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ADVANCING RACTOPAMINE DETECTION METHODS: IMMUNOAFFINITY COLUMN CLEANUP IN ELISA FOR SWINE TISSUES

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Abstract

The misuse of agonists as growth promoters in meat-producing animals has raised significant concerns over the past two decades. Ractopamine (RCT) has emerged as a prominent agonist substance, illicitly employed for enhancing leanness in animals. Despite being approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) and various authorities in Brazil, Venezuela, Colombia, Guatemala, Dominican Republic, and the Philippines for swine feed, phenethanolamine agonists like RCT have a history of unauthorized usage by livestock producers. This unauthorized use poses potential food safety risks due to the presence of drug residues in animal tissues, particularly when the compound is administered illegally or not in accordance with regulatory guidelines.

The positive impact of ractopamine hydrochloride on economically significant traits makes it an appealing product for swine growers and producers of other livestock species. However, concerns persist regarding the safety and legality of its use, particularly in the context of food safety. Thus, there is a pressing need for comprehensive research and regulatory measures to address the challenges associated with the unauthorized use of RCT and other agonists in meat production.

INTRODUCTION

Agonists have been misused as growth promoting agents in meat producing animals over 20 years. Ractopamine (RCT) has been developed to be the main agonist substance used illegally for this purpose. Ractopamine is a phenethanolamine leanness-enhancing agent that has been approved by U.S. Food and Drug Administration (FDA, 2000; Muirhead, 2000) and regulatory agencies in Brazil, Venezuela, Colombia,

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Guatemala, Dominican Republic, and the Philippines as a feed additive for swine (Shelver and Smith, 2002). Phenethanolamine -agonists have a history of being used for unauthorized purposes by livestock producers (Kuiper et al., 1998; Mitchell and Dunnavan, 1998; Shiu and Chong, 2001). The positive influence of ractopamine hydrochloride on these economically important traits should make the product attractive to swine growers, and to producers of livestock species for which ractopamine is not approve The presence of drug residues in animal tissues is a potential food safety concern, especially when the compound has been used illegally or in a manner not prescribed by regulatory official.

In an effort to combat the illicit use of-agonists compounds, regulatory organizations worldwide test animal tissues for the presence of illicit drugs through both screening and confirmatory assays. For residue analysis, the analyte is separated from the matrix by liquid-liquid or solid-phase extraction (SPE), followed by liquid or gas chromatography or immunological analysis. Sample preparations steps are often time consuming and tedious, making an efficient cleanup method very desirable when high throughput is required. High sensitivities of immunoassays are practically desirable for off-label drug monioring because it may be desirable to detect the analyte even after extended withdrawal periods. -agonists immunoassays have been developed for clenbuterol (Yamamoto and Iwata, 1982), fenoterol (Haasnoot et al., 1994) and ractopamine (Elliott et al., 1998; Ba, 1998).

MATERIALS AND METHODS

Reagents and instruments: CNBr-activated Sepharose 4B was obtained from Pharmacia Biotech. Protein A-Sepharose 4B was purchased from Sigma. Ractopamine was provided by Eli-lilly (USA). Liquid scintillation cocktail (EcoliteTM) was purchased from ICN Biomedicaal, Inc. Animal specimens including Swine live and muscle samples were obtained from swine known to be free of ractopamine and were pooled (Beijing Laboratory Animal Research center, China). Microplate Reader MK3 was from Finland LABSYSTEMS. All other chemicals or solvents were obtained from Sigma or Amerscreso and were analytical grade or better.

Immunogen and enzyme conjugate preparation: RCT (99.999%, Daming Company Henan, China) was coupled to a carrier, bovine serum albumin (BSA), and to an enzyme, horseradish peroxidase (HRP), using the coupling agent, butane-1,4-diol diglycidyl ether (Yamamoto and Iwata, 1982; Haasnoot et al., 1994).

