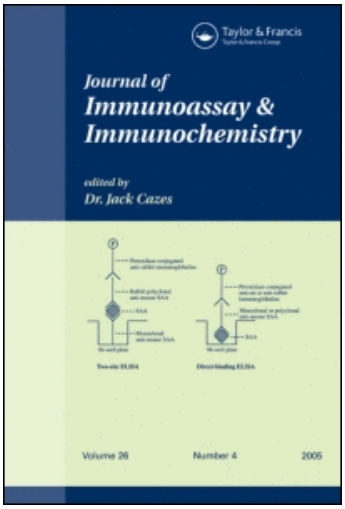

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DEVELOPMENT OF AN IMMUNOASSAY FOR THE β-ADRENERGIC AGONIST RACTOPAMINE

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ABSTRACT

Antibody generated from ractopamine-hemiglutarate-KLH was used to develop a ractopamine ELISA. The antibody showed good sensitivity in phosphate buffer, with an IC_{50} of 4.2 ng/ml (ppb) toward ractopamine and 16.2 ng/ml toward glucuronides of ractopamine conjugated to the phenethanolamine phenol of ractopamine. Phenylbutylamine phenol glucuronides of the (*RS, SR*) ractopamine diastereoisomers showed about 4% cross-reactivity, but the glucuronide of the (*RR, SS*) diastereoisomers conjugated at the same phenolic group showed no detectable reactivity with the antibody. The antibody generally had cross-reactivity towards compounds with bis-phenylalkyl amine structures rather than compounds with simple branched N-alkyl substituents. For example, the antibody showed little or no cross reactivity towards clenbuterol, isoproterenol, metaproterenol, and salbutamol, but cross-reacted with dobutamine. The system demonstrated a matrix effect similar to other enzyme immunoassays, dilution of urine decreased but did not eliminate the matrix effect.

Key Words: Ractopamine, immunoassay, ELISA, β-agonists, cross-reactivity

INTRODUCTION

β-Adrenergic agonists have been used as bronchiodilators in human medicine for over 30 years. Unfortunately, these agents have also been used illegally to improve performance of human athletes and show animals. Their illegal use in livestock production has led to toxic effects after human consumption of meat products (1). The European Union has banned the use of β-adrenergic agonists as growth promoters in livestock and for improving athletic performance (2). In the US, the FDA, in cooperation with the Food Safety Inspection Service (FSIS), has detected illicit β-adrenergic agonists in show animals (3). Consequently, the illicit use of these compounds represents a potential problem and analytical methods are needed for their detection.

Ractopamine (Figure 2) is reportedly under development for registration in the United States (4) and has a leanness enhancing action in many species. For example, lipolysis was stimulated by ractopamine in porcine adipose tissue (5), total and myofibrillar protein synthesis was increased by ractopamine in rat myotubes (6). Smith and Paulson investigated the repartitioning effect of ractopamine in rats and observed that when administered by the intraperitoneal route animals had increased weight gains with improved feed efficiency (7). At dietary treatment levels (at a ppm level), ractopamine treated swine had larger longissimus muscle, less rib fat, and leaner hams than control animals (8). If ractopamine became available through the black market there is adequate data available which shows its efficacy in livestock.

For regulatory purposes, an immunoassay may serve as a screen to detect the presence of an analyte which could be confirmed by instrumental methods such as GC-MS or LC-MS. Immunoassays have the advantage of rapidity, mobility, and high sensitivity with detection limits in the ng/ml range. High sensitivities of immunoassays are particularly desirable because they can be used to detect the analyte after extended withdrawal periods. Several immunoassays (IA) have been developed to measure clenbuterol (9) and structurally related phenethanolamines such as albuterol (10). However, few of the commercial available IAs have shown useful cross-reactivity toward bis-phenyl-substituted β-adrenergic agonists such as ractopamine (11).

