Production and Characterization of a Monoclonal Antibody against the β-Adrenergic Agonist Ractopamine

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A monoclonal antibody was generated toward the β-adrenergic agonist ractopamine hydrochloride {(1*R**,3*R**),(1*R**,3*S**)-4-hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]methyl]benzenemethanol hydrochloride}. Ractopamine−glutarate−keyhole limpet hemocyanin (KLH) was used as the antigen for antibody generation in mice. Clone 5G10, secreted antibody with isotype IgG1κ, was used for the development of an immunoassay. The selected antibody was specific for racemic ractopamine with an IC_{50} of 2.69 ± 0.36 ng/mL (n = 15). Antibody binding toward ractopamine was stereoselective with (1*R*,3*R*)-ractopamine having an IC_{50} of 0.55 ± 0.09 ng/mL (n = 3). IC_{50} values for the (1*S*,3*R*)-, (1*S*,3*S*)-, and (1*R*,3*S*)-ractopamine stereoisomers were 2.00 ± 0.37, 140 ± 23, and 291± 32 ng/mL (n = 3), respectively. Phenethanolamine β-agonists showed low cross-reactivity. Studies using a series of ractopamine metabolites and ractopamine analogues demonstrated structural requirements for the antibody binding. A free phenolic group on the *N*-butylphenol moiety was required for high-affinity binding because methoxylated analogues and metabolites glucuronidated at this phenol generally had IC_{50} values greater than 200 ng/mL. Ractopamine analogues methoxylated or glucuronidated at the ethanolamine phenol had IC_{50} values of 0.7−2.6 ng/mL. Lack of a benzylic hydroxyl group was of less importance to antibody binding than was the correct stereochemical orientation (3*R*) of ractopamine's *N*-phenylalkyl group. In conclusion, a highly specific monoclonal antibody to ractopamine hydrochloride was developed that could be of potential utility in screening assays.

Keywords: *β-Agonist; ELISA; monoclonal antibody; ractopamine; feed additive; residue*

INTRODUCTION

Ractopamine is a phenethanolamine leanness-enhancing agent recently approved as a feed additive for swine by the U.S. FDA Center for Veterinary Medicine (FDA, 2000; Muirhead, 2000). Hogs administered dietary ractopamine exhibit increased growth rates, feed efficiencies, and yields of boneless, closely trimmed retail cuts (Anderson et al., 1990; Stites et al., 1991; Watkins et al., 1990) relative to untreated control animals. The positive influence of ractopamine hydrochloride on these economically important traits should make the product attractive to swine growers, and perhaps to producers of livestock species for which ractopamine is not approved.

Phenethanolamine β-agonists have a history of being used for off-label purposes by livestock producers (Kuiper et al., 1998; Mitchell and Dunnavan, 1998) hoping to improve the economics of livestock production; additionally, they have been used extensively by body-builders hoping to modify their phenotypic characteristics (Prather et al., 1995; Hausmann et al., 1998; Ayotte and Goudreault, 1999). The most commonly abused β-agonist is clenbuterol hydrochloride, a highly potent phenethanolamine (Smith, 1998), which has not been approved for leanness-enhancing effects in livestock or in humans by any regulatory body worldwide.

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The presence of drug residues in animal tissues is a concern for food safety, especially when the compound has been used illegally or in a manner proscribed by regulatory officials (off-label use). In an effort to combat the illicit use of β-agonist compounds, regulatory organizations worldwide test animal tissues for the presence of illicit drugs (Elliott et al., 1996; Kuiper et al., 1998; Mitchell and Dunnavan, 1998). For regulatory purposes, both screening and confirmatory assays are used to detect illegal residues. Immunoassays (IA) are convenient screening tools used to detect the presence of an analyte in various matrixes and have wide application for determination of the presence of environmental toxins (Sanborn et al., 1998), herbicides (Clegg et al., 1999), insecticides/pesticides (Wang et al., 1999; Abad et al., 1999), and pharmaceuticals (Stanker et al., 1998; Brandon et al., 1998). A successful screening assay should be quick, reliable, and relatively inexpensive. Positive samples from screening assays may then be assayed by more costly and complex instrumental method such as GC-MS or LC-MS that unequivocally identify the analyte in the sample (eliminating false positives). However, for screening, immunoassays provide the advantages of high throughput, portability, and sensitivity (detection limits in the parts per billion range). High sensitivities of immunoassays are particularly desirable for off-label drug monitoring because it may be desirable to detect the analyte even after extended withdrawal periods. β-Adrenergic agonists immunoassays have been developed for clenbuterol (Yamamoto and Iwata, 1982), albuterol (salbutamol;

