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Determination of Ractopamine in Swine Feed and Urine Using an Indirect Competitive Immunoassay

[1]Pingli He, [1]Liying Zhang and [2]Tangbin Yang
[1]National Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100094, China
[2]Laboratory of Space Cellular and Molecular Biology, Institute of Space Medico-Engineering, Beijing 100094, China

Abstract: Anti-ractopamine polyclonal antisera were prepared by coupling ractopamine to bovine thyroglobulin and human serum albumin using two different methods. A systematic study of cross-reactions with eleven related compounds showed that the antibodies had a high specificity for ractopamine. Using the purified antiserum R3, an indirect competitive immunoassay for the determination of ractopamine in swine feed and urine was established and a typical competition calibration curve showed good sensitivity of 0.8 ng mL^{-1}, estimated as the value of IC_{50}, with a practical working range between 0.2 and 3 ng mL^{-1}, the limit of detection of 0.15 ng mL^{-1}. Finally, this assay was applied to the analysis of ractopamine in spiked swine feed and urine. The average recoveries of ractopamine were between 80-120% and the coefficients of variations were less than 10%. The results were also confirmed by liquid chromatography-mass spectrometry, which showed good agreement ($r^2 = 0.997$) with the established immunoassay.

Key words: Ractopamine, ELISA, liquid chromatography-mass spectrometry, feeds, urine

INTRODUCTION

β-agonists are related to catechol amines (Antignac *et al*., 2002). These compounds are phenyl ethanolamines with different substituents on the aromatic ring and on the terminal amino group. The family of β-agonists includes compounds such as clenbuterol, mabuterol, cimaterol, zilpaterol and salbutamol. In the livestock industry, β-agonists have been used as repartitioning agents and a large body of research has shown that β-agonists reduce carcass fat and increase muscle mass while improving growth rate and feed conversion when fed to calves (Andersom *et al*., 1989), pigs (Watkins *et al*., 1990) and poultry (Wellenreiter and Tonkinson, 1990).

Ractopamine is a β-agonist belonging to the phenolic group. This compound was developed commercially in the United States where it is authorized for use with cattle and swine (FDA, 2001). It is sold commercially under the brand names Optaflexx® and Paylean® (Eli Lilly and Company, Indianapolis, Indiana). However, in the Euopean Union and China the use of ractopamine is completely banned (Blanca *et al*., 2005).

The presence of drug residues in animal tissues is a food safety concern. Meat products obtained from agonist-fed animals may pose a potential risk to consumers' health, particularly to persons with muscular tremors, vomiting, nervousness and cardiac palpitations (Mitchell and Dunnavan, 1998; Martinez-Navarro, 1990). In addition, drug residues may negatively impact the export trade of edible animal products that result in nearly incalculable economic loss.

In an effort to combat the illicit use of β-agonists and related compounds, regulatory organizations worldwide are testing animal tissues and excreta for the presence of drug residues (Kuiper *et al*., 1998). Various analytical methods have been reported for the determination of ractopamine residues and its metabolites in animal tissues and body fluids. These include liquid chromatography with electrochemical detection (Turberg *et al*., 1995; 1996), liquid chromatography with mass spectrometry (Dickson *et al*., 1995; Shishan *et al*., 2003; Churchwell *et al*., 2002), gas chromatography with mass spectrometry (Bocca *et al*., 2003), immunoassay with polyclonal or monoclonal antibodies (Haasnoot *et al*., 1994; Wang *et al*., 2006; Elliott *et al*., 1998; Shelver and Smith, 2002, 2000; Shelver *et al*., 2000b) and immunosensor with surface plasmon resonance (Shelver and Smith, 2003).

Corresponding Author: Pingli He, National Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100094, China

Enzyme-linked immunosorbent assays (ELISAs), because of their high sensitivity, high throughput and rapid turnaround time, are the most convenient screening tools to detect the presence of an analyte in various matrices. In the present research, we describe the development and characterization of a polyclonal antibody-based ELISA against ractopamine. It showed very high sensitivity for ractopamine and was used for the detection and quantitative determination of trace amounts of ractopamine in spiked animal feed and swine urine samples. Moreover, a new pretreatment method was developed to optimize the extraction and purification for spiked feed and urine samples. In addition, a Liquid Chromatography-Mass Spectrometry (LC-MS) procedure was also developed which was able to act as a confirmatory procedure for the ELISA results.