Ractopmaine can be analyzed by HPLC with various detection methods or by GC-MS. Using HPLC incorporated with electrochemical detection, Turberg *et al.* were able to detect Ractopamine in animal feeds (12), tissues (13), monkey plasma, and swine serum (14), and were able to achieve a ng/ml level of sensitivity. However, the HPLC methods requireded extensive sample preparation. Haasnoot *et al.* reported an IA for fenoterol and ractopamine in calf urine (2). The antibody was derived from a fenoterol derivative and showed 20% cross-reactivity with ractopamine, but the analysis of ractopamine did not correlate well with GC-MS analysis. Elliott *et al.* (15) reported the generation of an antibody to detect ractopamine which correlated with LC-MS-MS (r^2 of 0.73) when samples were enzymatically hydrolyzed prior to analysis. The enzyme digestion steps were needed prior to extracting the parent ractopamine with organic solvent. Neither Elliot *et al.* or Haasnoot *et al.* specifically addressed the cross-reactivity toward ractopamine

metabolites. Because ractopamine is extensively metabolized (major metabolites include ractopamine-mono-glucuronides; a ractopamine glucuronide, sulfate diconjugate; and a ractopamine-sulphate-ester (16-17)), analysis of fluids or tissues must address the cross-reactivity of these metabolites. We report the development of a ractopamine immunoassay and the measurement of cross-reactivity toward a number of β-adrenergic agonists as well as purified ractopamine metabolites.

MATERIALS and METHODS

Chemical and Reagents:

[^{14}C]Ractopamine, 5.71 μCi/mg, was a gift from Lilly Research Laboratories, Greenfield, IN, USA. Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise. ECH-sepharose was obtained from Pharmacia Biotech (Uppsala, Sweden). All solvents were analytical grade or higher.

Synthesis of Ractopamine-hemiglutarate:

The hapten synthesis was similar to that of Brunswick (18). Several batches of hapten were made using the following general procedure. Ractopamine HCl (34 mg; 0.1 mmole), with a trace amount (3 x 10^5 dpm) of [^{14}C]ractopamine, was reacted with 12 mg (0.1 mmole) of glutarate anhydride in the presence of 2 ml pyridine. The reaction was stirred overnight at room temperature. The extent of the reaction was ascertained using silica gel thin layer chromatography (TLC) developed by 90%

CH_2Cl_2:10% methanolic NH_4OH (10%). The plates were scanned for ^{14}C activity using a TLC scanner (BIOSCAN, Inc. Washington, D. C., USA). After the reaction was complete, the pyridine was evaporated under a stream of nitrogen and the ractopamine hemiglutarate was used for carrier protein conjugation.

Synthesis of Immunogens:

The hapten (ractopamine hemiglutarate) was linked to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in separate reactions. The KLH conjugate was used for the immunization of rabbits and BSA conjugate was used as the coating antigen for the ELISA study. The use of different carrier proteins assures the assay is not complicated by recognition of the protein portion of the immunogen.

Immunogen was formed by dissolving ractopamine hemiglutarate (0.1 mmole) in 4 ml of DMF:1,4-dioxane (1:1) and adding 26.2 µl (0.11 mmole) of tributylamine. The mixture was stirred on ice for 10 min, isobutylchloroformate (0.11 mmole) was added, and the reaction was brought to room temperature and stirred for 1 hr. The mixture was added dropwise to an ice-cold protein solution (100 mg BSA, or 50 mg KLH dissolved in 0.1 M sodium borate, pH 8.5). The resulting solution was brought to room temperature and allowed to react overnight. The final solution was dialyzed against several changes of phosphate buffered saline (PBS) (each liter contained 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in H_2O) and the conjugation ratios were checked by measuring the radioactivity incorporated in the protein or by

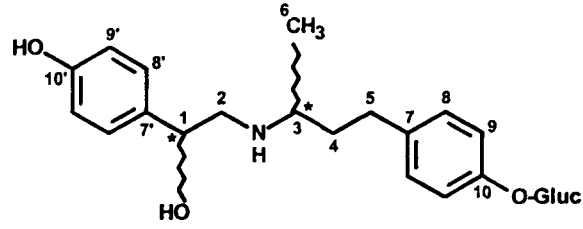
comparing the apparent molecular weight of conjugate to carrier protein on 10% SDS-PAGE.

