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Development of an enzyme-linked immunosorbent assay for the determination of hexoestrol*

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ABSTRACT

A competitive enzyme-linked immunoassay (ELSIA) was developed for the quantitative detection of the hexoestrol (HES). Polyclonal rabbit antisera, raised against protein conjugate hexoestrol-mono-caroxyl-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 urine samples.

KEY WORDS: hexoestrol, ELISA, immunoassay

INTRODUCTION

Hexoestrol (HES: 3, 4-Bis (p-hydroxyphenyl) hexane; CAS RN: 84-16-2; Figure 1), together with dienestrol and diethylstilbestrol belong to the group of stilbene oestrogens. When used illegally in cattle feed, HES improved growth rate and increased feed conversion (Dinusson et al., 1950; Aitken and Crichton, 1956; Gill et al., 1956; Berg et al., 1999; Piersma et al., 2002). However, in most countries, the

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use of HES has been banned currently due to its teratogenic (Hewitt et al., 2002), mutagenic (Yu et al., 2005; Rambaud et al., 2005) and carcinogenic properties (Li et al., 1983; Li and Li 1984; Liehr et al., 1985; Jan et al., 1998). So, it is quite necessary to control its abuse.

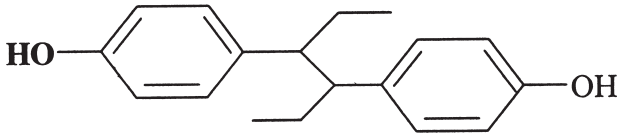

Figure 1. The structure of HES

The traditional method for the analysis of HES and other oestrogens in the present is gas chromatography (GC) with liquid chromatography (Tobioka and Kawashima 1978, 1981) and mass spectrometry (Bagnati et al., 1990; Sawaya et al., 1998; Talat et al., 1999; Leslie et al., 2003). Large-scale surveillance programs require a rapid analysis of synthetic nonsteroidal oestrogen; 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 ELSIA was applied to determine HES in bovine urine samples.

MATERIAL AND METHODS

Reagents

Hexoestrol, diethylstilbestrol, dienestrol, 17β-estradiol, *O*-phenylenediamine (OPD), complete Freund's adjuvant (CFA) and incomplet Freund's adjuvant (IFA) were obtained from Sigma (St. Lous, 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 Plan

(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).

Instruments

Vertical saturate tank, plates (10 × 20 cm) and ZF-90 dark box UV transilluminater (Gucun Apparatus Plant, Shanghai, China) were used. The LC-MS spectrometer used for analysing 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).

Synthesis and characterization of hexoestrol-MEBE derivative

The schematic principles of synthesis hexoestrol-MCPE is shown in Figure 2 as described by Winkler et al. (1971) with slight modifications (Winkler et al., 1971; Zhang et al., 1994). 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 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 concentrated evaporation

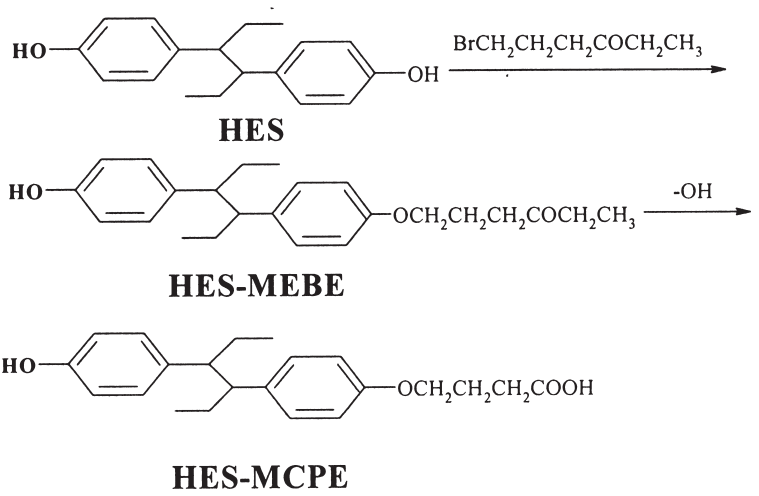

Figure 2. Principles of connecting of space-arm to HES

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× 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.

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 (1975). The hapten HES-MEBE was conjugated to BSA for forming immunogen, to OVA for coating conjugate preparation and to HRP for enzyme tracer. Briefly, approximate 15 mg of hapten were dissolved in the appropriate volume of *N*, *N*'-dimethylformamide (DMF) to the 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. Then, the mixture was centrifuged, and the supernatant 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 on 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.

Production of antisera against HES-MCPE-BSA

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.

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.

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 1:10000 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 3 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 (1h, 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.

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.