MATERIALS AND METHODS

Reagents and chemicals: Ractopamine hydrochloride was obtained from the National Feed Quality Evaluation Center (Beijing, China). Human serum albumin (MW 66000), bovine thyroglobulin (BTG, MW 670,000), Freund's complete and incomplete adjuvants and *o*-phenylenediamine were obtained from Sigma Company (St Louis, MO). Goat anti-rabbit IgG-horseradish peroxidae was obtained from Jackson Immuno-Research Laboratories (West Grove, PA). HPLC grade acetonitrile was obtained from Fisher Scientific International (Hampton, NH). Hydrogen peroxide (H_2O_2, 30%) was obtained from Beijing Chemical Engineering Plant (Beijing, China). All other chemicals were analytic grade.

Each liter of phosphate buffer contained 8 g NaCl, 0.2g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 and was adjusted to pH 7.4 with 0.2 M Na_2HPO_4 (Shelver *et al*., 2000a). The substrate solution containing *o*-phenylenediamine (OPD/H_2O_2) was prepared by dissolving 4 mg of *o*-phenylenediamine in 10 mL of 0.1 M citrate/phosphate buffer (pH 5.0) and adding 2 μL of 30% H_2O_2. Phosphate acid-methanol extraction solution (0.2 M) was prepared by dissolving 3.92 g phosphate acid in 200 mL water and adding methanol to 1 L. All water used was deionized water (Milli-Q, Millipore Corporation, Bedford, MA).

New Zealand White rabbits were purchased from the Institute of Genetics and Developmental Biology Chinese Academy of Sciences (Beijing, China).

Apparatus and procedures: The absorbance values for the ELISA were read in dual-wavelength mode (492-570 nm) using a TECAN SUNRISE Microplate Reader (Salsburg, Austria). Liquid chromatography-mass spectrometry (LC-MS) analysis was performed on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA) equipped with a Symmetry® C_{18} column (2.1 mm× 150 mm; 3.5 μm) coupled to a Macromass ZQ2000 Mass Spectrometer (Manchester, UK). OASIS® MCX columns (1cc), used for purification of samples, were purchased from Waters Corporation (Milford, MA). The solid phase extraction system was a vacuum manifold processing station obtained from Agilent Technologies (Palo Alto, CA).

Preparation of antigens: The immunogens were synthesized as described by Shelver *et al* (Shelver *et al*., 2000). Briefly, ractopamine HCl was reacted with glutarate anhydride (1:1 molar ratio) in the presence of pyridine. The reaction was stirred at room temperature for 20 h and then evaporated to dryness under a stream of nitrogen. The ractopamine hemiglutarate (0.1 mM), produced above, was dissolved in 4 mL of dimethylformamide and 1,4-dioxane (1:1, v/v) and then 26.2 μL (0.11 mM) of tributylamine was added. The mixture was stirred on ice for 10 min and then isobutylchloroformate (0.11 mM) was added and the reaction was brought to room temperature and stirred for 1 h. This mixture was added dropwise to an ice-cold bovine thyroglobulin solution (50 mg BTG dissolved in 1 mL of 0.1 M sodium borate, pH 8.5). The final solution was brought to room temperature and allowed to react overnight.

The synthesis of coating antigen followed the procedures of Elliott *et al*. (1998). Human serum albumin (30 mg) was dissolved in 0.5 mL of distilled water and the pH was adjusted to 10.8. A 6 μL solution of butane-1,4-diol diglycidyl ether was added and the solution was incubated at ambient temperature for 20 h. Ractopamine-HCl (15 mg) was dissolved in 0.5 mL of 0.1 M sodium hydorxide. Dimethylformamide (200 μL) was added to increase solubility and this mixture was then added to the epoxy-activated human serum albumin solution. The reaction mixture was incuated at ambient temperature for 20 h.

All reactions were conducted under a nitrogen atmosphere and the synthesized antigens were dialyzed against phosphate buffer and stored at -20°C, under desiccation, until needed.

Polyclonal antibody production: Four New Zealand White rabbits were used to produce antibody. Each rabbit was immunized subcutaneously with 500 μg of the ractopamine-bovine thyroglobulin immunogen, emulsified in Freund's complete adjuvant. Four weeks after the initial dose, 3 other booster shots were administered at 2 week intervals, using the half dose but emulsified in Freund's incomplete adjuvant. Ten days after the last