Ractopamine-Glucuronide Isolation and Identification:

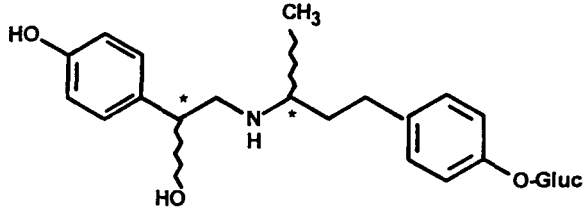
[^{14}C]Ractopamine-glucuronides were synthesized according to Smith *et al.* (16) and the diastereoisomers were purified using liquid chromatography. A known amount of [^{14}C]Ractopamine-glucuronides were injected into a Prodigy ODS-3 column (4.6 x 250 mm, 5μ) (Phenomenex; Torrence, CA) and eluted with an isocratic system consisting of 87.5% of 0.05 M ammonium acetate buffer (pH 4.5) and 12.5% acetonitrile at a flow rate of 1 ml/min. Peaks corresponding to individually resolved ractopamine-glucuronide diasteroisomers were collected into vials as they eluted from the column. The amount of isomer in each peak was quantitated by liquid scintillation counting. Ractopamine-glucuronide peaks A, B, and C, have been unambiguously identified (16) and their structures shown in Figure 1. Ractopamine glucuronide A was conjugated to the C-10 phenol of the (*RS, SR*) isomers. Ractopamine glucuronide B was glucuronidated to the C-10 phenol of the (*RR, SS*) isomers. Conjugate C was glucuronidated at the C-10' position and was a mixture of all ractopamine stereoisomers (*RR, RS, SR, SS*).

Antibody Preparation and Characterization:

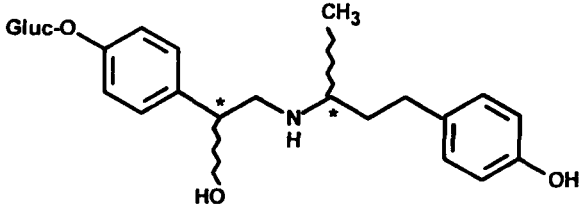
Antibodies were generated using New Zealand white rabbits. Three rabbits were obtained from Mytle's Rabbitry Inc. (Thompson Station, TN). Each rabbit



Peak A (RS, SR)



Peak B (RR, SS)



Peak C (RR, RS, SR, SS)

FIGURE 1: Structure of Ractopamine glucuronides, arabic numbers represent the nomenclature system. Glucuronidation occured at either C-10 or C-10'. The asterisks indicates chiral carbons.

received 200 μg of immunogen in Complete Freund's Adjuvant (CFA) for the first immunization followed by 7 additional monthly boosters with 200 μg of Incomplete Freund's Adjuvant. The antiserum used for the development of a competitive ELISA was obtained after the fifth boost.

The antiserum was purified by a ractopamine-ECH-Sepharose column. This column was generated by reacting 84.5 mg (0.25 mmole) of ractopamine HCl overnight with 5 ml of ECH-Sepharose (Pharmacia Biotech, Uppsala, Sweden) in the presence of 576 mg (3 mmole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) at pH 6 (4 °C). Unreacted ractopamine was washed off by alternating buffer changes between 0.1 M Tris (pH 8) and 0.1 M HAc (pH 4). Antiserum was diluted 1:2 with PBS and was loaded onto the ractopamine-ECH-Sepharose column. The column was washed with PBS and the antibody was eluted with 5% CH_3CN/95% 0.1 M propionic acid (v/v). Buffer exchange was performed using a desalting column (KwikSepa Dextran Plastic Desalting Columns; Pierce; Rockford, IL), and the final antibody solution was stored in PBS/ 0.2% BSA at –20 °C until used. The protein concentration was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and the purity of the antibody was confirmed by 10% SDS-PAGE run under reducing conditions.