Date analysis

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

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

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

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:

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

Application of the ELISA method on real samples

Once the method was optimized, it was applied to bovine urine. Urine samples were usually analysed directly without any clean up procedure.

RESULTS AND DISSCUSION

Analysis of HES and its derivatives

In the following HPLC, the retention time at 4.10, 10.47 and 18.05 min are corresponding to HES, HES-MEBE and HES-DEBE, respectively, which were

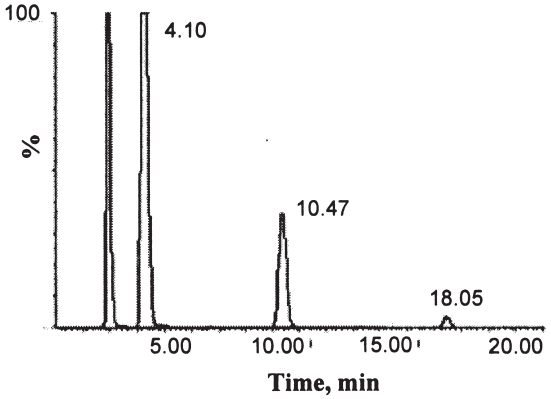

Figure 3. HPLC illustration of solution after reaction

shown on Figure 3. The conversion rate of HES-MEBE is 23 %. Mass spectrums are presented on Table 1.

Table 1. Presentation of HES and its derivates

HES and its derivates	Peak of HPLC /min	Characteristic Ions /(mass to charge ratio m/z)
HES	4.10	269.5[M-H]$^-$
HES-MEBE	10.47	383.7[M-H]$^-$ 297.6[M-($C_2H_5)_3$]$^-$ 407.8[M+Na]$^+$
HES-DEBE	18.05	521.8[M+Na]$^+$ 407.9[M+Na-$(CH_2)_3COOC_2H_5$+H]$^+$

Antigenic of the BSA-HES conjugate

Antigenic of BSA-HES was evaluated by indirect non-competitive ELISA. As show in Figure 4, the titer value for R1, R2 and R3 was 1: 640,000, 1:16, 0000 and

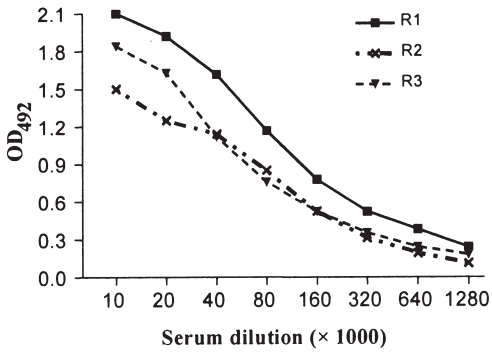

Figure 4. Titration curves obtained with 3 rabbit antisera using OVA-HES as coating antigen

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 (Szurdoki et al., 2002). Because R1 displayed the highest titer value our subsequent experiments were carried out with it.

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 Figure 5. The general trend of Amax 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.

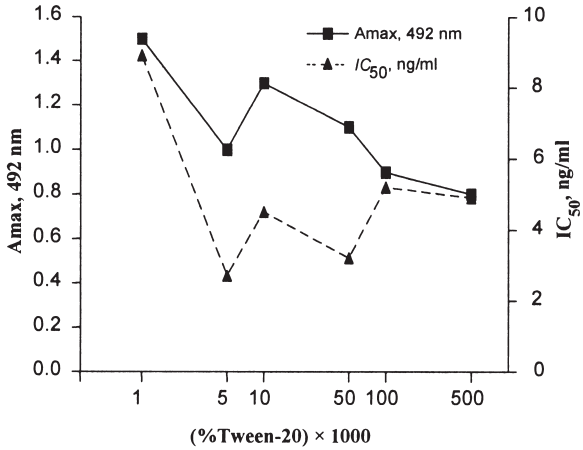

Figure 5. Influence of Tween-20 concentration on the maximum signal (*A*max) and assay sensitivity (IC_{50}). Each point represents the mean of three replicates

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 on Figure 6, 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

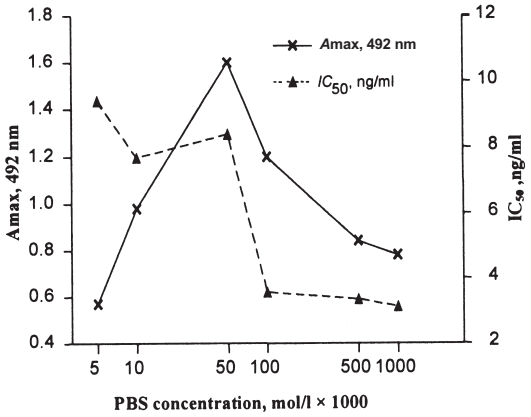

Figure 6. Influence of buffer concentration (PBS) on the maximum signal (*A*max) and assay sensitivity (IC50). Each point represents the mean of three replicates

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.