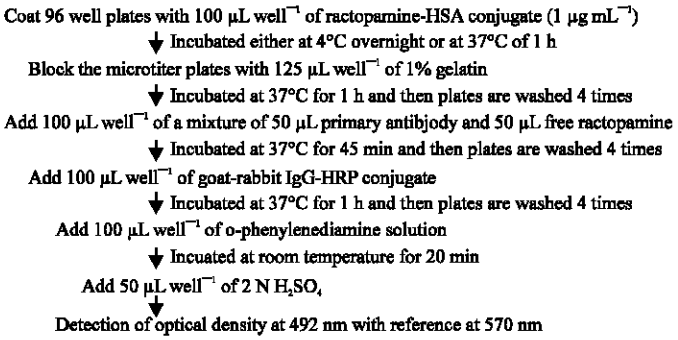

Fig. 1: Flow chart of competitive ELISA for free ractopamine

injection, blood samples were collected directly from the heart and were centrifuged at 4000 rpm for 20 min. The antibody was purified from the antiserum by ammonium sulfate precipitation. The purified protein concentration was determined using a BCA™ Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions and the purity of the antibody was confirmed by 10% SDS-PAGE, conducted under reducing conditions.

Enzyme immunoassay procedure: The concentrations of coating antigen (0.125, 0.25, 0.5, 1 and 2 μg mL^{-1}) and the dilution of purified polyclonal antibody (serial dilutions from 1:4000 to 1:512,000, with a dilution factor of 2) were optimized by a checkerboard procedure. A competition ELISA as shown in Fig. 1 was utilized to measure ractopamine binding and cross reactivity to related compounds. A competition calibration curve was prepared using serial dilutions ranging from 0.10-100 ng mL^{-1} of free ractopamine. The resulting curves were fitted with a four-parameter logistic equation to determine the IC_{50}, which was defined as the concentration (ng mL^{-1}) of competitor which produced 50% of the absorbance when no competitor was used.

Preparation of feed and urine samples: Stock standard solutions (1 mg mL^{-1}) were prepared by dissolving 10 mg of ractopamine in 10 mL of methanol. A commercially prepared feed sample typically used for growing-finishing pigs (based on corn and soybean meal) was made at the Pilot Workshop of the Ministry of Agricultural Feed Industry Center (Beijing, China). The feed sample was spiked with free ractopamine dissolved in methanol at concentrations of 0.1, 0.2, 1, 2, 10 and 20 mg kg^{-1} and mixed to maintain uniformity. Urine samples obtained from swine that had not been fed β-agonists were filtered through qualitative filter paper and centrifuged at 3000 rpm for 15 min. A 10 mL aliquot of the supernatant was spiked with ractopamine at concentrations of 0.5, 1.0, 5.0, 10.0 and 20.0 ng mL^{-1}. All spiked feed and urine samples were freshly prepared immediately before analysis.

Extraction of feed and urine samples: A 5 g sample of finely ground (1 mm) feed, spiked with ractopamine, was accurately weighed into a 50 mL polytetrafluoroethylene tube and 40 mL of freshly prepared phosphate acid-methanol extraction solution (0.2 M) was added. The mixture was shaken vigorously for 30 minutes and was then centrifuged at 3000 rpm for 10 min. The supernatant was placed into a 100 mL volumetric flask. The residue was extracted using 40 mL of the phosphate acid-methanol solution immediately followed by an extraction using 20 mL of the same solution. The supernatant was collected and made up to 100 mL by the addition of the extraction solution. A 1 mL aliquot of the supernatant was decanted into a 5 mL tube and the solution was evaporated under a stream of nitrogen in a water bath at 55°C. The extract was reconstituted in 1 mL of 2% acetic acid solution and purified using solid phase extraction.

A 10 mL sample of swine urine, spiked with ractopamine, was diluted with 40 mL of phosphate acid-methanol extraction solution, shaken and centrifuged at 4000 rpm for 20 min at room temperature. A 1 mL aliquot of the supernatant was taken and followed the same procedures as that for the spiked feed.

Purification of samples by solid phase extraction: The OASIS® MCX column was preconditioned by passing 1 mL of methanol, followed by 1 mL of double-deonized water through the column. Then, 1 mL of the extract solution was slowly passed through the OASIS® column at a flow rate of 1 mL min^{-1}. After washing with 1 mL of 0.1 M hydrochloric acid and 1 mL of methanol, respectively, the analyte was eluted with 1 mL of ammonia-methanol solution (2.5%, v/v). The solutions were evaporated in a 55°C water bath, under nitrogen and the extract was dissolved in 1 mL of phosphate buffer (pH 7.4) for ELISA or 2% aqueous acetic for LC-MS analysis.