Enzyme immunoassay procedure:

The checker board procedure was used to optimize the coating antigen and the primary antibody concentrations. To each well of a 96 well ELISA plate, 100 ng

of ractopmaine-hemiglutarate-BSA in 100 µl of 0.1 M bicarbonate buffer (pH 9.6) was incubated at room temperature for 2 hrs and then at 4 °C overnight. The excess binding sites were blocked by 1% fish gelatin for 1 hr. After removing the blocking solution, 100 ng of primary antibody was added to each well followed by buffer or competitor in buffer, and the plate was equilibrated at room temperature for 1.5 hrs. Alkaline phosphatase conjugated goat anti-rabbit IgG was diluted to 1:50,000 with PBS/Tween and 100 µl was pipetted into each well and incubated at room temperature for 1 hr. Color was developed by adding p-nitrophenyl phosphate and incubated at room temperature for 30 min and the color reaction was stopped by adding 50 µl of 3 M NaOH to each well. The plate was read using a Bio-Rad plate reader equipped with a 415 nm filter.

Antibody Cross-Reactivity Study:

Competitors used for the competition studies are shown in Figures 1, 2 and 3. These included both typical β-phenylethanolamine and bisphenylalkylamine β-agonists, as well as the three ractopamine metabolites. Cross-reactivity was measured by comparison of the IC_{50} produced by the competitor with ractopamine. Competitive ELISA was performed using an indirect assay format with competitor concentrations ranging from 0.1 ng/ml to 1 µg/ml, except for compounds with lower activity for which the upper concentration was 100 µg/ml. Compounds that produced sigmoidal like curves were fitted to the four parameter logistic equation using SAS (SAS

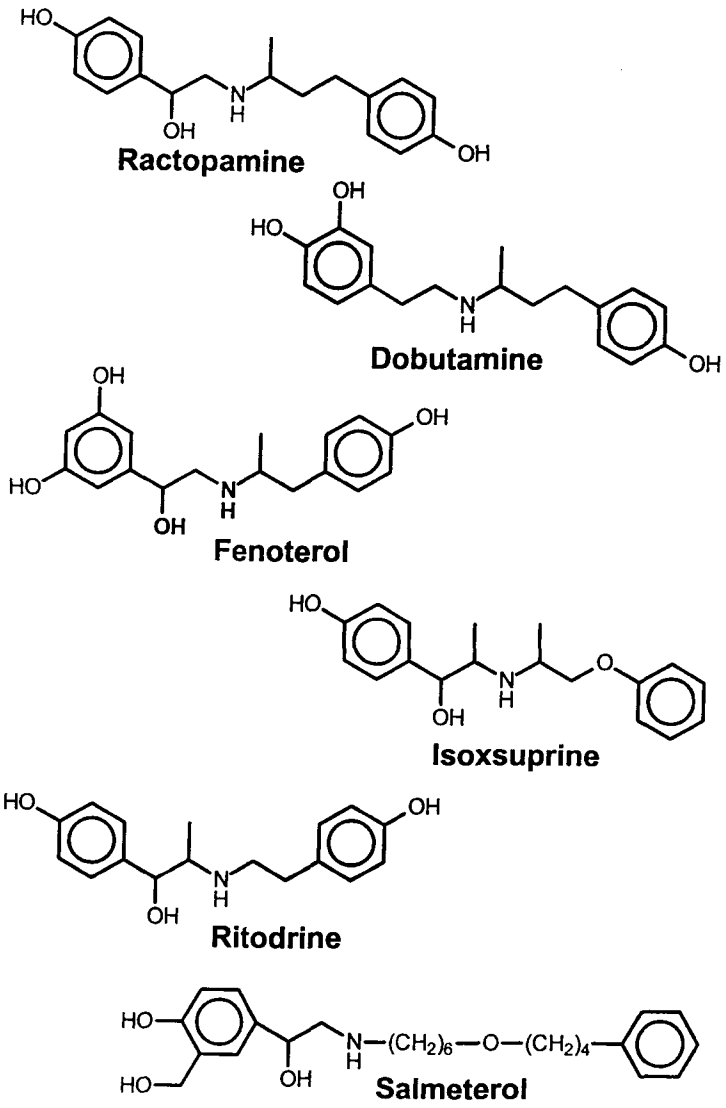


FIGURE 2: Structure of Ractopamine and related phenethanolamine β-adrenergic agonists with N-alkylphenyl substituents.

