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Development and evaluation of immunoassay for zeranol in bovine urine*

LIU Yuan[1], ZHANG Cun-zhen[1], YU Xiang-yang[1], ZHANG Zhi-yong[1], ZHANG Xiao[1], LIU Rong-rong[1], LIU Xian-jin[†‡1], GONG Zhen-ming[2]

([1]*Institute of Food Safety, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China*)

([2]*School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200030, China*)

[†]E-mail: jaasliu@jaas.ac.cn

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Abstract: A high affinity polyclonal antibody-based enzyme linked immunosorbent assay (ELISA) was developed for the quantification of zeranol in bovine urine. On the basis of urine matrix studies, the optimized dilution factors producing insignificant matrix interference were selected as 1:5 in pretreatment. In the improved ELISA, the linear response range was between 0.02 and 1 μg/ml , and the detection limit was 0.02 μg/ml for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 82%~127% and 3.5%~8.8%, respectively. Thirty-six bovine urine samples spiked with zeranol (ranging from 0.2 to 10 μg/ml) were detected by the ELISA and liquid chromatography (LC) method, and good correlations were obtained between the two methods (R^2=0.9643). We conclude that this improved ELISA is suitable tool for a mass zeranol screening and can be an alternative for the conventional LC method for zeranol in bovine urine.

Key words: Zeranol, Enzyme linked immunosorbent assay (ELISA), Bovine urine
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INTRODUCTION

Zeranol [2,4-dihydroxy-6-(6α,10-dihydroxyundecyl) benzoic acid μ-lactone] (ZER) is a semi-synthetic oestrogenic growth-promoting agent banned in EU and China (Tuomola *et al*., 2002; Wang *et al*., 2004). It is generated by chemical reduction of zearalenone, a mycotoxin derived from *Fusarium* spp. (Richardson *et al*., 1985). Zeranol is implanted in cattle for promoting rapid weight gains (Daniel and Robert, 1983). However, the residue of zeranol can exert estrogenic effects on mammalian endocrine system (Baldwin *et al*., 1983). Therefore, reliable analytical methods are required to monitor zeranol residue.

Various techniques have been developed for the detection of zeranol including gas chromatography/mass spectrometry (GC-MS) (Dickson *et al*., 2003), high performance liquid chromatography/mass spectrometry (HPLC-MS) (Sorensen and Elbaek, 2005; Fang *et al*., 2002; Taguchi *et al*., 2001; Horie and Nakazawa, 2000) and immunoassay (Dioxn and Russell, 1983; Daniel and Robert, 1983; Jansen *et al*., 1986). Immunoassay is a good alternative to instrumental analysis for the determination of veterinary residues in various samples. However, the presence of impurities in the sample can affect the results of analysis. In previous studies, immunoaffinity chromatography columns and C18 columns were used for cleanup of bovine urine before applied in immunoassay (Tuomola *et al*., 2002; Wang *et al*., 2004), which increases the cost and time of detection. Here we study the urine matrix effects to

‡ Corresponding author
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immunoassay, report an improved enzyme linked immunosorbent assay (ELISA) method for determination of zeranol in bovine urine without any pretreatment other than dillution, and compare the results of this ELISA method with the conventional HPLC approach.

MATERIALS AND METHODS

Reagents and instruments

Anti-ZER antiserum was developed by immunizing New Zealand white rabbits with the conjugate of carboxypropyl ether derivatives at C-16 (16-CPE) of zeranol and the immunogenic carrier protein bovine serum albumin (BSA) (Liu *et al*., 2006; Dioxn and Russell, 1983). The coated antigen ZER-ovalbumin (ZER-OVA) was synthesized using the mixed anhydride method (Chen *et al*., 1990). Drug-free urine was obtained from the dairy of the Jiangsu Academy of Agricultural Sciences, China. Zeranol (97%, w/w) was provided by Guilin Jiqi Biotech Co., Ltd., China. Goat antirabbit IgG-horseradish peroxidase, tetramethylbenzidine (TMB), zearalenone and β-glucuronidase were all purchased from Sigma Chemical Co., USA. All other chemicals used in the present study were of analytical grade.

