medium, incubate 45 min), a typical competitive displacement binding curve of the HES–horseradish peroxidase complex as tracer by increasing concentrations of HES from 0.05 to 31.25 ng/ml is shown in Fig. 4. The binding curves analyzed by non-linear regression using a four-parameter logistic equation were characterized by a slope factor of 0.997 and an IC_{50} of 2.4 ng/ml, with a minimum detectable and maximum concentration of 0.07 and 30.5 ng/ml, respectively.

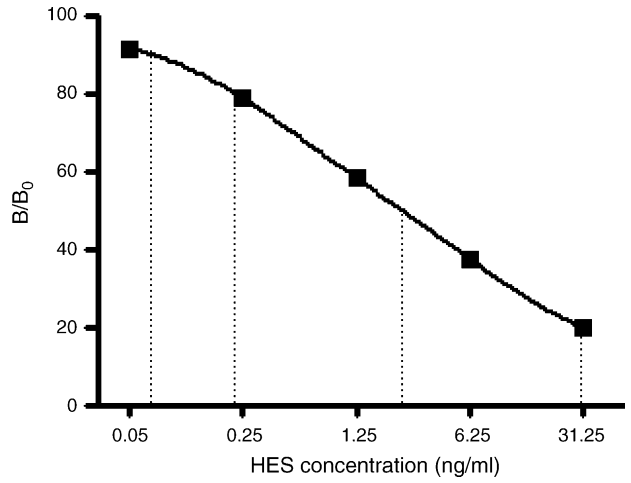

Fig. 4. Calibration curve obtained for HES using optimized antibody-coated format. Each point represents the mean ± S.D. of three plates with three replicates per plate.

Table 1
Cross reactivity of HES antisera with its analogs

Hexoestrol analog	Cross-reactivity (%)
Hexoesterol	100
Diethylstilbestrol	23
Dienestrol	<1.0
17β-Estradiol	<0.1
Zeranol	<0.1
19-Nortestosterone	<0.1
Testosterone	<0.1
Cortisol	<0.1

3.5. Assessment of the specificity of the antisera generated

The cross reactivity of the antisera generated with estrogen analogs was carried out by competitive ELISA. The ratio of the does at 50% displacement of HES relative to its analogs tested is presented in Table 1. The result showed the ratio cross reactivity of diethylstilbestrol was more than 20%, dienestrol was less than 1%, and 17β-estradiol, zeranol, 19-Nortestosterone, testosterone and cortisol was less than 0.1%. The antisera demonstrated good specificity.

3.6. Precision and accuracy

To assess the precision and accuracy, three spiked samples at low, medium and high concentrations corresponding to 0.2, 4, 8 μg/kg were studied. As shown in Table 2, the intra-assay precision (measured as CV (%)) were all below 8%, demonstrating an acceptable level of precision. By assaying the same group of spiked samples on four different days, the CV of the inter-assay was found to be less than 15%. The accuracy ranged from 104.0% to 120.0% for HES concentrations at different spiked samples. This indicated a reasonable parallelism and accuracy of the assay when it was applied to real samples. Accuracy here was evaluated by adding an increasing amounts of HES (0.2, 4, and 8 μg/kg) to pork samples, by measuring the percentage of the recovery.

3.7. Comparison with LC/MS/MS

The calibration curves for detection of the target compounds were obtained by performing a linear regression analysis on standard solution using the ratio of standard area to internal standard area (hexestrol for D_8-diethylstilbestrol) against analyte concentrations ranging from 0.50 to 500.00 μg/l containing 20 μg/l internal standard. Fig. 5 presented the chromatograms of the substances at 0.1 μg/kg of spiked level. The chromatograms indicate that even in the low level the target compounds are separated from the interference of biological extract.

Good linearity was obtained for analytes, with correlation coefficients of $r > 0.99$. The instrumental detection limits (IDL) with 10 μl injection was 0.19 pg for HES, which was estimated at a signal-to-noise (S/N) ratio of 3. The analyte recovery of this procedure was evaluated by spiking 1, 10 and 20 ng of standard analyte and 20 ng internal standard to 10 g sample at three levels

Table 2
Intra- and inter-assay accuracy and precision for HES determination in spiked pork samples

Spiked pork samples (μg/kg)	Intra-assay ($n = 4$)			Inter-assay ($n = 4$)		
	Mean ± S.D.	CV (%)	Accuracy (%)	Mean ± S.D.	CV (%)	Accuracy (%)
0.2	0.22 ± 0.02	9.1	110.0	0.23 ± 0.03	13.1	115.0
4	4.08 ± 0.11	2.7	102.0	4.16 ± 0.24	5.8	104.0
8	8.23 ± 0.13	1.6	102.9	8.57 ± 0.41	4.8	120.0

Table 3
Recoveries and precision of the LC/MS/MS method for hexestrol ($n = 6$)