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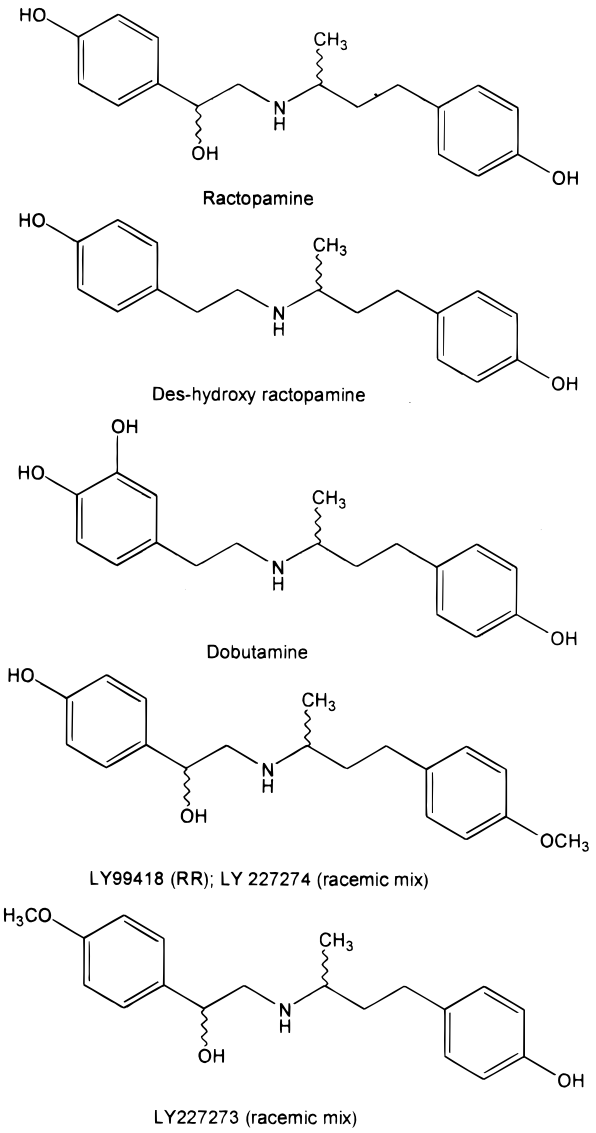

Figure 1. Structures of ractopamine and ractopamine analogues tested for cross-reactivity to the monoclonal antibody generated against ractopamine. LY99418 was the (1*R*,3*R*) stereoisomer only; LY227274 and LY227273 were racemic mixtures.

Adam et al., 1990), and fenoterol (Haasnoot et al., 1994), as well as ractopamine (Haasnoot et al., 1994; Elliott et al., 1998; Shelver and Smith, 2000a). Currently available IA kits cross-react poorly with ractopamine (Wicker et al., 1995), and the IA previously reported for ractopamine was generated from polyclonal antibodies. In this report, we describe the development and characterization of a monoclonal antibody against ractopamine.

MATERIALS AND METHODS

Chemicals and Reagents. Ractopamine hydrochloride {(1*R**,3*R**),(1*R**,3*S**)-4-hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]methyl]benzenemethanol hydrochloride}, LY227273, LY227274, and LY99418 (Figure 1) were gifts from Lilly Research Laboratories, Greenfield, IN. (*S*)-Deshydroxyractopamine, and (*R*)-deshydroxyractopamine (Figure 1) were synthesized by Dr. V. J. Feil (USDA−ARS Biosciences Research Laboratory, Fargo, ND). Ractopamine stereoisomers were synthesized as described previously (Ricke et al., 1999). Ractopamine glucuronide conjugates (Figure 2) were synthesized and isolated as described by Smith et al. (1993); each of the ractopamine glucuronide conjugates has been shown to be present in urine or tissues of test animals (Dalidowicz et al., 1992; Smith et al., 1993, 1995, 2000). Ractopamine glucuronide A was composed of 66% and 33% (1*S*,3*R*) and (1*R*,3*S*) ractopamines respectively; ractopamine glucuronide B had equal quantities of the (1*R*,3*R*) and (1*S*,3*S*) stereoisomers. Ractopamine glucuronide C was composed of 27%, 19%, 28%, and 26% (1*R*,3*R*), (1*S*,3*R*), (1*S*,3*S*), and (1*R*,3*S*) ractopamine stereoisomers, respectively. Clenbuterol hydrochloride (Figure 3), dobutamine hydrochloride (Figure 1), fenoterol hydrochloride, isoxuprine hydrochloride, ritodrine hydrochloride, bamethane sulfate, salmeterol 1-hydroxy-2-naphthoate, salbutamol hemisulfate (Figure 3), poly(ethylene glycol) 1500, complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), pristane (2,6,10,14-tetramethylpentadecane), and goat anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit and monoclonal antibody isotyping kit 1 were purchased from Pierce (Rockford, IL). RPMI 1640, HT, and HAT supplement were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT) and was heat-inactivated at 56 °C for 30 min prior to use. Hybridoma cloning factor and DMSO freeze medium were obtained from Fisher Scientific (Pittsburgh, PA). Murine myeloma cell line Sp2/0Ag14 (ATCC CRL-1581) was purchased from American Type Culture Collection (Manassas, VA).