Immunogen: A 30 mg amount of BSA was dissolved in 0.5 mL of de-ionized water and the pH was adjusted to 10.8 using 0.5 mol/L sodium hydroxide. A 6 uL volume (30 umol) of butane-1,4-diol diglycidyl ether was added and the mixture was incubated for 20 h at room temperature under a nitrogen atmosphere. A 15 mg (50 umol) amount of RCT was dissolved in 0.5 ml of 0.1 mol/L sodium hydroxide, 200 uL dimethylformamide being added to help solubility. This solution was degassed using nitrogen then added to the epoxy-activated BSA solution. The reaction mixture was incubated for 20 h at room temperature under a nitrogen atmosphere. Purification of the RCT-BSA immunogen was achieved using extensive dialysis (10 000 molecular mass off) against 0.15 mol/L^{-1} sodium hydroxide. The immunogen was diluted with 0.15 mol/L sodium hydroxide to give a final protein concentration of 1 mg/mL and stored at –20ºC until required.

Enzyme conjugate: A 20 mg amount of HRP was dissolved in 0.5 mL of de-ionized water and the pH was adjusted to 10.8 using 0.5 mol/L sodium hydroxide and the solution was de-gassed with nitrogen. A 50 L volume (5 mol) of a solution of butane-1,4-diol diglycidyl ether was added and the mixture was incubated for 20 h at room temperature under a nitrogen atmosphere. Unreacted butane1,4-diol diglycidyl ether was removed by gel filtration (Sephadex G25-M, equilibrated with 1 mmol/L sodium acetate). A 20 mg amount of RCT was added to the activated HRP solution, 200 uL dimethylformamide being added to increase solubility. This solution was degassed using nitrogen. The enzyme-labelled conjugant was then purified by gel filtration (Sephadex G25-M, equilibrated with 1 mmol/L sodium acetate) and by use of dextran-coated charcoal.

The HRP-RCT was stored at –20ºC until required.

Production and purification of polyclonal antibodies. The polyclonal antibodies against RCT-BSA were obtained from New Zealand White rabbits (Beijing laboratory research center, China). In brief, immunogen was emulsified with an equal volume of Freund's complete adjuvant to give a final concentration of 0.5 mg/mL. This mixture was given in four intradermic injections. Boosting injections of Freund's incomplete adjuvant were made at 2-week intervals. Blood samples were collected 1-week after the third booster injections and tested for titer determination. Blood collected from rabbits was first allowed to stand overnight at 4ºC, then centrifuged at 200 × g for 15 min.

The obtained antiserum was purified in three steps by firstly using a saturated ammonium sulfate method (Elliott et al., 1998). The 20 mL antiserum was purified by the saturated ammonium sulfate method. At last the precipitates were dissolved in 2 mL of PBS and dialyzed against PBS until no sulfate ion could be detected in the dialysis solution with 0.5 M $BaCl_2$ acidified with HCl. The obtained immunoglobulin (IgG) was purified in the second step by the way of protein A-Sepharose 4B (Zhao et al., 2003). The eluates (0.1 M glycine buffer pH 7.2, 0.5 mL) were collected in tubes containing 50 mL of 1 M Tris (pH 8.0) and mixed gently to bring the pH back to neutral. At last, the antiserum was purified to remove the anti-BSA antibodies by adsorption of anti-BSA antibodies to a column containing BSA cross-linked to an Affiprep 10 matrix (Bio-Rad) and the column could be regenerated by elution with 50 mM glycine (pH 2.3)-0.5 M NaCl-0.02% Triton X-100 (Giraudi et al., 1998). The IgG concentration in the obtained solution was calculated based on the UV absorption difference between 280 and 260 nm (Chen et al., 1993). The formula used for the calculation was $C_{protein}$ (mg/mL) = $1.45 \times A_{280\ nm} - 0.74 \times A_{260\ nm}$. The purity of the purified IgG was checked on SDS-PAGE gel.