Spiked Samples ($n = 6$)	Spiked level (0.1 μg/kg)		Spiked level (1 μg/kg)		Spiked level (2 μg/kg)		LOD (ng/kg)	LOQ (ng/kg)
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)		
Pork tissues	103.4	13.7	98.2	9.2	98.3	9.7	5.0	15.0
Liver tissues	96.0	16.9	83.4	10.1	92.1	13.4	20.0	50.0

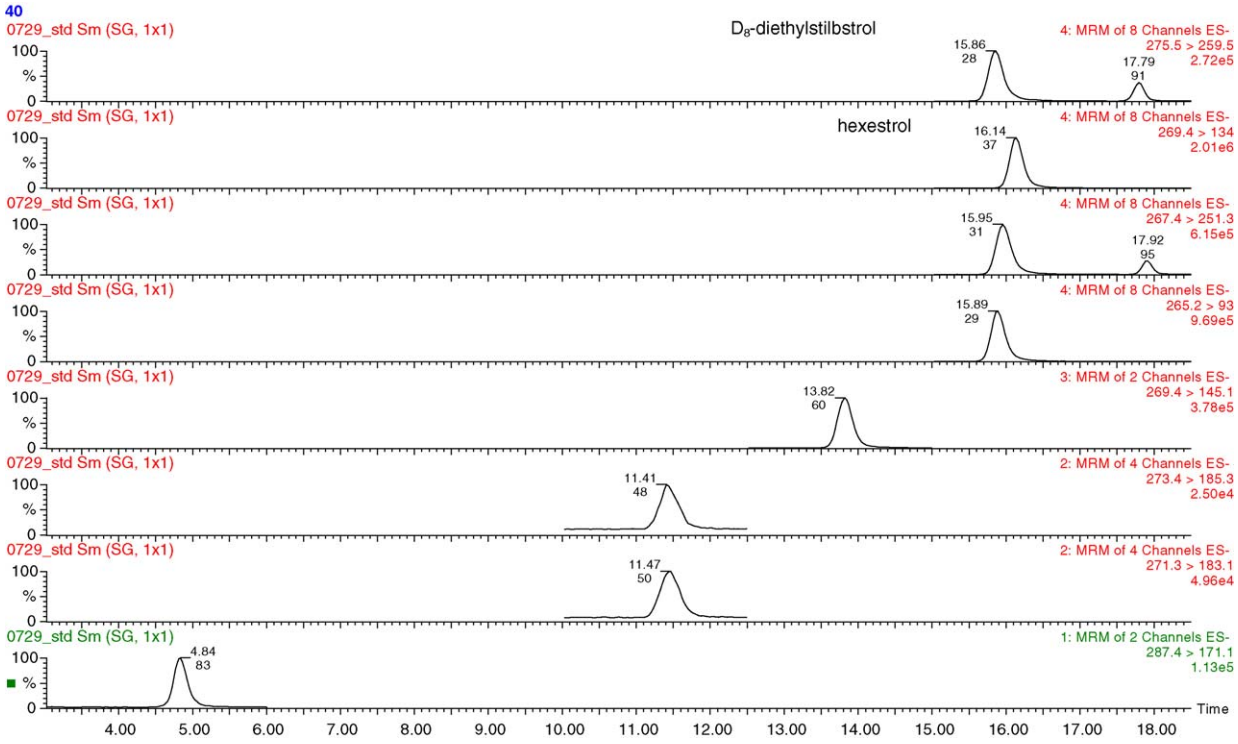

Fig. 5. LC/MS/MS chromatograms of spiked sample containing 0.1 ng/g.

Table 4
Comparative results between ELISA and LC/MS/MS for HES in pork and liver tissues

	Methods							
	ELISA				LC/MS/MS			
	Pork[a]		Liver[a]		Pork[a]		Liver[a]	
No. of determined samples	6	6	6	6	6	6	6	6
Spiked (μg/kg)	1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0
Determined (μg/kg)	0.68	1.52	0.62	1.49	0.65	1.50	0.58	1.46
C.V. (%)	8.9	11.4	10.3	12.8	9.5	12.1	9.8	13.2

[a] Samples.

in replicates of six. The results are listed in Table 3; the average recoveries ranged from 83.4% to 103.4%. The limits of detection, defined as the concentration which yield an S/N equal to 3, was 5 ng/kg for pork, 20 ng/kg for liver. The limits of quantification (LOQ), defined as the concentration which yields an S/N equal to 10, was 15 ng/kg for pork, 50 ng/kg for liver. Table 4 showed the comparative results between ELISA and LC/MS/MS for HES in pork and liver tissues samples. This method clearly demonstrated good linearity, accuracy, and precision.

4. Conclusion

We have devised a fast and reliable immunomethod based on direct competitive ELISA format, to determine HES in animal tissues samples at the 0.07 ng/ml detection limit. The method described in this paper presents advantages over the existing chromatographic techniques and allows sensitive, quick, simple assessment of HES. This assay was verified by LC/MS/MS. The two analytical methods can be applied to measure HES at subnanogram levels, and practicable for monitoring HES residues in edible foods. The immunoassay can complement chromatography techniques in field assay conditions or/and screening procedures.

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