Liquid chromatography-mass spectrometry analysis: Chromatographic separation of ractopamine was achieved at room temperature on a symmetry C_{18} (2.1 mm × 150 mm; 3.5 μm) column. The mobile phase delivered at a flow rate of 0.2 mL min^{-1}, consisted of A: 0.01 M aqueous ammonium formate (pH 3.8) and B: acetonitrile. A gradient program was used, starting at an A/B (v/v) composition of 98/2. This composition was changed linearly to reach 70/30 within 5 min and then to reach 50/50 from 5-15 min. A 10-min equilibration time was needed before the next injection. The injection volume was 20 uL and the run time was 25 min.

The optimized ionization conditions for mass spectrometric detection were determined using a cone voltage of 25 V, a extractor voltage 5 V and a capillary voltage 3.0 kV. The ion source and desolvation temperatures were 120 and 350°C, respectively. The ionization was tested in ESI positive (ESI^+) mode and high-purity nitrogen was used as the Electrospary Ionization (ESI) nebulizing gas.

RESULTS AND DISCUSSION

Characteristics of the polyclonal antibody: With the checkerboard procedure, the optimum reagent concentrations were defined under which it gave the maximum absorbance around 1.0, in the absence of analyte with minimum reagent used. Thus, the optimum concentration of coating antigen was 1.0 μg mL^{-1}. Based on the titer and IC_{50} of the antibodies obtained from the different rabbits, we found that the antibody of rabbit No.3 (R3) was better than those of the other rabbits and therefore R3 antibody was selected for use in further study. In the present study, the dilution of the used purified primary antibody of R3 was 1:40000. We also listed the IC_{50} values of the antibodies with the same protocol against ractopamine in the previous reports for comparison. It was shown that R3 performed a good sensitivity in phosphate buffer with an IC_{50} of 0.8 ng mL^{-1}, which is considerably better than others (Table 1).

Cross-reactivity studies are used to reveal the structural specificity of the antibody and serve as an important guide to select compounds that may cross-react with the antibody and compounds that are unlikely to bind to the antibody. The cross-reactivities of R3 antibody with ractopamine-related compounds, defined as the ratio of the competitor at IC_{50} to that of ractopamine at IC_{50}, were investigated by an indirect competitive immunoassay following the same procedures shown in Fig. 1. Due to the structure similarity, dobutamine showed higher cross-reactivity (~1.5%) and IC_{50} value of 53.3 ng mL^{-1}, but the other compounds, including terbutaline, salbutamol, metaproterenol, fenoterol, ritodrine, bamethane, clenbuterol, isoproterenol and isoxsuprine, had negligible cross-reactivities (<0.01%). A similar result was also reported by Shelver *et al.* (2000a), but Haasnoot *et al.* (1994), showed significantly high cross-reactivity between ractopmine and fenoterol.

Table 1: Sensitivity comparison of different antibodies with IC_{50}

Type of antibody	IC_{50}[a] (ng mL^{-1})	Application in matrices	Ref
Polyclonal	0.8	Feed and urine	tw[b]
Polyclonal	0.2	urine	Elliott *et al.* (1998)
Polyclonal	4.2	urine	Shelver *et al.* (2000a)
Monoclonal	2.7	urine	Shelver *et al.* (2000b)
Monoclonal	4.7	urine	Shelver *et al.* (2003)
Monoclonal	5.3	feed	Wang *et al.* (2006)

[a]IC_{50}: the concentration of ractopamine which produced 50% of the absorbance compared to no ractopamine, [b]tw: this work

Table 2: Intra-assay, inter-assay variance and recovery of competitive ELISA for antibody

Ractopamine (ng mL^{-1})	Intra-assay (ng mL^{-1})	C.V. (%)	Inter-assay (ng mL^{-1})	C.V (%)
10.0	10.12±0.21	2.1	10.18±0.74	7.4
5.0	5.13±.021	4.2	4.85±0.14	2.8
2.5	2.41±0.18	7.2	2.43±0.08	3.2
0.6	0.57±0.02	3.3	0.64±0.03	5.0
0.3	0.32±0.02	6.7	0.31±0.05	16.7

Determination of inter- and intra-assay variation: Serial dilutions of free ractopamine (ranging from 0.3-10 ng mL^{-1}) were prepared in phosphate buffer (pH 7.4). The ractopamine concentration was calculated by using a ractopamine calibration curve. The intra-assay (within a day) variation was measured for each concentration of the ractopamine. After a week the same measurement was performed for the purpose of determining the inter-assay (between days) variation (Table 2). The established indirect competitive immuno-assay showed a little bit higher of the variations at the low concentration of ractopamine and less than 10% of variations in all other concentrations.