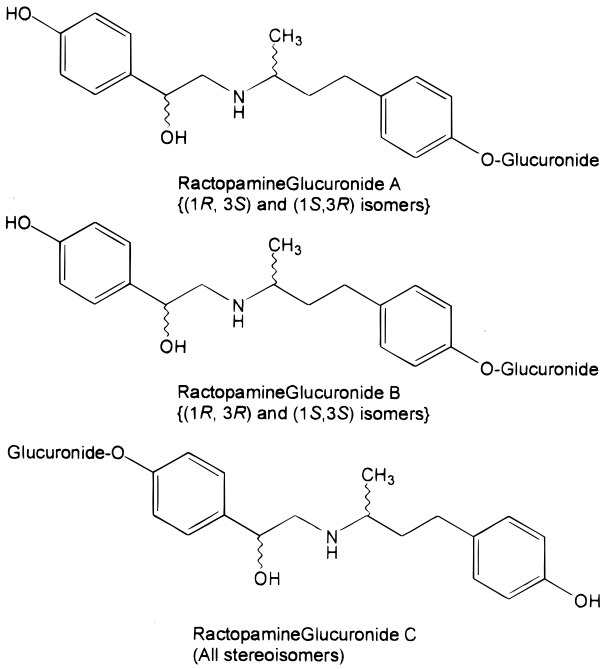

Figure 2. Structures of ractopamine glucuronides that were tested for cross-reactivity to the monoclonal antibody generated against ractopamine. Ractopamine glucuronide A was conjugated to the (1*R*,3*S*) and (1*S*,3*R*) stereoisomers of ractopamine at its butylamine phenol; ractopamine glucuronide B was conjugated to the (1*R*,3*R*) and (1*S*,3*S*) stereoisomers of ractopamine at its butylamine phenol; ractopamine glucuronide C was conjugated to each of the four ractopamine stereoisomers at its ethanolamine phenol.

Instrumentation and Supplies. ELISA assays were conducted and absorbance data were collected on a Bio-Rad model 550 ELISA plate reader from Bio-Rad Laboratories (Hercules, CA) with Microplate Manager 4.0. Immunoassays were conducted with the aid of an EX50 auto strip washer from Bio-Tek Instruments, Inc. (Winooski, VT); and Costar 96 well Easywash polyvinyl high-binding EIA/RIA plates were obtained from Corning Inc. (Corning, NY).

Preparation of Mice. Animal handling was in accordance with institutional guidelines. Four female BALB/c mice, 8