Incubation time

The influence of the incubation time on the competitive step was investigated because it can affect seriously the ELISA response (Tijssen, 1985). Standard curves at different (from 15 min to 1 h incubation times), were performed using the optimal immunoreagent concentrations as shown in Figure 7. In all cases, *A*max increased and sensitivity decreased as incubation time expanded. The optimum

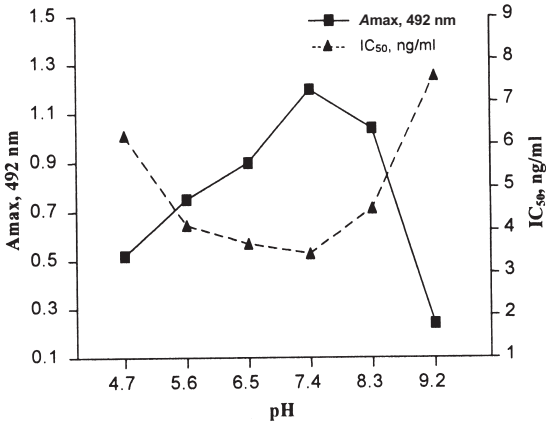

Figure 7. Variation of the immunoassay performance (*A*max, IC50) as a function of the competition time step. Each point represents the mean of three replicates

time chosen was 45 min since there was equilibrium between *A*max (1.12) and IC_{50} (3.2 ng/mL).

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. Figure 8 shows the result of pH on assay performance. Maximal signal intensity was seen at neutral pH (7.4). Figure 8 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.

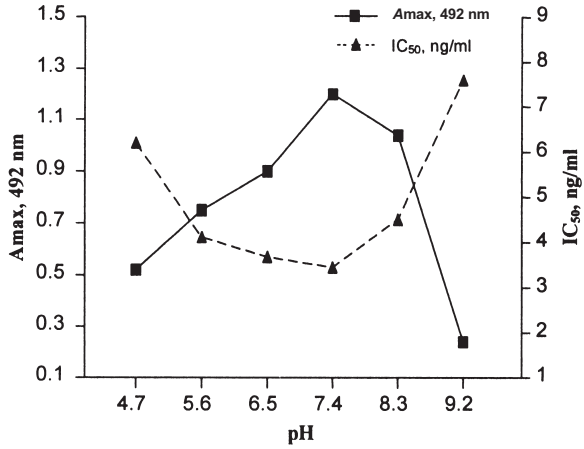

Figure 8. Variation of maximum signal (*A*max) and assay sensitivity (I50) at different pH values of the assay medium. Each point represents the mean of three replicates

Analytical parameters of the optimized ELSIA

Under the optimized conditions (PBS (0.1 mol/l, pH7.4) 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 Figure 9. The binding curves analysed 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 ng/mL and 30.5 ng/mL, respectively.

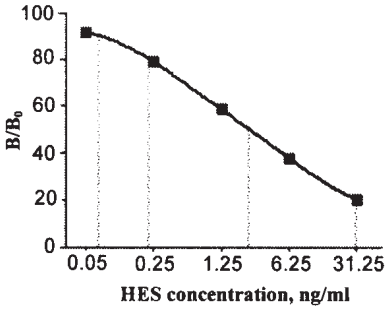

Figure 9. Calibration curve obtained for HES using optimised antibody-coated format. Each point represents the mean±SD of three plates with three replicates per plate

Assessment of the specificity of the antisera generated

The cross reactivity of the antisera generated with oestrogen 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 2. 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.

Table 2. 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

Precision and accuracy

To assess the precision and accuracy of the assay, three spiked HES in urine samples at low, medium and high concentrations corresponding to 0.2, 4, 8 ng/mL was studied. As shown in Table 3, 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. Thus indicating a reasonable

parallelism and accuracy of the assay when applied to real samples. Accuracy here was evaluated by adding an increasing amounts of HES (0.2, 4, 8 ng/mL) to urine samples, by measuring the percentage of the recovery.

Table 3. Intra-assay and inter-assay accuracy and precision for HES determination in urine samples

Spiked sample ng/mL	Intra-assay (n=4)			Inter-assay (n=4)		
	mean	CV %	accuracy %	mean	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

CONCLUSIONS

We have devised a fast and reliable immunomethod based on direct competitive ELISA format, to determine HES in bovine urine 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, and without sample manipulation. This immunoassay can complement chromatography techniques in field assay conditions or/and screening procedures, nevertheless, more studies should be performed for its application to bovine and fowl tissues.

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