Analysis of spiked feed samples: Feed samples typically contain a significant amount of salt and proteins that can interfere with the detection of ractopamine. However, a significant amount of metal ions and proteins present in feeds are precipitated in the presence of phosphoric acid and organic solvent. Therefore, the phosphoric acid-methanol solution was used to pretreat the samples prior to analysis. Any additional interfering compounds could be removed in the subsequent clean-up procedure using solid phase extraction. To clean-up spiked samples, several solid phase extraction columns, such as Mixed Cation-Exchange (MCX), Hydrophilic-Lipophilic Balance (HLB) and reversed-phases C_{18} columns, were tested.

Ractopamine possesses amine group at its chemical structure which is easily protonated in relatively acidic environment and charged positively. Based on this property of ractopamine, we found that the cation-exchange or mixed mode column, containing cation-exchange and hydrophobic groups was the suitable for the purpose of purification.

The competitive ELISA established above was used to detect ractomamine in spiked feed samples. Our preliminary work indicated that the determination of ractopamine in feed samples requires a sample clean-up process to remove interfering compounds, in order to allow trace levels of the analyte to be determined in such a complex with ELISA. Especially at low concentrations of ractopamine, we found the OD_{492} values of samples were often very low when the phosphate acid-methanol extraction solution was used directly to determine ractopamine by ELISA. Recoveries sometimes exceeded 200%. Blank samples also showed very low OD values. Even when the phosphate acid-methanol extraction solution was adjusted to pH 7.4 using 1 M sodium hydroxide, similar poor results were obtained. Therefore, the additional purification step involving solid phase extraction is essential for both methods, just as that in previous reports (Pickett and Sauer, 1993).

We also studied whether the solution used to dissolve ractopamine influenced the immunological reaction. Three solutions were compared namely 2% acetic acid, 2.5% ammonia-methanol solution or phosphate buffer (pH 7.4). The results suggested that there were significant differences between the solutions on the recovery of ractopamine. For detail, the value of OD_{492} was very low when 2% acetic acid or 2.5% ammonia-methanol was used, but it showed normal with the phosphate buffer at pH 7.4. Therefore, standardization of buffer and pH is necessary to enhance the sensitivity of the assay.

The calibration curve for free ractopamine, ranged from 0.2-3.0 ng mL^{-1} with the limit of detection of 0.15 ng mL^{-1} for free ractopamine, was obtained using optimized competitive ELISA (Fig. 2). The recovery of ractopamine from feed samples spiked with 0.1, 0.2, 1, 2, 10 and 20 mg kg^{-1} were also measured (Table 3). The statistical results showed good recoveries ranged from 80-115% and coefficients of variation less than 10%. Therefore, combined with the extraction and clean-up procedure, this competitive immunoassay can be employed to accurately determine ractopamine in feed samples.

Those ractopamine-spiked feed samples were also analyzed by LC-MS using select ion recording mode for identification and quantification purposes. The ionization conditions for ractopamine were optimized mainly by