The main instruments include HPLC (Agilent 1100, USA), Multiskan Ascent 1.0 (Thermo), Wellwash plus (Thermo, USA), and 96-well polystyrene microplates (Corning, USA).

Preparation of zeranol standard and sample pretreatment

Stock solutions (200 μg/ml) of zeranol and zearalenone were prepared in methanol and stored at 4 °C in the dark. Zeranol standard solutions (0.002~20 μg/ml) were diluted from the 200 μg/ml stock solution with 10% methanol-phosphate buffer saline (PBS, 50 mmol/L, pH 7.4) for ELISA in buffer. Drug-free urine was spiked with zeranol from stock solution in methanol to reach defined concentrations.

After the spiked urine samples were hydrolyzed with 500 units β-glucuronidase per 10 ml, the hydrolyzed samples were then subjected to the improved ELISA without any pretreatment other than dilution with 10% methanol-PBS. For HPLC determination, the spiked urine samples were extracted with 40 ml of dichloromethane. The organic layer was evaporated under a stream of nitrogen at 50 °C. The residue was re-dissolved with 10 ml of methanol and directly analyzed by HPLC.

ELISA procedure

Competitive indirect ELISA (CI-ELISA) (Dong *et al*., 2001) was applied. Briefly, the standard solutions or pretreated samples were individually added to 1/5000 dilution antiserum in 1:1 volume ratio, and they were incubated overnight at room temperature. On the following day, ELISA plates were coated with 2 μg/ml ZER-OVA (100 μl/well) in carbonate-bicarbonate buffer saline (CBS, 50 mmol/L, pH 9.6) and incubated for 2 h at 37 °C. The plates were washed three times with PBST (PBS containing 0.05% Tween-20, pH 7.4) by automated microplate washer and were blocked by incubating with 1% OVA in PBS (200 μl/well) for 1 h. After another washing step, 100 μl/well of the mixture of analyte and antiserum was added. After incubation for 2 h, the plates were washed. Subsequently, 100 μl/well of a diluted goat antirabbit IgG-horseradish peroxidase was added and incubated for 1 h, and after another washing step, 100 μl/well of a tetramethylbenzidine (TMB) solution (120 μl of 10 mg/ml TMB-DMSO and 30 μl of 0.65% (v/v) H_2O_2 diluted with 11.85 ml of citrate-acetate buffer (CPBS), 25 mmol/L citrate and 62 mmol/L sodium phosphate, pH 5.5) was added. The reaction was stopped after 15 min by adding 50 μl/well of 2 mol/L H_2SO_4, and absorbance was read by a microplate reader at 450 nm.

HPLC procedure

The HPLC separation was performed on Waters Sphrisorb® S5 ODS (USA) (4.6 mm×20 mm analytical column) using acetonitrile-water (40:60, v/v) as the mobile phase. The flow-rate is 1.2 ml/min and UV detection at 262 nm.

Data analysis

Dose-response curves were analyzed by using software Sigmaplot 6.0. ELISA mean absorbance values (Y) of standards were plotted against the logarithm of analyte concentrations (X) and fitted to the following four-parameter logistic equation (Wortberg *et al*., 1995):

$$Y=D+(A-D)/(1+10^{(C-X)}B),$$

where A is the high asymptote, B is the slope, C is the IC_{50}, and D is the low asymptote.

Note that the concentrations in the calibration curve were referred to actual concentrations of the analyte in the assay. For example, in Fig.1 the practical spiked concentrations of zeranol in the standards were 0.002~20 μg/ml. In the next CI-ELISA, the diluted urine was pre-mixed with antiserum of equal volume. The final zeranol concentrations in the assay were between 0.001 and 10 μg/ml.