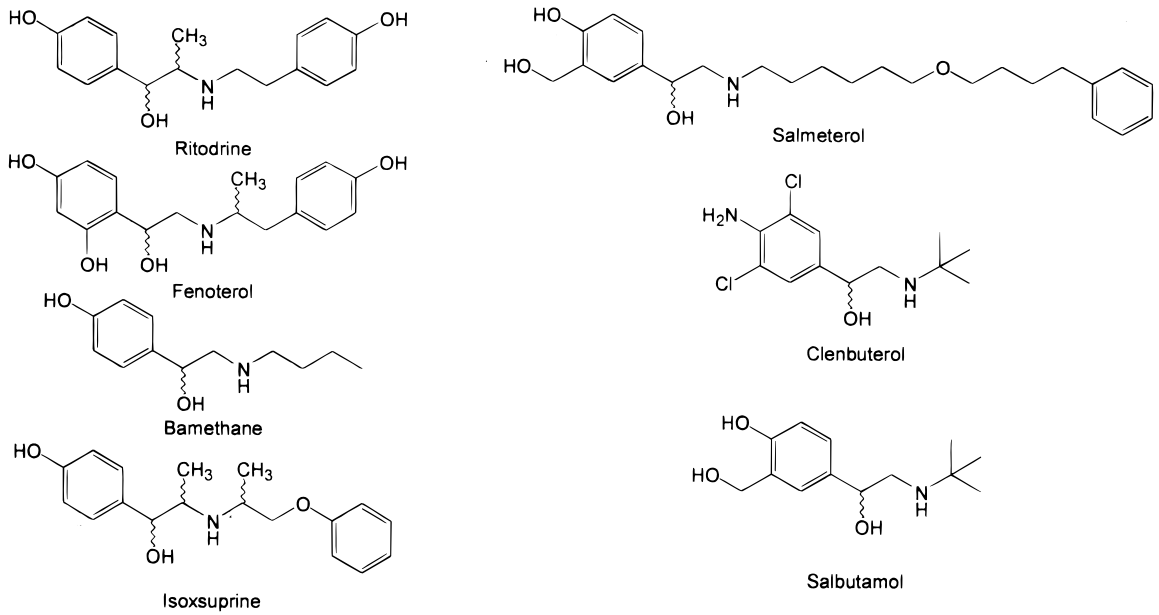

Figure 3. Structures of phenethanolamines tested for cross-reactivity to the monoclonal antibody generated against ractopamine.

weeks old, were initially immunized by intraperitoneal injection with 100 μg of ractopamine−glutarate−KLH (Shelver and Smith, 2000) mixed with complete Freund's adjuvant. Four booster immunizations were administered at 30-day intervals, consisting of antigen emulsified with incomplete Freund's adjuvant. Blood was collected 10−14 days after the last booster immunization in order to check for antibody titers. Titers were checked by both indirect ELISA and competitive ELISA that employed ractopamine−glutarate−BSA as the coating antigen and racemic ractopamine as competitor. Two mice that produced high titers (1:16 000) after the 4th boost, and showed competition toward ractopamine, were used for fusion experiments. Four days prior to splenocyte harvest, mice were injected with 50 μg of antigen through their tail veins.

Monoclonal Antibody Generation. Murine myeloma cells Sp2/0Ag14 were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (designated as complete medium). The culture medium supernatant from the Sp2/0Ag14 was used as the conditioned medium and replaced feeder cells in the fusion and cloning experiment. Splenocytes were fused with Sp2/0Ag14 murine myeloma cells by fusion, selection, cloning, expansion, and ascites generation procedures described by Barrett (1994). Briefly, splenocytes from two mice were harvested and fused with Sp2/0Ag14 cells by PEG 1500. Fused cells were suspended in complete medium supplemented with 30% conditioned medium and plated into a total of 13 96-well cell culture plates. The HAT selection was performed from days 3 to 14 postfusion by supplementing 10 μM sodium hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine to the complete medium. Two weeks after cell fusion, hybridomas were screened for their ability to produce antibodies that recognize ractopamine by indirect ELISA. A noncompetitive and a competitive indirect ELISA were performed simultaneously with ractopamine−glutarate−BSA as coating antigen. The protocol of ELISA procedure is essentially the same as described in the specificity determination section. Positive hybridomas were expanded into 24-well cell culture plates followed by culturing in T-flasks and were cloned twice by a limiting dilution technique. Four hybridomas, namely, 4B7, 5G10, 7D12, and 13B2, were chosen for ascites generation. Nomenclature of hybridomas was based on the plate number and particular well from which the hybridoma originated. Mice were primed with pristane (ip) 12 days prior to the injection of 1×10^6 (4B7, 5G10, and 13B2) or 5×10^5 (7D12) cells. Ascites were collected 1 week after injection of the cloned cells and were frozen at −20 °C until used. Hybridomas were pelleted by centrifuging at 400*g* and were resuspended into DMSO freeze medium and cryopreserved in liquid nitrogen.

Monoclonal Antibody Isotyping. Antibody type was determined following the manufacturer's instructions supplied with a commercial kit (Pierce, Rockford, IL). Briefly, 50 μL/well of a 5 μg/mL ractopamine−glutarate−BSA solution was coated on a 96-well ELISA plate in 50 mM sodium bicarbonate buffer (pH 9.6) and the plate was kept overnight at room temperature. After the removal of coating antigen, the plate was blocked with 125 μL/well 0.5% BSA at 37 °C for 1 h. The plate was washed four times with PBST, 50 μL of ascites 1:1000 was added to each well, and the plate was incubated at 37 °C for 1 h. After the plate was washed four times with PBST, subclass rabbit anti-mouse immunoglobulins (IgG) were added to each well and incubated at 37 °C for 1 h. The plate was washed, horseradish peroxidase-conjugated goat anti-rabbit IgG was added, and the plate was incubated at 37 °C for 1 h. After the plate was washed, ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate solution (100 μL/well) was added, and the plates were incubated 20 min at room temperature and then read at 415 nm.