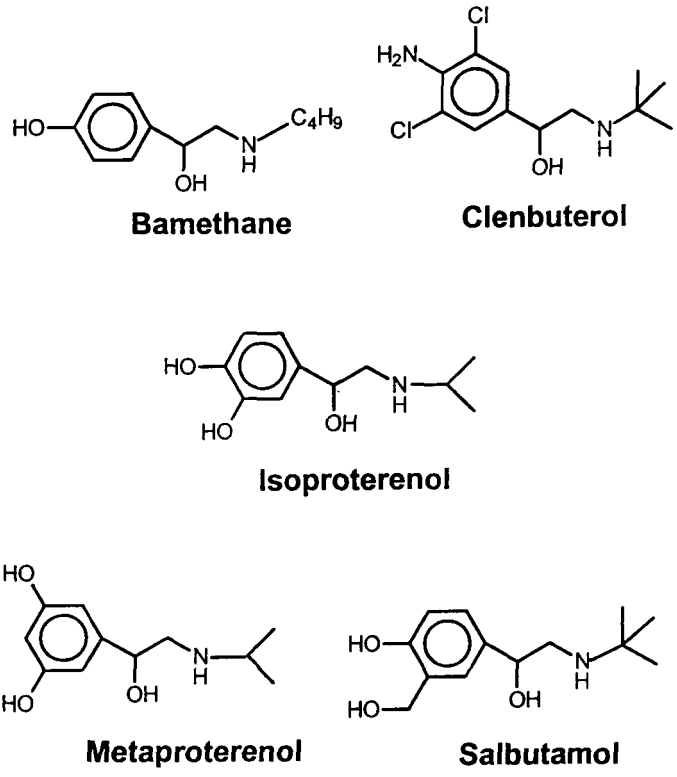


FIGURE 3: Structures of phenethanolamine β-adrenergic agonists with linker or bronchial chain N-alkyl substitution.

Institute Inc. Cary, NC) or Microplate Manager®/PC ver 4.0 (BIO-RAD Laboratories, Hercules, CA).

$$\%B/Bo = (A-D)/ [1+ (x/B)^C]+ D$$

Where A and D are the upper and lower asymptotes of the curve, B is the concentration which corresponds to the inflection point of the curve (IC_{50}), and C is the slope of the curve at the inflection point.

The Study of Matrix Effect:

Ractopamine (0.1 to 100 ng) was dissolved in PBS/0.05% Tween 20/0.2% BSA, or various dilutions of urine in buffer (1:2, 1:5, 1:10, and 1:20). Urine was collected from a Holstein cow being used for an unrelated study, but which had not received any ractopamine treatment. The competition curves were run as described for the immunoassay in order to determine the matrix effect of urine.

Assay Parameter Determination:

Known amounts of ractopamine were spiked into urine diluted to 1:10 by PBS/Tween and were analyzed using a twelve point calibration curve (0 -1 μg/ml). The ractopamine concentrations were calculated from the standard curve. Twelve replicates were used to define within-day variability and recovery. For inter-assay experiments, the concentrations were computed using a twelve point calibration curve and repeated on five different days to determine between-day variation and recovery.

RESULTS AND DISCUSSION

Antibody Preparation and Characterization:

The antibody titer appeared to plateau after the 3rd booster injection. Antibodies generated from ractopamine-hemiglutarate-KLH produced high ELISA backgrounds and were therefore purified using affinity columns. Purified antibodies produced sigmoidal competition curves with high sensitivities and little background.

Competition Curve:

Figure 4 illustrates typical competition curves produced by ractopamine and the ractopamine-glucuronide peak C using the affinity purified antibody. Both compounds produced sigmoidal dose-response curves. Clenbuterol, the β-adrenergic agonist documented to have been used illicitly, showed no competition. The sigmoidal curves were fit to a four parameter logistic curve indicating classical competition.