RESULTS AND DISCUSSION

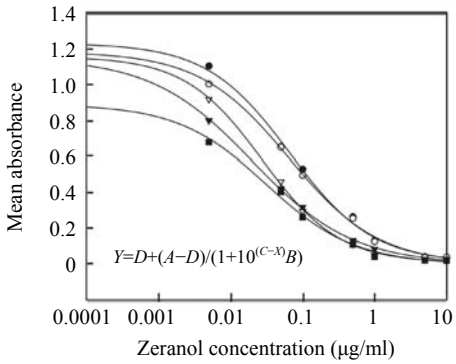
Study of bovine urine matrix effects

Initially in the present study, the tolerance to methanol in the zeranol ELISA was investigated. The results show that 10% methanol in PBS did not significantly affect the calibration curve but attributed to dissolubility of zeranol in buffer. Therefore, we selected 10% methanol-PBS as the basic working buffer for ELISA. Subsequently, results of ELISA establishment experiments for standards in buffer showed that the zeranol response ranged from 0.018 to 1.744 μg/ml (calculated as the concentrations of analyte giving 20%~80% inhibition), and the detection limit was 0.008 μg/ml (10% of the highest absorbance, I_{10}). The I_{50} (50% of the highest absorbance) value of the ELISA was 0.179 μg/ml and cross reactivity of the antibodies with zearalenone was 1.35%.

To determine the influence of the bovine urine matrix on the ELISA, zeranol standard curves were established in 10% methanol-PBS and in 10% methanol-urine, and quality control (QC) spikes (0.02, 0.5 and 1 μg/ml) in the working range were fortified. As a result, the urine matrix depressed the response of the assay from a maximum absorbance of approximately 1.2 optical density (OD) units in PBS to around 0.8 OD units in urine. The recoveries of zeranol in bovine urine were between 139% and 170% and the coefficients of variation (CVs) were between 1.8% and 9.0%. The high level of zeranol in ELISA was attributed to interferences from the urine matrix, suggesting that either sample preparation or sample dilution is needed.

Determination of dilution factors of urine for ELISA

Matrix effects are quite common in applications of immunoassay. Since the method usually requires no cleanup pretreatment for aqueous samples, appropriate dilution factors producing insignificant matrix interference should be tested. Fig.1 shows the calibration curves with different urine concentrations. The inhibition curves of zeranol (0~10 μg/ml) were constructed using 0%, 20%, 30%, 50% and 70% urine in 10% methanol-PBS, assayed in the same plate. The concentrations of zeranol in the assay were 0, 0.005, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml. Three levels of zeranol (0.02, 0.5 and 1 μg/ml) were also spiked in these solutions to study the accuracy and precision in different urine concentrations.



	A	*B*	*C*	*D*	*R*
● 0% urine (10% methanol-PBS)	1.237	0.751	0.066	0.008	0.998
○ 20% urine (10% methanol-PBS)	1.189	0.696	0.064	0	0.999
▽ 30% urine (10% methanol-PBS)	1.162	0.807	0.027	0.007	0.999
▼ 50% urine (10% methanol-PBS)	1.152	0.620	0.020	0	0.999
■ 70% urine (10% methanol-PBS)	0.894	0.706	0.031	0	0.998

Fig.1 Calibration curves for zeranol with 0% (•), 20% (○), 30% (▽), 50% (▼) and 70% (■) of urine concentration in 10% methanol-PBS

The max absorbance is around 1.2 for 10% methanol-PBS, but around 0.9 for 70% concentration blank urine. The mean absorbance decreased along with the increase of the bovine matrix concentration. It seems to be a negative correlation between the two factors, suggesting that the interfering component in urine reduced the abilities of binding antibodies to immobilized antigens. For three QC spike levels (0.02, 0.5 and 1 μg/ml), satisfactory recoveries were obtained, from 84% to 117% with urine concentrations

from 0% to 30%, and the CVs were between 3.5% and 10.3%. However, with the urine concentrations from 50% to 70%, the recoveries and CVs were 76%~137% and 4.6%~13.8%, respectively. So the urine concentration from 10% to 30% has little interference with the assay maximum absorbance and reliability. Finally, the 20% urine concentration was selected for the improved ELISA method (The urine dilution factor producing insignificant matrix interference was 1:5 in pretreatment).