Specificity Determination. The checkerboard method was used to determine the optimum amount of coating antigen−ractopamine−glutarate−BSA (0.1−1 μg/well), primary antibody (1:4000−1:14 000), and secondary antibody (1:5000−1:50 000) required for the indirect competition ELISA. After optimization, a competitive ELISA procedure was used to determine the inhibition concentration (IC_{50}) for ractopamine and structurally related compounds described below. Ninety-six-well flat-bottom ELISA plates were coated with 500 ng/well ractopamine−glutarate−BSA in 50 mM bicarbonate buffer, pH 9.6, and allowed to shake for 2 h on an orbital shaker, after which the plates were washed 5 times with PBST. Excess binding sites in each well were blocked by incubation with a 1% solution of Teleostean cold water fish gelatin for 1 h, after which each well was washed 5 times with PBST. Fifty microliters of primary antibody (5G10 ascites batch 10/7/98, diluted to 1:14 000) per well was added to competitor (100 μL/well) and the plate was incubated for 1.5 h. Competitors were incubated at concentrations of 0, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000, and 10 000 ng/mL. A racemic ractopamine calibration curve was used in every experiment as a quality control. Washing wells five times with PBST terminated the reactions. Goat anti-mouse IgG peroxidase conjugate, diluted 1:50 000 (100 μL/well), was incubated for 1 h. A TMB substrate solution (0.2 g/L TMB dissolved in 0.01% citric acid buffer in the presence of peroxidase) was used during color development (30 min); the color developments were

stopped by adding 50 μL/well 2 N H_2SO_4. All incubations were conducted at 37 °C. The plates were read at 450 nm and the data were analyzed with a four-parameter logistic equation for the competitors that produced competition curves.

Matrix Effects and Assay Parameter Determinations. Ractopamine (0, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000, and 10 000 ng/mL) was added to bovine urine samples diluted 1:2, 1:5, 1:10, and 1:20 with PBST. Urine was collected from a Holstein cow being used for an unrelated study that had not received any ractopamine treatment. Immunoassays in diluted matrixes were run according to the procedure described above. The resulting competition data were fit to a four-parameter logistic equation by use of the program manager provided by the plate reader (Bio-Rad Laboratories, Hercules, CA) and the IC_{50} values were determined. The IC_{50} and B_0 from each diluted urine curve were compared with IC_{50} and B_0 values generated from the PBST standard curve. Ractopamine in a 1:10 urine dilution at concentrations of 1, 5, 10, and 20 ng/mL were divided and stored at −20 °C until used. For determination of intraassay variation, each concentration of the spiked urine samples was pipetted into 12 wells, along with the appropriate standard curve (0−10 μg/mL, 12 levels) and the data were processed with the spiked samples as replicated unknowns. Interassay variation was determined in a similar manner on five separate days with duplicate wells for each levels of spiked sample. Results from intra- and interassay experiments were used to test the precision and accuracy.

RESULTS AND DISCUSSION

Monoclonal Antibody Generation and Isotyping Determination. Sera obtained from immunized mice were used to determine antibody titers and the ability of the antibody to recognize ractopamine. Because mice were immunized with ractopamine−glutarate−KLH and the ELISA used ractopamine−glutarate−BSA as the coating antigen, the assay measured the ability of the antibody to recognize ractopamine. The use of different carrier proteins for immunization and ELISA provided a facile method to identify hapten-active antibodies.

After HAT selection, microscopic inspection revealed cell growth in 137 out of 1248 wells. Of the active wells, 23 tested positive toward the ractopamine hapten by ELISA methodology. Hybridomas that showed continuous growth, the ability to withstand liquid nitrogen freezing and subsequent thawing, and the ability to continuously secrete antibody able to compete for ractopamine were selected for cloning. Four hybridomas were chosen for cloning and isotype determination: 4B7, IgMλ; 5G10, IgG1κ; 7D12, IgG1κ; and 13B2, IgG1κ. Antibody sensitivities, based on their ability to compete with racemic ractopamine, were 5G10 > 13B2 > 4B7 > 7D12. Because the antibody produced from the clone, 5G10, was the most sensitive toward ractopamine, it was chosen for further evaluation of antibody specificity and subsequent immunoassay development.