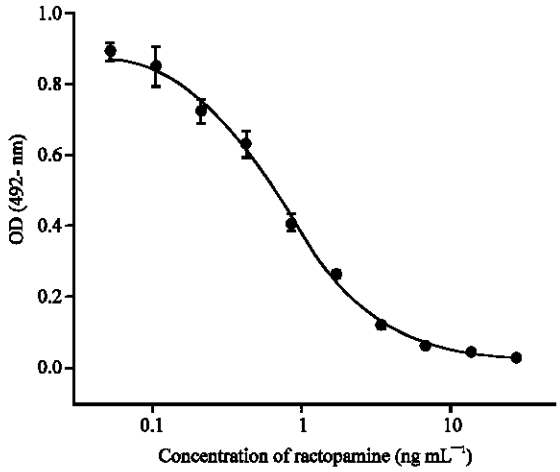

Fig. 2: The calibration curve of ractopamine using competitive ELISA

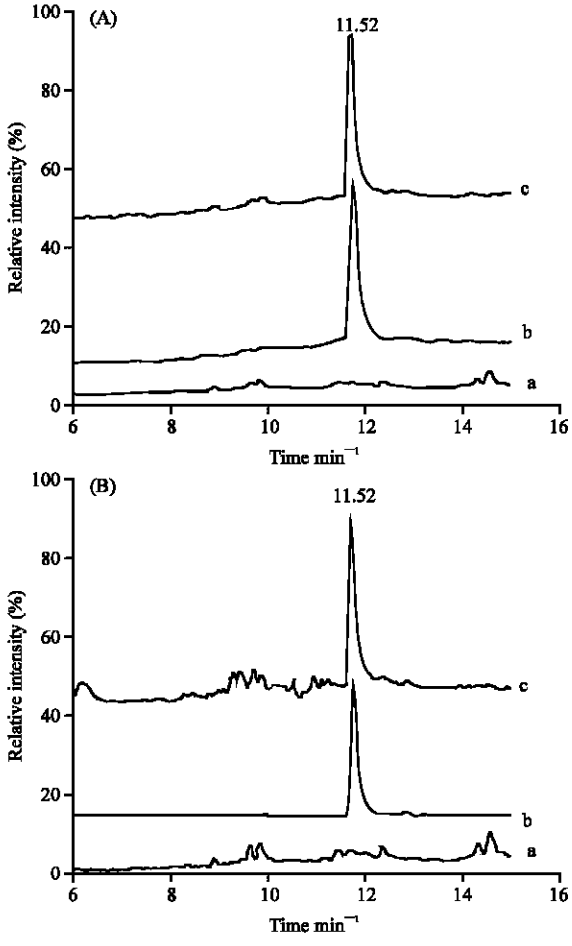

Fig. 3: Chromatograms at select ion recording mode for (A) feed samples and (B) swine urine samples: (a) blank sample, (b) ractopamine standard, (c) spiked sample

Table 3: Recovery of ractopamine from spiked feed samples determined by ELISA and LC-MS

	ELISA (n^a=6)			LC-MS (n^a=6)		
Amount added (mg kg^{-1})	Amount measured (mg kg^{-1})	Recovery (%)	C.V. (%)	Amount measured (mg kg^{-1})	Recovery (%)	C.V. (%)
20.0	19.34±1.7	96.7	8.5	17.43±0.62	87.2	3.1
10.0	8.28±0.48	82.8	4.8	9.17±0.08	91.7	0.8
2.0	2.03±0.13	101.5	6.5	1.73±0.04	86.6	2.0
1.0	1.12±0.08	112.0	8.0	0.88±0.03	88.0	3.1
0.2	0.22±0.008	110.0	4.0	0.19±0.005	95.0	2.5
0.1	0.097±0.002	97.0	2.0	0.10±0.003	100.0	3.0

[a]The number of determined sample

Table 4: Recovery of ractopamine from the spiked urine samples determined by ELISA and LC-MS

	ELISA (n^a=6)			LC-MS (n^a=6)		
Amount added (ng mL^{-1})	Amount Measured (ng mL^{-1})	Recovery (%)	C.V. (%)	Amount Measured (ng mL^{-1})	Recovery (%)	C.V (%)
20.0	20.86±1.46	104.3	7.3	19.46±0.62	97.3	3.1
10.0	10.24±0.45	102.4	4.5	9.05±0.34	90.5	3.4
5.0	4.88±0.28	97.6	5.6	4.64±0.26	92.8	5.2
1.0	0.92±0.03	92.2	3.0	0.83±0.07	83.0	7.0
0.5	0.59±0.02	118.4	4.0	0.41±0.03	82.0	6.0

[a]The number of determined sample

varying the cone voltage (range 15-40 V) and the source block temperature (range 100-150°C). Figure 3A shows an ion chromatogram for the analysis of a feed sample spiked with ractopamine at a concentration of 2 mg kg^{-1}. The chromatograms of the ractopamine and a blank sample were also demonstrated (Fig. 3A). The results show a peak at about 11.52 min for both the ractopamine standard and the spiked feed sample which should be attributed to a pseudo-molecular ion peak of ractopamine. The peak area at 11.52 min illustrated a linear relationship with the amount of ractopamine spiked in the range of 0.1-20 mg kg^{-1} and a correlation coefficient of 0.999. The recoveries piked were shown in Table 3, which indicated recoveries ranging from 85-100% and coefficients of variation less than 5% and were similar to those obtained by ELISA.