Antibody cross-reactivity study:

IC_{50}'s and cross-reactivities of the ractopamine antibodies towards competitors are shown in Table 1. Three general groups emerged for the competition curves. Group I consisted of those compounds eliciting S-shape competition curves and having an IC_{50} of less than 20 ng/ml (ractopamine, ractopamine-glucuronide peak C, and dobutamine). Group II compounds generated S-shape competition curves, but had IC_{50} values greater than 100 ng/ml (ractopamine-glucuronide peak A, fenoterol, ritodrine, salmeterol, bamethane, isoxsuprine and salbutamol). Group III included compounds that did not generate classical S-shape competition curves (clenbuterol, metaproterenol, (-) isoproterenol, and (+) isoproterenol). The IC_{50} of ractopamine was 4.2 ng/ml and indicates a useful sensitivity.

Cross-reactivity studies revealed the structural specificity of the antibody, and served as an important guide to the anticipation of compounds that may cross-react

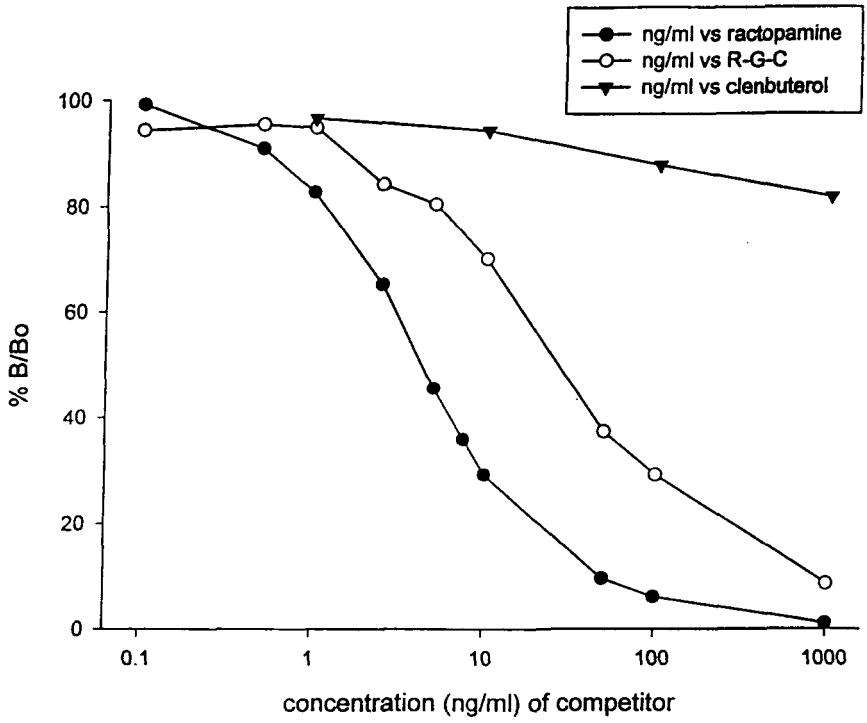


FIGURE 4: Representitive competition curves for ractopamine, ractopamine-glucuronide peak C, and clenbuterol.

and compounds that are unlikely to bind to the antibody. Cross-reactivity to glucuronides was diastereoisomer specific; for ractopamine glucuronided at C-10, the (*RS, SR*) diastereomers bound tighter than the (*RR, SS*) diastereomer glucuronide conjugates, although neither compound bound strongly. This indicated the C-10 phenolic group contributed significantly to antibody binding. The mixture of C-10' diastereomeric conjugates had close to 30% cross-reactivity demonstrating a strong interaction with the antibody. This indicated that blocking the C-10' phenolic group

TABLE 1

Cross-reactivity profile toward beta-adrenergic agonists from antiserum raised to R-glutarate-KLH.