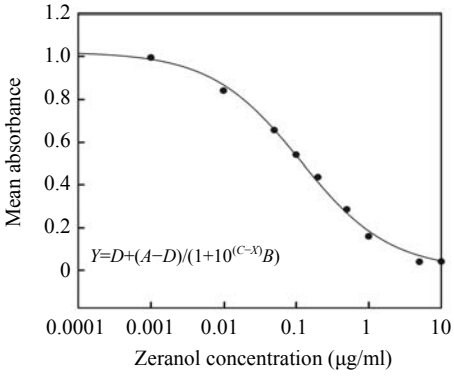
Improved ELISA method for zeranol in urine

As shown in Fig.2, an improved standard curve with the zeranol concentrations range from 0.001 to 10 μg/ml in the assay was established with 20% urine in 10% methanol-PBS. The zeranol concentration that reduced 50% of the highest absorbance (I_{50}) was approximately 0.115 μg/ml. To more accurately determine the linear response range of the ELISA curve, urine samples fortified at 8 different levels of zeranol were analyzed in three separate assays conducted on different days (Table 1). When the fortified concentration in the assay ranged from 0.02 to 1 μg/ml, the mean recoveries were between 82% and 127%, and the CVs were between 3.5% and 8.8%. However, when the fortified concentration in the assay was 0.01 μg/ml, the recovery was very poor (58%). This result confirmed that the analyte concentration in the assay (0.01 μg/ml) was too low for a reliable determination. The QC spikes (5, 6 and 7.5 μg/ml) were also out of the working range of the standard curve. The overall recoveries and CVs were in the range of 119%~178% and 10.4%~21.6%, respectively. In summary, the linear response range was between 0.02 and 1 μg/ml, and the detection limit was 0.02 μg/ml for the assay.

Table 1 Accuracy and precision of the zeranol ELISA for fortified urine

Spike conc. (g/ml)	Recovery (%)			CV (%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
0.01	58	73	69	7.8	6.8	4.3
0.02	87	82	78	4.1	4.8	3.7
0.05	101	116	109	4.2	4.5	3.8
0.20	113	107	121	3.5	7.2	5.7
1.00	117	127	102	4.8	8.8	4.2
5.00	178	155	128	12.9	14.4	21.2
6.00	145	172	133	20.3	18.8	10.4
7.50	119	138	169	21.6	14.3	19.2

Data were obtained from six determinations (n=6); CV: Coefficient of variation



	A	B	C	D	R
● Calibration curve with 20% urine in 10% methanol-PBS	1.024	0.705	0.115	0	0.998

Fig.2 Improved calibration curve for zeranol with 20% urine in 10% methanol-PBS

Comparison of ELISA and HPLC data

The reliable HPLC method for zeranol was established in our lab previously (Fang *et al*., 2003) (Fig.3). The detection limit based on the method validation was 0.1 μg/ml and the working range between 0.1 and 10 μg/ml. The standard curve was:

$$Y=25.213X-0.596 \quad (R^2=0.999).$$

To determine the correlation between the ELISA and HPLC methods, 36 urine samples spiked with zeranol (ranging from 0.2 to 10 μg/ml) were detected by the two methods.

Fig.4 displays the correlation between the ELISA and HPLC data of 36 spiked urine samples. In general, there appears to be good correlation between the ELISA and HPLC methods in this range (R^2=0.9643). And the recoveries of zeranol by ELISA and HPLC in urine samples were 72%~126% and 93%~107%, respectively. A comparison between the ELISA and HPLC methods (in terms of accuracy, precision, sample preparation and sample throughput) is described in Table 2. The ELISA and HPLC methods have similar response ranges and detection limits. Although the ELISA method was not as good as HPLC method in terms of detection accuracy and precision, it has some advantages, such as cost-effective and more rapid detection for a large number of samples.