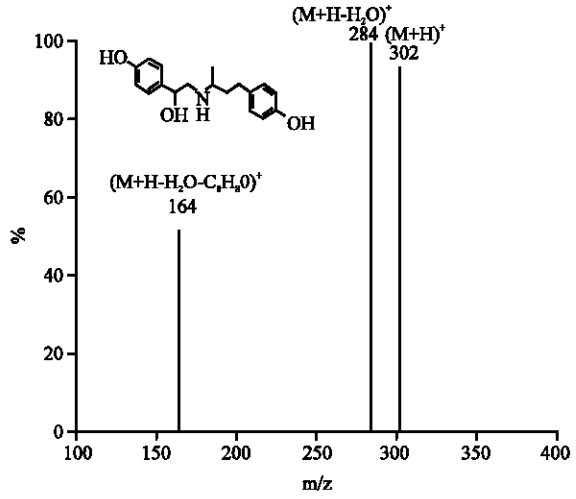

Fig. 4: ESI^+ mass spectrum of ractopamine ions and its diagnostic fragmentation pattern for feed sample spiked ractopamine. Voltage: capillary, 3.00 kV; cone, 25 V; extractor, 5 V

Analysis of spiked urine samples: The competitive immunoassay established above was also applied to detect ractopamine spiked in urine samples. Ractopamine was spiked in swine urine at 0.5, 1, 5, 10 and 20 ng mL^{-1} with 6 replicates for each concentration. Spike levels, spike recoveries and correlation coefficients were calculated. A good correlation between the spiked quantity and the actually determined result in the urine matrice was shown, with recoveries ranging from 90-120% and coefficients of variation less than 10% (Table 4).

The purified ractopamine-spiked samples were also analyzed by LC-MS using select ion recording mode. Figure 3B shows an ion chromatogram for a spiked urine sample at a concentration of 5 ng mL^{-1} of ractopamine. The chromatograms of a blank feed sample was also shown in Fig. 3B as a control. The results showed that the peak at about 11.52 min for the spiked urine sample had same shape and position to that of the ractopamine standard. The peak area demonstrated a linear relationship with the amount of ractopamine spiked in urine in the range of 0.5-20 ng mL^{-1}. The statistical data for the quantitation of ractopamine by LC-MS are also shown in Table 4, which indicated recoveries ranging from 80-100% and coefficients of variation less than 10%.

The results obtained with both the competitive immunoassay and the confirmatory LC-MS procedures showed a good agreement for the spiked swine feed (r^2 = 0.997) and urine samples (r^2 = 0.999). Thus, both analytical techniques are suitable for the estimation of ractopamine after optimization of the isolation parameters from feed and urine samples.

LC-MS confirmation: In accordance with the European Union guidelines (Draft SANCO/1085, 2000), at least 2 diagnostic ions are recommended to identify veterinary drugs and contaminants in one method. Under moderate ionization conditions, the selected parent ion *m/z* 302 and the daughter mass ions *m/z* 284 and *m/z* 164 of ractopamine spiked in feed (Fig. 4) and urine samples were monitored and it was determined that *m/z* 284 is the most abundant ion. The selected ions of standard ractopamine were also monitored as a control (data not shown). The ion *m/z* 302 is attributed to the pseudo-molecular ion of ractopamine and the *m/z* 284 and *m/z* 164 would be attributed to the fragments of $(M+H-H_2O)^+$ and $(M+H-H_2O-C_8H_8O)^+$, respectively. Furthermore, the ratios of the parent and daughter ions for both spikes and standards obtained from mass spectrum were tested to confirm the presence of ractopamine. The ratios show a good correlation between the spikes and the standards with differences less than 10%. The results suggest that the LC-MS procedure can act as a confirmatory procedure for the ELISA results.

CONCLUSION

Antibodies generated from the ractopamine-bovine thyroglobulin immunogen have high sensitivities and are suitable for a screening assay for ractopamine at the ppb level. The specificity of the antibody was excellent and other common phenethanolamine β-agonists showed only slight cross-reactivity with the antibody. Combination of phosphate acid-methanol extraction and a MCX clean-up column can reduce the matrix effect of the sample. Both competitive immunoassay and LC-MS methods was found to be satisfactory for the purpose of determining ractopamine in animal feed and urine samples, particularly in terms of specificity and sensitivity.

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REFERENCES

Andersom, D.B., E.L. Veenhuizen, J.F. Wagner, M.I. Wray and D.H. Mowrey, 1989. The effect of ractopamine hydrochloride on nitrogen retention, growth performance and carcass composition of beef cattle. J. Anim. Sci., 67: 222-225.

Antignac, J., P. Marchand, B. Bizec and F. Andre, 2002. Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography-positive electrospray tandem mass spectrometry. J. Chromatogr. B., 774: 59-66.

Blanca, J., P. Muñoz, M. orgado, N. Méndez, A. Aranda, T. Reuvers and H. Hooghuis, 2005. Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry. Anal. Chim. Acta, 529: 199-205.