Antibody Specificity. Table 1 shows the selectivity of the antibody produced by hybridoma 5G10. In absolute measures, the antibody was most selective toward the (1*R*,3*R*) stereoisomer of ractopamine with a cross-reactivity of 489% relative to the racemic mixture. With the α-methyl group on the butylamine moiety in the (*S*)-configuration (i.e., 1*R*,3*S*-ractopamine), antibody binding decreased by a factor of 100, indicating the stereochemical configuration at this carbon was critical to efficient binding. Although the stereochemical orientation of the β-hydroxyl group (the benzylic alcohol) affected antibody binding, its effect was less pronounced than that of the α-methyl group. For example, the (1*S*,3*R*) and (1*S*,3*S*) stereoisomers had IC_{50} values that were approximately 134% and 1.9% of racemic ractopamine, respectively. Relative to the (1*R*, 3*R*) stereoisomer, the (1*S*,3*R*) stereoisomer reducing binding by a factor of 4. The (1*S*,3*S*) stereoisomer of ractopamine, with a stereochemistry "opposite" that of the most efficiently bound isomer, also exhibited poor binding relative to stereoisomers with the 3-carbon in the *R* configuration. These results clearly indicate the antibody binds most efficiently to ractopamine isomers in the *R* configuration at carbon 3.

Table 1. IC_{50} and Percentage Cross-Reactivities of Ractopamine, Ractopamine Stereoisomers, Ractopamine Metabolites, Ractopamine Analogues, and Selected Phenethanolamine β-Agonists

compound	IC_{50}[a]	cross-reactivity[b] (%)
ractopamine (racemic)	2.69 ± 0.36	100
(1*R*,3*R*)-ractopamine	0.55 ± 0.09	489
(1*R*,3*S*)-ractopamine	291 ± 32	0.9
(1*S*,3*R*)-ractopamine	2.00 ± 0.37	134
(1*S*,3*S*)-ractopamine	140 ± 23	1.9
R-des-OH-ractopamine	1.59 ± 0.09	169
S-des-OH-ractopamine	25.2 ± 1.9	10.7
dobutamine	50.3 ± 8.1	5.3
LY99418	232 ± 26	1.2
LY227274	250 ± 40	1.1
LY227273	2.62 ± 0.08	103
ractopamine glucuronide A	263 ± 20	1
ractopamine glucuronide B	87.8 ± 5.0	3.1
ractopamine glucuronide C	0.70 ± 0.04	384
ritodrine	73.8 ± 4.4	3.6
fenoterol	2682 ± 350	0.1
bamethane	831 ± 11	0.3
isoxsuprine	1391 ± 127	0.2
salmeterol	1519 ± 116	0.2
clenbuterol[c]		<0.1
salbutamol[c]		<0.1

[a] Data represent three separate experiments run on three different days. The value for racemic ractopamine represents the average of 15 experiments performed over a 3-month period. The IC_{50} was the competitor concentration where absorbance value was decreased in half compared to the absorbance value of no competitor. [b] Percentage cross-reactivity is defined as the ratio of the test compound's IC_{50} to that of racemic ractopamine. [c] Clenbuterol and salbutamol did not produce competition curves.

The importance of the stereochemical configuration of the α-methyl group (C-3), and the less significant effect of the β-alcohol (C-1) configuration, was confirmed with the ractopamine analogue deshydroxyractopamine (Figure 1), which completely lacks a β-hydroxy group but maintains the stereochemical configuration at C-3. The IC_{50} of *R*-deshydroxyractopamine (1.59 ng/mL) was comparable to that of (1*S*,3*R*)-ractopamine. In contrast, *S*-deshydroxyractopamine had an IC_{50} of 25 ng/mL, confirming that the *R* configuration is favored at the 3-carbon. These data demonstrate that with the proper configuration of the α-methyl group, the β-hydroxyl group does not contribute a great deal to binding. When the configuration of the α-methyl group was *S*, the presence of a β-hydroxyl (ractopamine stereoisomers) decreased binding further to the extent that more efficient binding was observed for the deshydroxy compounds.

Data obtained with dobutamine (Figure 1), a structural isomer of ractopamine, confirmed observations made with deshydroxyractopamine. Dobutamine, having the same *N*-alkyl substituent as ractopamine, showed significant binding to the antibody and confirmed the importance of the *N*-alkyl group in antibody

binding. Because the dobutamine used in this study was racemic, the effect of its stereochemistry was not assessed.