Table 2 Comparison of ELISA and HPLC methods for zeranol in urine[a]

	Recovery (%)	CV (%)	Quantification limit (μg/ml)	Sample preparation	Sample throughput
ELISA	72~126	3.9~13.2	0.2[b]	Hydrolyzation and dilution	Around 22 samples for 8 h
HPLC	93~107	3.6~6.8	0.1	Hydrolyzation and organic extraction	10 samples for 8 h

[a]Data were obtained from three determinations (n=3); [b]The quantification limit of ELISA for zeranol is 0.02 μg/ml, but considering the dilution factors (1:5 in pretreatment, 1:10 in the assay) to the urine, the practical detection limit of ELISA in urine is 0.2 μg/ml

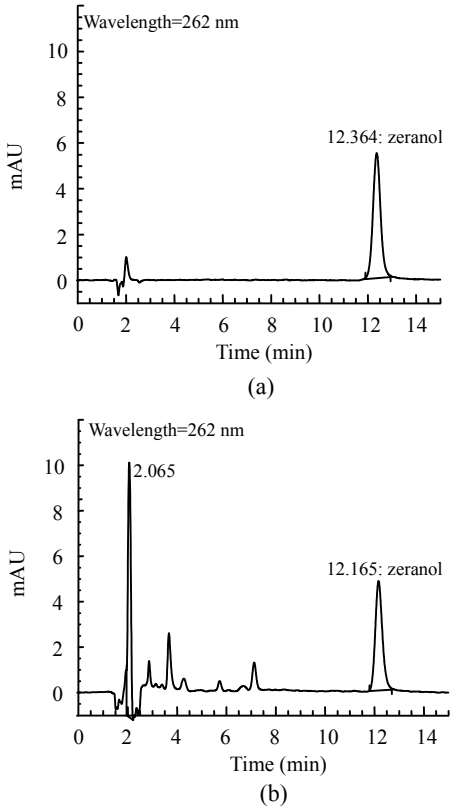


Fig.3 HPLC of (a) a standard solution of zeranol (5 μg/ml) and (b) a fortified urine sample extracted with dichloromethane (5 μg/ml)

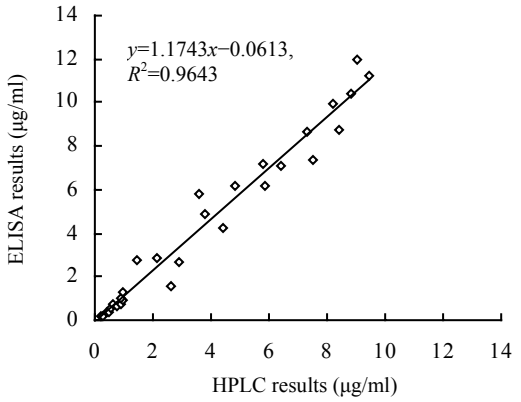


Fig.4 Comparison of ELISA and HPLC results for 36 spiked samples

CONCLUSION

It is concluded that the ELISA method developed in the present study is reliable and relatively economical and rapid for screening zeranol residues in bovine urine, so it could be used as an alternative or supplement for the conventional HPLC method to monitor the zeranol residue, especially for the routing screening of a large number of urine samples.

References

Baldwin, R.S., Williams, R.D., Terry, M.K., 1983. Zeranol: a review of the metabolism, toxicology, and analytical methods for detection of tissue residues. *Regul. Toxicol. Pharmacol.*, **3**(1):9-25. [doi:10.1016/0273-2300(83)90046-6]

Chen, X.J., Liu, H.C., Meng, F.J., 1990. Direct enzyme-linked immunoassay for zearalenone. *Plant Physiol. Commun.*, **16**(1):70-76 (in Chinese).