Compound	IC_{50}[a]	% CR[b]	Compound	IC_{50}[a]	% CR[b]
Ractopamine	4.2	100	Bamethane	2,900	0.1
R-G-PK A[c]	155	2.7	Isoxsuprine	6,200	0.1
R-G-PK B	>1000[d]	<0.1	Salbutamol	79,000	<0.01
R-G-PK C	16.2	26	(-) Isoproternol	>100,000	<0.01
Dobutamine	12.7	33	(+) Isoproternol	>100,000	<0.01
Fenoterol	310	1.3	Metaproterenol	>100,000	<0.01
Ritodrine	577	0.8	Clenbuterol	>100,000	<0.01
Salmeterol	1800	0.2			

[a]IC_{50} was defined as the concentration (ng/ml) of competitor which produced 50% of the absorbance compared to no competitor.
[b]% CR is % cross-reactivity from the ratio of the competitor at IC_{50} to that of Ractopamine at IC_{50}.
[c]Abbreviation for Ractopamine Glucuronide Peak A (B or C).
[d]R-C-PK B has IC_{50} greater than 1μg/ml, but does not has enough mass to generate a sigmoid curve for IC_{50} calculations.

did not affect binding to the antibody showing that the C-10' phenolic group does not play a critical role in antibody binding.

Cross-reactivity with metabolites is important because glucuronides are major metabolites of ractopamine. Only 8% of urinary radioactivity was recovered as unconjugated ractopamine in turkey urine (16). Although the C-10 glucuronides are

the major metabolites found in turkey urine and in rat bile (17) significant amounts of the C-10' metabolites are also found. Thus this antibody could be used in screening assays to detect animals which had been treated with ractopamine because it interacts with both free ractopamine and its metabolites.

Dobutamine is structurally similar to ractopamine, but has an catecholic group and lacks the benzylic hydroxyl group of ractopamine (Figure 2). Our antibodies cross-reacted with dobutamine extensively, with an IC_{50} about 33 percent of ractopamine. This high cross-reactivity indicates that the benzylic hydroxyl group of ractopamine, as well as the catechol of dobutamine play a minor role in binding. Both compounds are bisphenylalkyl amines, but the two phenylalkyl groups do not interact in the same manner with the antibody. Consistent with this observation is the fact that ractopamine-glucuronide C, conjugated at the ethanolamine phenol of ractopamine (C-10'), showed the greatest cross-reactivity with the antibody generated toward ractopamine. Collectively, these data suggest that the 4-hydroxyphenyl isobutylamine region is of primary importance in antibody binding.

Fenoterol and ritodrine are less similar to ractopamine (Figure 2) than dobutamine and show less cross-reactivity with IC_{50}'s of 310 and 577 ng/ml respectively. For ritodrine and fenoterol, the N-substituents of the phenethanolamine place the aromatic group two carbons away from the nitrogen rather than the three found in ractopamine and dobutamine. The presence of alkyl groups containing oxygen (isoxuprine and salmeterol) futher decreases antibody binding.

Phenethanolamines showing no cross-reactivity contained terminal nitrogen substituents consisting of a small branched alkyl group (i-propyl, or t-butyl) with no aromatic group included in the substituent. Overall, the structural requirements for binding the ractopamine antibodies were specific and the antibody did not tolerate any structural changes in the general shape of the molecule.

In comparison with the results of Elliot *et al.* (15), our antibody showed a similar pattern of low cross-reactivity toward fenoterol (0.2% vs 1.3%) and isoxsuprine (0.1% for both groups). In both Elliot's work and the present work no cross-reactivity (less than 0.01%) of ractopamine antibodies was observed toward clenbuterol and salbutamol. Haasnoot *et al.* (2), using an antibody generated toward fenoterol, showed significantly greater cross-reactivity between fenoterol and ractopamine (20%) than did we or Elliot *et al.* Haasnoot *et al.* and Elliot *et al.* used the same linker (1,4-butanediol diglycidyl ether) to generate their antigens; in the current study hemiglutarate was used as the linker. The linker apparently has only a minor role in the antibody cross-reactivity.