The importance of ractopamine's phenolic groups in antibody binding was assessed by use of a series of methylated analogues (Figure 1) as well as synthetic ractopamine−glucuronide metabolites (Figure 2). LY99418, LY227273, and LY227274 were each used for competition assays, of which only LY227273 exhibited appreciable binding with an IC_{50} of 2.6 ng/mL. Because LY227273 has a free *N*-alkyl phenolic group and the other two analogues are methoxylated at this position (Figure 1), the disparate IC_{50} values obtained (2.6 vs 233 and 250 ng/mL for LY227273, LY99418, and LY227274, respectively) clearly indicate the advantage for antibody binding of a free *N*-alkyl phenol.

The specificity of the antibody for the free *N*-alkyl phenol was unequivocally confirmed by binding experiments with synthetic ractopamine glucuronides. Ractopamine glucuronides A and B were both conjugated to the *N*-alkylphenol of ractopamine, whereas ractopamine glucuronide C was conjugated to the ethanolamine phenol. Relative to the (1*R*,3*R*) stereoisomer of ractopamine, ractopamine glucuronides A and B were over 100 times poorer ligands for the monoclonal antibody. In contrast, ractopamine glucuronide C bound to the antibody with an affinity almost as great as that of (1*R*,3*R*)-ractopamine. These results are consistent with results obtained from studies with the ractopamine analogues indicating the importance of a free *N*-alkylphenol for antibody binding and are also consistent with binding studies using individual ractopamine stereoisomers. For example, ractopamine glucuronide A, which exhibited the least affinity among the glucuronides for the monoclonal antibody, is composed only of the (1*R*,3*S*) and (1*S*,3*R*) stereoisomers (66% and 33% of total, respectively); glucuronide conjugates of these stereoisomers would not be expected to have low IC_{50} values. The ractopamine glucuronide conjugate containing the (1*R*,3*R*) stereoisomer (B), however, had an IC_{50} (87 ng/mL) value considerably lower than that for ractopamine glucuronide A. Presumably, the 3-fold increase in affinity of the antibody for ractopamine glucuronide B, relative to A, was due to the presence of the (1*R*,3*R*) isomer in B. Ractopamine glucuronide conjugate C, which had a stereochemical composition of 27%, 19%, 28%, and 26% (1*R*,3*R*), (1*S*,3*R*), (1*S*,3*S*), and (1*R*,3*S*) stereoisomers, respectively, had an affinity for the antibody almost as great as that of (1*R*,3*R*)-ractopamine, having a cross-reactivity relative to racemic ractopamine of 384%. At this time we do not have a ready explanation of why ractopamine glucuronide conjugates composed of all four stereoisomers have a binding affinity greater than unconjugated racemic ractopamine.

Regardless of the mechanism of antibody recognition for the individual stereoisomer conjugates, the fact that the antibody is extremely sensitive to this metabolite is an excellent property for off-label screening purposes. Dalidowicz et al. (1992) and Smith et al. (1993, 1995, 2000) have each reported that ractopamine glucuronides are the major metabolites of ractopamine in various species; these glucuronides, including glucuronide C, are present in edible tissues (Dalidowicz et al., 1992) and excreta of animals dosed experimentally with ractopamine hydrochloride. Thus, it is highly likely that animals dosed with ractopamine in an off-label manner can be tested and the presence of either the parent ractopamine or its metabolite can be detected. These concepts are currently being validated in this laboratory.

As the *N*-alkyl substituents of β-agonists diverged from that of ractopamine, the antibody binding decreased substantially (Table 1). Ritodrine and fenoterol (Figure 3), which have *N*-β-(4-hydroxyphenyl)ethyl substituents, rather than ractopamine's *N*-γ-(4-hydroxyphenyl)propyl substituent, had binding affinities 30−1000 times lower than that of racemic ractopamine. With further divergence in structure from ractopamine, antibody binding became negligible or nearly so. The β-agonist most commonly associated with illegal use, clenbuterol, did not cross-react with the 5G10 antibody.

Matrix Effects and Assay Parameter Determinations. The linear range of the percentage B/B_0 vs log ractopamine concentration competition curve generated in PBST was 0.8−12 ng/mL. The limit of detection for racemic ractopamine with a cutoff criterion of 80% B/B_0 was 0.5 ng/mL. Although in most cases the affinity constants for monoclonal antibodies are generally lower than for polyclonal antibodies, we found the 5G10 monoclonal antibody had an affinity for racemic ractopamine comparable to that for polyclonal antibodies generated from rabbits (IC_{50} 2.7 vs 4.2 ng/mL) (Shelver and Smith, 2000). The major difference is that the 5G10 monoclonal antibody is more selective than the polyclonal antibody. This does not surprise us, because monoclonal antibodies recognize a single epitope while polyclonal antibodies respond to multiple epitopes. In addition, the monoclonal antibody had excellent sensitivity toward a single stereoisomer of ractopamine, having a detection limit of 0.1 ng/mL in PBST. This sensitivity, in addition to its stereoselectivity, is a consequence of the single-epitope-recognizing nature of monoclonal antibodies.