Daniel, T., Robert, F.M., 1983. Radioimmunoasssay for zearalenone and zearalanol in human serum: production, properties and use of porcine antibodies. *Appl. Environ. Microbiol.*, **45**(1):16-23.

Dickson, L.C., MacNeil, J.D., Reid, J., Fesser, A.C., 2003. Validation of screening method for residues of diethylstilbestrol, dienestrol, hexestrol, and zeranol in bovine urine using immunoaffinity chromatography and gas chromatography/mass spectrometry. *J. AOAC Int.*, **86**(4):631-639.

Dioxn, S.N., Russell, K.L., 1983. Radioimmunoassay of the anabolic agent zeranol II. Zeranol concentrations in urine of sheep and cattle implanted with zeranol (Ralgro). *J. Vet. Pharmacol. Therap.*, **6**:173-179.

Dong, J., Liu, X.J., Han, Z.J., 2001. Hapten design and antibodies preparation of fipronil. *Jiangsu J. Agric. Sci.*, **17**(3):172-175 (in Chinese).

Fang, X., Chen, J., Guo, D., Wang, G., 2002. Detection and identification of zeranol in chicken or rabbit liver by liquid chromatography-electrospray tandem mass spectrometry. *J. AOAC Int.*, **85**(4):841-847.

Fang, X.M., Chen, J.H., Tang, Y.F., 2003. Determination of zeranol in chicken livers by high performance liquid chromatography with ultraviolet detection. *Chin. J. Chromatogr.*, **21**(2):158-161 (in Chinese).

Horie, M., Nakazawa, H., 2000. Determination of trenbolone and zeranol in bovine muscle and liver by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A*, **882**(1-2):53-62. [doi:10.1016/S0021-9673(00)00205-3]

Jansen, E.H.J.M., Vanden Berg, R.H., Zomer, G., Willemsen, C.E., Stephany, R.W., 1986. A chemiluminescent immunoassay for zeranol and its metabolites. *J. Vet. Pharmacol. Therap.*, **9**:101-108.

Liu, Y., Liu, X.J., Yu, X.Y., Zhang, C.Z., Gong, Z.M, Zhou, Q., 2006. Synthesis of artificial antigen and preparation of polyclonal antibodies against zeranol. *J. Anal. Sci.*, **22**(1):1-4 (in Chinese). [doi:10.2116/analsci.22.1]

Richardson, K.E., Hagler, W.M., Mirocha, C.J., 1985. Production of zeralenone, α- and β-zearalenol and α- and β-zearalanol by *Fusarium* spp. in rice culture. *J. Agric. Food Chem.*, **33**(5):862-866. [doi:10.1021/jf00065a024]

Sorensen, L.K., Elbaek, T.H., 2005. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **820**(2):183-196. [doi:10.1016/j.jchromb.2005.03.020]

Taguchi, S., Yoshida, S., Tanaka, Y., Hori, S., 2001. Simple and rapid analysis of trenbolone and zeranol residues in cattle muscle and liver by stack-cartridge solid-phase extraction and HPLC using on-line clean-up with EC and UV detection. *Shokuhin Eiseigaku Zasshi*, **42**(4):226-230 (in Japanese).

Tuomola, M., Cooper, K.M., Lahdenperä, S., Andrew Baxter, G., Elliott, C.T., Glenn Kennedy, D., Lövgren, T., 2002. A specificity-enhanced time-resolved fluoroimmunoassay for zeranol employing the dry reagent all-in-one-well principle. *Analyst*, **127**:83-86. [doi:10.1039/b108671p]

Wang, H.J., Chao, Z., Sheng, J.Z., 2004. Preparation and identification of monoclonal antibody to zeranol. *Chin. J. Vet. Med.*, **40**(10):3-5 (in Chinese).

Wortberg, M., Kreissig, S.B., Jones, G., Rocke, D.M., Hammock, B.D., 1995. An immunoarray for simultaneous determination of multiple triazine herbicides. *Analytica Chimica Acta*, **304**(3):339-352.