The Study of Matrix Effect:

Figure 5 shows the reduction in absorbance was dependent on urine dilution. As the dilution of the urine increased from 1:2 to 1:20 the absorbance gradually increased to approach the buffer control values. The IC_{50s} of standard curves generated from diluted urines deviated from the IC_{50} generated from phosphate buffer

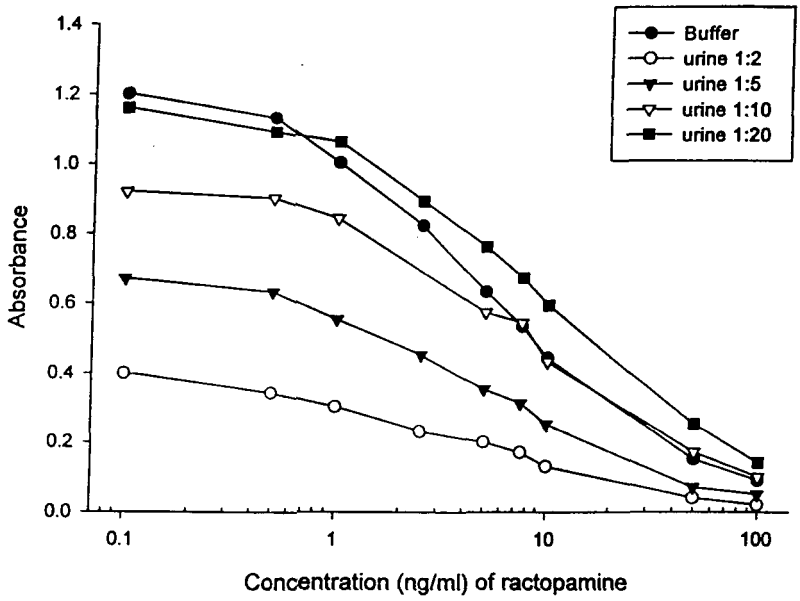


FIGURE 5: Effect of dilution of bovine urine on immunoassay results.

standard, but these deviations were small (data not shown). Matrix effects are commonly observed for immunoassays, and proper control is needed to ensure accurate results for individual matrices (urine, serum, tissue, etc...).

Assay Precision Determination:

Table 2 indicates the precision and accuracy of the assay. As expected, the between day variation is greater than the within day. The within day assay coefficient of variation was well below 10%. The between day coefficient of variation was more

TABLE 2

Precision and variable of inter-assay and intra-assay of ractopamine.

n	Expected[a]	Observed[a]	% Recovery	%CV
	Inter-assay	(between	days)	
5	1	1.98±1.00	198	50.5
5	5	4.63±0.64	92.6	13.8
5	10	9.28±2.58	92.8	27.8
5	20	17.5±3.24	87.5	18.5
	Intra-assay	(within	days)	
12	1	0.9±0.03	90	3.6
12	5	5.06±0.30	101	5.9
12	10	8.84±0.27	88.4	3.0
12	20	18.5±1.18	92.5	6.4

[a] Expected and observed values are based on ng/ml.

variable with the assay being reliable down to 5 ng/ml but not at 1 ng/ml.

Recoveries of the within days assay were good at all levels. The recoveries for the between days are high at the 1 ng/ml range but are acceptable at higher concentrations. The 1 ng/ml is close to the assay's detection limit of 0.3 ng/ml (based on a detection limit of B_0 minus two times the standard deviation) hence the larger variability and decreased accuracy are to be expected. Consequently, excellent recovery and accuracy are demonstrated up to the 1 ng/ml (1ppb) level.

CONCLUSIONS

Antibodies generated from ractopamine-hemiglutarate-KLH have sensitivities at the ppb level and are suitable for a screening assay for ractopamine. The specificity of the antibody was excellent, with common phenethanolamine β-adrenergic agonists showing only slight cross-reactivity. The exception was dobutamine, a mixed α-, β-adrenergic agonist that is a structural isomer of ractopamine. The significant cross-reactivity between ractopamine and ractopamine-glucuronide is beneficial for screening because ractopamine is extensively metabolized and this cross-reactivity would increase the sensitivity of the screening assay.

The distance between the nitrogen and the aromatic group in the alkylphenyl side chain was the most critical factor in determining cross-reactivity. Alteration of this distance or substitution of oxygen for carbon decreased cross-reactivity. The phenethanolamine group found in beta-adrenergic agonists was not sufficient to introduce significant cross-reactivity.

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