Bovine urine was used for the determination of the immunoassay's ruggedness and its potential application in a real-world situation. We chose to use bovine urine instead of swine urine for this study to simulate the off-label usage for cattle. Racemic ractopamine was used in this study instead of (1*R*,3*R*)-ractopamine because the feed additive used the racemic mixture. The mean B_0 (antibody binding with no competitor present) for urine dilutions 1:2, 1:5, 1:10, and 1:20 had absorbances of 0.17, 0.34, 0.63, and 0.95 ($n = 3$ per dilution), respectively, compared to 1.27 for antibody in PBST. The IC_{50} values, with racemic ractopamine as the competitor, were 11.58, 4.62, 3.16, and 3.02 ng/mL compared with 2.49 ng/mL for buffer. Because urine diluted 1:10 caused only a small effect on the assay based on the deviation of the IC_{50} from PBST, a 1:10 dilution was used to generate the precision and accuracy data. Although the 1:20 dilution showed somewhat less of a matrix effect, the loss of sensitivity caused by the dilution makes the use of this dilution unattractive. Table 2 indicates intraassay variation was with a coefficient of variation below 6%. Recoveries were within 20% of theoretical values, indicating acceptable accuracy. Coefficients of variation values ranging from 16% to 38% were obtained for the interassay variation experiments. As might be expected, variation was greatest for the low-concentration (1 ng/mL) samples. These results demonstrate that the immunoassay could be used with no sample preparation (no organic solvent extraction is needed) other than simple dilution. Further validation studies are underway to test the effectiveness

Table 2. Intra- and Interassay Variations for the Determination of Racemic Ractopamine Spiked into Diluted (1:10) Urine

spike level (ng/mL)	found	% recovery	% CV
Interassay Variation ($n = 5$)			
1	1.1 ± 0.4	110	38
5	3.9 ± 0.6	78	16
10	9.5 ± 1.7	95	18
20	22.4 ± 5.6	112	25
Intraassay Variation ($n = 12$)			
1	1.2 ± 0.06	120	4.6
5	4.2 ± 0.2	84	4.8
10	10.4 ± 0.6	104	5.8
20	20.6 ± 0.7	103	3.2

of the antibody in samples collected from ractopamine-treated animals.

The monoclonal antibody has sufficient sensitivity for determination of ractopamine in tissues. In swine, the tolerance for the marker residue (parent ractopamine) is 150 ppb in liver and is 50 ppb in muscle. Because the antibody cross-reacts with parent ractopamine and ractopamine metabolites, quantitative determinations of ractopamine residues in tissues may be best accomplished after enzymatic or chemical hydrolysis of the metabolites. Smith et al. (1995) have demonstrated that ractopamine metabolites are readily hydrolyzed by hydrolytic enzymes, unlike the glucuronide conjugates of clenbuterol (Shelver and Smith, 2000b), so an accurate quantitation is possible. Dalidowicz et al. (1992) have demonstrated that total residues of [^{14}C]ractopamine were 60 and 20 ppb in livers and kidneys, respectively, of hogs 7 days after the last administration of 1.5 times the approved level of 20 ppm dietary ractopamine. Because the antibody detects both parent ractopamine and its metabolites, and because of its excellent sensitivity to parent ractopamine and its metabolites, we anticipate that detection of off-label use by monitoring liver or kidney tissues is possible, even with at least a 7-day withdrawal period. We are currently testing this hypothesis on a number of species.

Monitoring urine for ractopamine or ractopamine metabolites might also be viable for monitoring for off-label ractopamine use, especially in situations in which the subject will not be immediately slaughtered (horses or animals at livestock competitions) or in instances of suspected use by athletes or body builders. Urine is a major route of ractopamine excretion for swine (Dalidowicz et al., 1992), turkeys (Smith et al., 1993), and humans (FDA, 2000) with both parent ractopamine and conjugated metabolites being excreted in each. With the use of hydrolytic enzymes to convert ractopamine conjugates to parent ractopamine, a quantitative assay could be developed.

Positive results obtained with screening assays require subsequent confirmatory mass spectrometric assays. The utility screening assays are thus somewhat dependent upon the availability of flexible confirmatory assays. To this end, confirmatory assays for ractopamine in edible tissues of several species are currently being validated.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); BSA, bovine serum albumin; CV, coefficient of variation; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine−aminopterine−thymidine; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine.

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