Enzyme immunoassay procedure: A direct competition ELISA format was utilized to measure ractopamine binding and cross reactivity to related compounds. The checker board procedure was used to optimize the enzyme tracer and the anti-RCT antibody concentrations. After optimization, the ELISAs were processed as follows: Microtiter plates were coated with 200 uL diluted antiserum in bicarbonate buffer (0.05 M, pH 9.6) overnight, 4ºC or 2 h at room temperature. The plate was washed with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) three times. Serial dilutions (50 uL) of the analyte or sample solution in PBS-organic solvent, together with 50 uL of the tracer, was added to the wells and incubated for an appropriate time. After another washing step, 100 uL per well of TMB solution (400 uL of 0.6% TMB-DMSO and 100 uL of 1% H_2O_2 diluted with 25 mL of citrate-acetate buffer, pH 5.5) was added. Followed by incubation at 37ºC for 30 min. Color development was stopped by adding 50 uL/well of 2 M sulfuric acid. The plates were read at 450 nm with Microplate Reader MK3 (Thermo Labsystems, Vantaa, Finland) and the resulting curves fitted with a four-parameter logistic equation to determine the IC_{50}. The IC_{50} was defined as the concentration of inhibitor required to inhibit color development by 50% compared to control wells containing no competitors.

Antiserum cross-reactivities: Competitors used for the competetion studies are fenoterol, isoxsuprine, salmeterol, fomoterol and clenbuterol, which have comparable structure to ractopamine. The sensitivity of the purified rabbit antiserum was determined by constructing calibration curves and calculating the 50% inhibition of control index (IC_{50}), that is, the mid-point of the calibration curve. Competitive ELISA was performed using direct assay format with competitor concentration of 1 ug/mL. Compounds that produced sigmoidal like curves were fitted to the four parameter logistic equation using SAS; $y\ (\%B/B_0) = (A - D)/ [1 + (x/B)^C] + D$ (Raab, 1983).

Coupling antibody to CNBr-activated sepharose (Primot et al., 2000): Purified antibodies against RCT were coupled to CNBractivated Sepharose to generate the immunoaffinity column. The IgG solution was dialyzed

against 0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, 0.2 M NaCl, pH 8.3, and was added to the Sepharose beads activeted with 1 liter of 1 mM HCl under constant rotation at 4ºC overnight at a concentration of 2 mg/mL (IgG/Sepharose beads). Then the supernatant was removed and the beads were incubated with 1 M ethanolamine pH 9.0 to deactivate the remaining active sites for 2 h under constant rotation at 4ºC. Beads were washed with 0.1 M acetate, pH 4.0, 0.5 M NaCl, then with bead buffer (PBS pH 7.4, 0.02 NaN_3) and stored in 20% suspension in bead buffer at 4ºC until further use.

Negative rabbit antiserum containing no antibody against RCT was applied to generate the blank column as a negative control (Xu et al., 2005).

Determination of the optimal conditions of ractopamine on the immunoaffinity column (Xu et al., 2005): IACs were subjected to multiple loadings and elutions to determine column stability. Columns were loaded with 5 ug ractopamine, washed with both 10 mL 10% MeOH and 10 mL PBS (pH 7.4), and eluted with either 10 mL 100% MeOH or 10 mL 0.2 M glycine buffe (pH 2.5). All eluants were measured by ELISA and LC to determine recovery. Between uses, columns were stored at 4ºC in PBS-0.02 NaN_3 (pH 7.4).