Bocca, M., B. Fiori, C. Cartoni and G. Brambilla, 2003. Simultaneous determination of zilpaterol and other beta agonists in calf eye by gas chromatography/ tandem mass spectrometry. J. AOAC Int., 86: 8-14.

Churchwell, M.I., C.L. Holder, D. Little, S. Precce, D.J. Smith and D.R. Doerge, 2002. Liquid chromatography/electrospray tandem mass spectrometric analysis of incurred ractopamine residues in livestock tissues. Rapid Commun. Mass Sp., 16: 1261-1265.

Dickson, L.C., J.D. Macneil, S. Lee, A.C.E. Fesser, 2005. Determination of beta-agonist residues in bovine urine using liquid chromatography-tandem mass spectrometry. J. AOAC Int., 88 (1): 46-56.

Draft SANCO/1085/2000 Rev. 1, Eruopean Commission.

Elliott, C.T., C.S. Thompson, C.J. Arts, S.R. Crooks, M.J. van Baak, E.R. Verheij and G.A. Baxter, 1998. Screening and confirmatory determination of ractopamine residue in calves treated with growth promoting doses of the β-agonist. Analyst, 123: 1103-1107.

FDA, Federal Register, 2001. Rules and Regulations, 66 (83): 21283-21284.

Haasnoot, W., P. Stouten, A. Lommen, G. Cazemier, D. Hooijerink and R. Schilt, 1994. Determination of fenoterol and ractopamine in urine by enzyme immunoassay. Analyst, 119 (12): 2675-2680.

Kuiper, H.A., M.Y. Noordam, M.M.H. van Dooren-Flipsen, R. Schilt and A.H. Roos, 1998. Illegal use of β-adrenergic agonists: European Community. J. Anim. Sci., 76: 195-207.

Martinez-Navarro, J.F., 1990. Food poisoning related to consumption of illicit beta-agonist in liver. Lancet, 336 (8726): 1311.

Mitchell, G.A. and G. Dunnavan, 1998. Illegal use of b-adrenergic agonists in the United States. J. Anim. Sci., 76: 208-211.

Pickett, R.J.H. and M.J. Sauer, 1993. Determination of clenbuterol in bovine urine by enzyme immunoassay following concentration and clean-up by immunoaffinity chromatography. Anal. Chim. Acta., 275 (1-2): 269-273.

Shelver, W.L. and D.J. Smith, 2000a. Development of an immunoassay for the β-adrenergic agonist ractopamine. J. Immunoassay, 21: 1-23.

Shelver, W.L., D.J. Smith and E.S. Berry, 2000b. Production and characterization of a monoclonal antibody against the β-adrenergic agonist ractopamine. J. Agric. Food Chem., 48: 4020-4026.

Shelver, W.L. and D.J. Smith, 2002. Application of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for the Determination of Ractopamine in Incurred Samples from Food Animals. J. Agric. Food Chem., 50 (10): 2742-2747.

Shelver, W.L. and D.J Smith, 2003. Determination of ractopamine in cattle and sheep urine samples using an optical biosensor analysis: Comparative study with HPLC and ELISA. J. Agric. Food Chem., 51: 3715-3721.

Shishani, E., S. Chai, S. Jamokha, G. Aznar and M. Hoffman, 2003. Determination of ractopamine in animal tissues by liquid chromatography-fluorescence and liquid chromatography/tandem mass spectrometry. Anal. Chim. Acta, 483: 137-145.

Turberg, M.P., T.D. Macy, J.J. Lewis and M.R. Coleman, 1995. Determination of ractopamine hydrochloride in swine and turkey tissues by liquid chromatography with coulometric detection. J. AOAC Int., 78: 1394-1402.

Turberg, M.P., J.M. Rodewald and M.R. Coleman, 1996. Determination of ractopamine in monkey plasma and swine serum by high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B Biomed. Applied, 675 (2): 279-285.

Wang, J.P., S.X. Zhang and J.Z. Shen, 2006. Technical Note: A monoclonal antibody-based immunoassay for determination of ractopamine in swine feeds. J. Anim. Sci., 84 (5): 1248-1251.

Watkins, L.E., D.J. Jones, D.H. Mowrey, D.B. Andersom and E.L. Veenhuizen, 1990. The effect of various levels of ractopamine hydrochloride on the performance and carcass characteristics of finishing swine. J. Anim. Sci., 68: 3588-3595.

Wellenreiter, R.H. and L.V. Tonkinson, 1990. Effect of ractopamine hydrochloride on growth performance of turkeys. Poult. Sci., 69 (Suppl. 1): 142-148.