To select the optimal pH condition for elution of RCT. 1 mL solution of a mixture of antibody RCT-Sepharose beads were washed with 5 mL of bead buffer. The matrix was incubated with 2 mL of RCT solution (0.25 mg/mL) and 2 m of OVA (0.25 mg/mL) for 40 min under constant rotation at 4ºC. After 7 times washing, the column was eluted with 0.2 M glycine buffer (2 mL) at various pH conditions (pH 5.5, 5.0, 4.5, 4.0, 3.5, 3.0 and 2.5). The collected fractions were assayed for RCT by ELISA. Column capacity and reusability of IAC: Column capacity for RCT was determined by passing 10 ug RCT in 10 mL PBS through an affinity column at 1 mL/min. To ensure maximum binding, the effluent was loaded to the column once more and washed with 10 mL of 10% MeOH (v/v) and 10 mL PBS (pH 7.4). Bound RCT was eluted with 0.2 M glycine buffer (pH 2.5) at 0.5 mL/min. RCT in the glycine eluate was determined by ELISA and LC. To determine column reusability, 1mL samples (100 ppb) were loaded onto 2 mL IAC for ractopamine. After the column was washed and eluted as the above, the column regenerated with equilibrating buffer and allowed to stand for various periods, from 10 min to 24 h. The capacity of the regenerated column was then determined by reloading with standards. This process was repeated ten times. RCT both in the washing eluate and in the glycine eluate was determined by ELISA and LC. Precision and accuracy of IAC. Samples of 100, 1000, 2500, and 5000 ng ractopamine mixture were fortified in 10 mL urine and loaded onto a 1 mL IAC. These represent 10, 100, 250, and 500 ppb fortified ractopamine samples. Columns were washed with 10 mL of 10% MeOH (v/v) and 10 mL 0.1 M sodium phosphate buffer (pH 7.4), and then eluted with 0.2 M glycine buffer (pH 2.5). Ractopamine in the glycine eluant was quantified by ELISA and LC. The procedure was repeated 3 times for each fortification level.

Preparation of urine, muscle, kidney, and liver samples, and application of IAC-ELISA: A 10 g test portion of tissue was added to 10 mL PBS (pH 7.4) and the mixture was homogenized for 1 min with a Tissumizer (Tekmar Company, Cincinnati, USA) set a high torque. Samples were then cooled in an ice bath. This was repeated until no large particles were visually observed (usually 2 - 4 homogenization cycles). Supernatants were separated from tissue debris by centrifugation at 12 000 × g for three times and stored at –20ºC until used. Ractopamine (10 and 50 ng) was dis-solved in PBS (pH 7.4) or various supernatants as mentioned above from various issues of swine. These samples were to be assayed by ELISA and IAC-ELISA in order to determine the matrix effect and to calculate the detection limit of various issues. Validation of ractopamine LC/MS (Kootstra et al., 2005). All the samples were evaporated to dryness at 55 under a stream of nitrogen. The residue is dissolved in 100 uL 10:90 (v/v) methanol: water. Calibration curve was set up with ten-fold dilution of Ractopamine in 10:90 (v/v)

methanol:water, from 100 to 0.1 ng/mL. These solutions were transferred to a LC vial and 20 uL is injected onto the LC-MS system.

Equipment

The MS system is a Agilent LCQ Classic-system equipped with an APCI+ interface. The LC system consists of a P4000 Spectra SYSTEM quaternary pump and an AS3000 SpectraSYSTEM autosampler (Agileng, Wilmington, DE, USA).

Chromatographic separations are carried out on a Phenomenex Luna 5 m C18 250 mm × 4.6 mm column at an oven temperature of 50. The LC gradient uses two solvents: 95% methanol 10 mM ammonium acetate (A) and 5% methanol 10 mM ammonium acetate (B). Flow rate 0.20 mL/min, linear gradient from 6% A-80% A in 20 min. After 20 min the system is reconditioned for 10 min at 6% A.

MS detection and conditions

The analytes are detected in MRM (MS2) mode. The mass frag- ments are used for the screening method. The instrument was tuned and calibrated according to manufacturer's specifications.

RESULTS AND DISCUSSION

Production and purification of polyclonal antibodies

The polyclonal antibody against RCT was firstly purified through a saturated ammonium sulfate and a protein A sepharose 4B affinity column. This two-step purification using 20 mL of antiserum resulted in a 70 mg of pure antibody. The antibody purity was checked by SDSPAGE, which showed one strong band (the heavy chain) and one weak band (the light chain) under the reducing conditions (data not shown). The purified antibodies were repeatedly flown through the column for over three times and the anti-BSA antibodies were efficiently separated from the anti-RCT antibodies. Both polyclonal antibody and monoclonal antibody were used in immunoaffinity chromatography. The most usable antibody was the polyclonal antibody (James et al., 2004). Polyclonal antibodies, obtained by immunizing a rabbit or goat and purifying the immunoglobulin fraction from the resulting serum. One can use quite pure antigen to avoid raising unwanted antibodies to minor impurities in the protein preparation. The obtained antiserum can be purified to remove many impurities and other unwanted antibodies by methods such as saturated ammonium sulfate, protein A sepharose 4B affinity column or Affiprep 10 matrix.

Control column

When the blank column was loaded with RCT spiked water using the modified immunoaffinity protocol as the above, RCT was not specifically adsorbed with the blank column containing the control IgG that not generated toward ractopamine. The amount of RCT in the washing and elution fractions was the same as that of columns containing anti-RCT antibodies. The above result demonstrated that the retention of RCT was primarily due to the anti-RCT antibody. The presence of RCT in the blank column eluate indicated that the RCT was only slightly retained by the blank column. This might possibly be due to insufficient blocking of active sites or might indicate the wash volume was not adequate. These results show that ractopamine was specifically retained by the ractopamine IAC, but not by the control column.

Determination of the optimal conditions of ractopamine on the immunoaffinity column

Elution of ractopamine from the IAC with glycine resulted in excellent recovery of 80 - 100% and offered two main advantages over elution with methanol. Firstly, elution with glycine buffer resulted in cleaner chromatograms of parent ractopamine after fortification in samples. Elution with methanol resulted in the co-elution of fluorescent compounds that interfered with chromatographic analysis of ractopamine (Shelver and Smith, 2002). Secondly, IACs were stable after glycine buffer was used, whereas columns eluted sequentially

with solvents containing a high percentage of methanol rapidly degraded (discussed below). The major disadvantage of using glycine as the eluting buffer is the increased amount of time required to concentrate it (that is by evaporation) comparing to methanol.

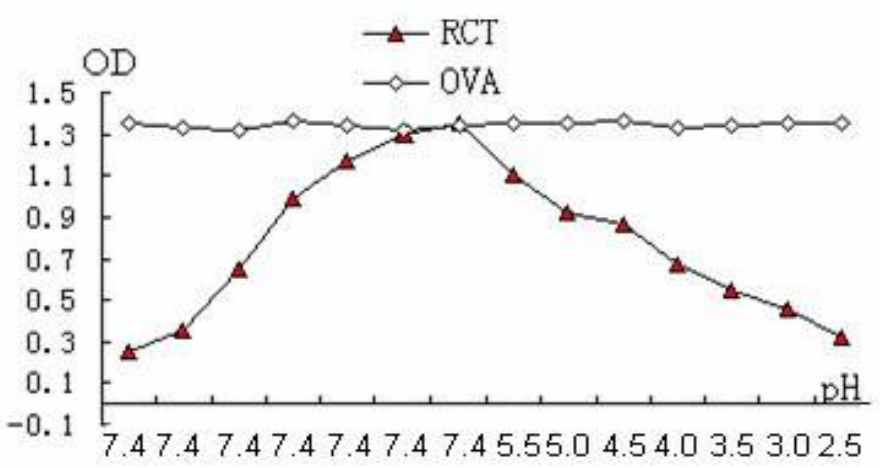

Figure 1. pH reducing elution of antibody against RCT-sepharose beads with 0.2 M glycine buffer. The eluting amount of RCT increased with the decrease of eluting pH.

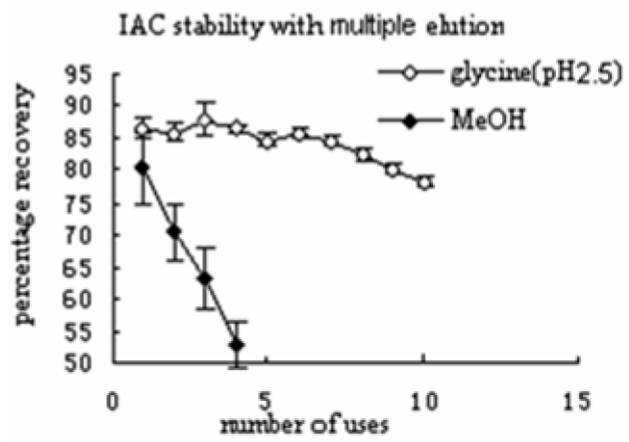

Figure 2. IAC stability with multiple elution using 0.2 M glycine, pH 2.5 or 100% MeHO. Results are expressed as percentage of loaded ractopamine in the elution fraction (n = 3). Almost half of the error bars for the glycine elution are too small to be visible. Ractopamine IACs quickly degraded when eluted with > 50% methanol and showed marked performance problems in the second use. However, when 0.2 M glycine, pH 2.5, was used to eluate the analytes, only minimal decreases in column capacity occurred after 10 uses.

Figure 1 showed that the antibody (anti-RCT)-sepharose beads was specifically combined with RCT, and the eluting amount of RCT increased with the decrease of eluting pH. If the eluting pH was too low, the antibody (against RCT)-sepharose beads were unstable which affected the combination between antibody and antigen, so the prime pH of elution buffer was selected as pH 2.5. In stability tests, ractopamine IACs quickly degraded when eluted with >50% methanol and showed marked performance problems in the second use. However, when 0.2 M glycine, pH 2.5, was used to eluate the analytes, only minimal decreases in column capacity occurred after 10 uses (Figure 2). Furthermore, ractopamine IACs stored at 4ºC in PBS-0.02% sodium azide were stable over 3 months. Because of the apparent advantages of glycine elution, subsequent studies on the effects of matrix (urine, muscle, kidney, and liver) were performed with glycine as the elution solvent.

Compound	Ractopamine	Fenoterol	Isoxsuprine	Salmeterol	Fomoterol	Clenbuterol
IC50	3.3	650	>80.000	>80.000	>80.000	>80.000
% CR	100%	0.5	<0.05	<0.05	<0.05	<0.05

Spiked ractopamine urine samples were used to determine the precision and recovery at various ractopamine levels. The recoveries of ractopamine from the urine samples for 10, 50, 100, 250, 500 ng/mL were 81.6, 87.3, 90.8, 86.5 and 83.4%, respectively. The standard errors of the measurements were 6.5, 7.8, 10.3, 8.4, and 5.6 ng/mL respectively. The IAC gave excellent recovery and reproducibility. The clean sample from IAC allows the sensitivity to be easily controlled by concentrating the eluant to obtain greater sensitivity. The method was confirmed useful over a very wide range of concentrations with only minor alterations in the LC procedure.

Matrices such as urine, tissues, and serum have great impact on the results of ELISA when these samples were to be detected directly by ELISA. But after these samples were purified by IAC, the effect can be eliminated. So IAC-ELISA enhances not only the sensitivity but also the accuracy of detection for ractopamine.

Antiserum cross-reactivity

Under these conditions, cross-reactivity to-wards other agonists was determined. A number of -agonists, which were fenoterol, isoxsuprine, salmeterol, fomoterol and have comparable structure to ractopamine. Fenoterol clenbuterol, tested for their cross-reactivity because they showed a cross-activity of 0.5% and other -agonists reacted 0.05 % (Table 1). Thus, this ELISA can only recognize ractopamine of the compounds tested. Detection limit and standard curve: The results obtained from the analysis of residue-free urine and other samples were used to estimate the detection limit. Figure 3 showed a representative standard curve for ractopamine. The detection limit was 0.2 ng/mL of ractopamine in urine, based on the mean response for the control sample extracts plus three times the standard deviation with IC50 of 3.3 + 0.25 ng/mL (Figure 3). In addition, IAC-ELISA allowed 0.5 ng/mL of RCT to be detected in other various tissues of swine.

Table 1. Cross-reactivity profile toward -adrenergic agonists from antiserum raised to ractopamine.

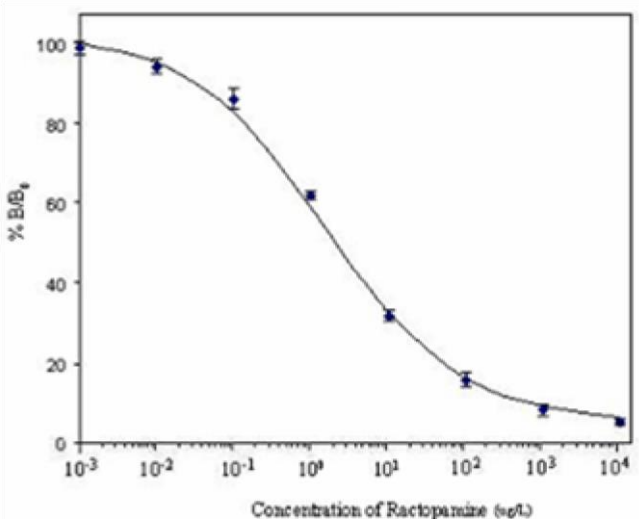

Figure 3. Representative standard curve for RCT by IAC-ELISA. Each point represents the mean of 5 determinants. Vertical bars indicate ± SD about the mean.

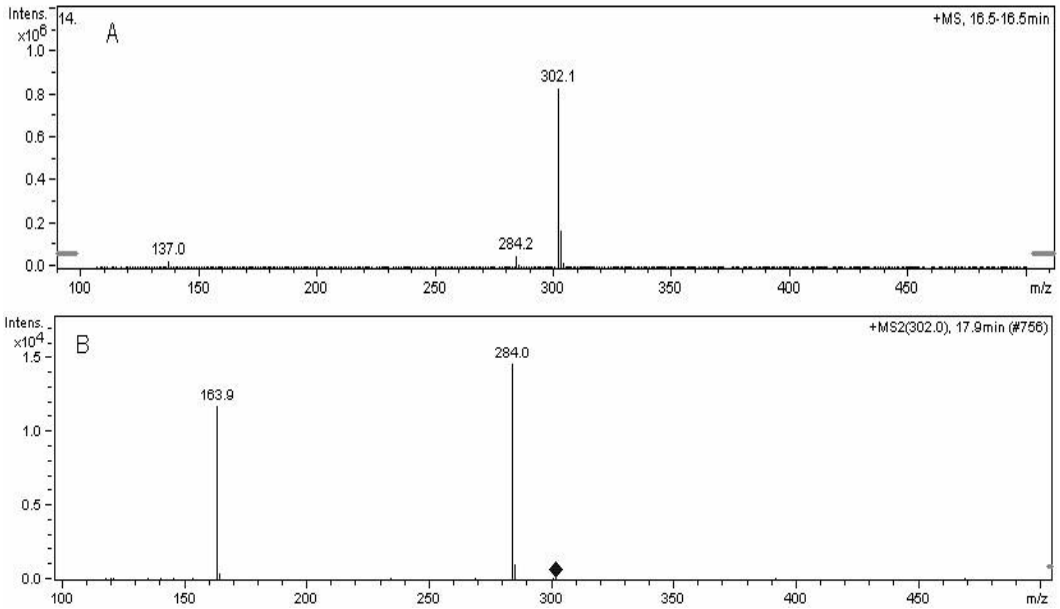

Figure 4A and 4B. The precursor ion and the daughter ions of ractopamine in all samples detected and qualified by MS-MS. The precursor ion of ractopamine is 302.1 and the daughter ions of ractopamine are 284 and 164.

Validation of IAC-ELISA by LC-MS

The validation indicated that all the eluates contain the ractopamine (The precursor ion of ractopamine is 302.1 and the daughter ions of ractopamine are 284 and 164) (Figure 4A and 4B) and the correlations between the results from LC-MS and those from IAC-ELISA were all high (above 0.8943). The amounts of ractopamine in the samples measured by IAC-ELISA agree with the results from LC-MS but the concentrations of RCT found with the IAC-ELISA were more or less higher compared with those obtained using LC-MS. This might be owing to the presence of metabolites of RCT in the samples, which could be detected by IAC-ELISA but could not be detected by LC-MS. The method of IAC-ELISA has been proven to be a simple, practical and reliable method for screening ractopamine.

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