2742 J. Agric. Food Chem. 2002, 50, 2742–2747

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Application of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for the Determination of Ractopamine in Incurred Samples from Food Animals

WEILIN L. SHELVER* AND DAVID J. SMITH

Biosciences Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 5674 University Station, Fargo, North Dakota 58105

A monoclonal antibody-based ractopamine immunoassay has been applied to incurred samples from sheep and cattle. Results obtained by immunoassay were compared with those from high-performance liquid chromatography (HPLC). Three sets of sample extracts containing primarily unmetabolized ractopamine were analyzed. Correlation of HPLC with enzyme-linked immunosorbent assay (ELISA) for beef liver samples gave an $r^2 = 0.98$ despite rather low ractopamine concentrations (range 1.1−13.4 ng/mL, $n = 6$). Ractopamine concentrations in cow urine samples treated by solid phase extraction, to remove ractopamine metabolites, also showed a high correlation between the HPLC and the ELISA results ($r^2 = 0.95$, range 1.0−275 ng/mL, $n = 61$). In contrast, HPLC and ELISA analyses of ractopamine in sheep urine were not well-correlated ($r^2 = 0.58$, range 0.85−51 ng/mL, $n = 34$). When ractopamine conjugates in urine samples were hydrolyzed with hydrolytic enzymes, ELISA and HPLC methods were highly correlated [$r^2 = 0.94$ for sheep (range 123−10 554 ppb, $n = 60$) and an $r^2 = 0.98$ for cattle (range 14−8159 ppb, $n = 62$)]. Tissues contained only minute amounts of ractopamine, and after 7-day withdrawal periods, less than 1 ppb of free ractopamine was detected. Ractopamine was rapidly metabolized in both cattle and sheep. The difference in ractopamine concentration of urine samples before and after hydrolysis indicated that only 1−5% of ractopamine was excreted unmetabolized. Results from this study indicate that the monoclonal antibody-based ELISA could be useful for a sensitive, quantitative, or qualitative ractopamine screening assay.

KEYWORDS: β-Agonist; ELISA; monoclonal antibody; ractopamine; residue

INTRODUCTION

Ractopamine HCl (**Figure 1**) is an β-adrenergic agonist leanness-enhancing agent, recently approved as a swine feed additive by the U. S. Food and Drug Administration (FDA) Center for Veterinary Medicine (*1*, *2*) and by regulatory officials in Brazil, Venezuela, Colombia, Guatemala, the Dominican Republic, and the Philippines (*3*). Swine treated with 4.5−18 g/t (5−20 ppm dietary concentrations) of ractopamine have increased weight gains, improved feed efficiencies, and leaner carcasses (*4*). In addition, ractopamine has also been approved by the U. S. FDA Center for Veterinary Medicine for combination with tylosin in swine feeds to promote growth as well as to prevent ileitis (*5*).

β-Adrenergic agonists have a history of being illicitly utilized as growth promoters, and human food poisonings have resulted from the consumption of meat from illegally treated animals (*6*−*9*). Clenbuterol, in particular, has been associated with illicit β-agonist use (*10*, *11*). With the recent approval of ractopamine as a feed additive in the U. S., South America, and Asia, there is a great potential for its proscribed use. Therefore, a rapid survey method would be useful in order to determine animal exposure to ractopamine.

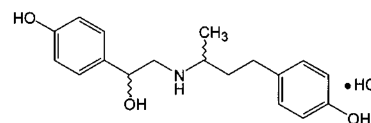

Figure 1. Ractopamine HCl.

Immunoassays are excellent survey tools because of their high throughput, user friendliness, and field portability. These important characteristics make immunoassays an attractive tool for food testing by regulatory agencies to ensure food safety. Several groups have generated immunoassays potentially useful for ractopamine analysis. Hassnoot et al. (*12*) generated a polyclonal-based ELISA for fenoterol that cross-reacted with

* To whom correspondence should be addressed. Tel.: 1-701-239-1425. Fax: 1-701-239-1430. E-mail: shelverw@fargo.ars.usda.gov.

10.1021/jf011372+ This article not subject to U.S. Copyright. Published 2002 by the American Chemical Society
Published on Web 02/07/2002

ractopamine, although with poor sensitivity (cross-reactivity of ractopamine was only about 20% that of fenoterol); matrix interference hindered further development of the assay (*13*). Elliott et al. (*14*) also generated a polyclonal antibody-based competition ELISA for ractopamine. Incurred urine samples were digested in order to convert metabolites into parent ractopamine, and ELISA results were compared with a liquid chromatography (LC)−mass spectrometry (MS)−MS confirmatory method. The correlation between the two methods was moderate, at $r^2 = 0.73$. A report using commercially available β-adrenergic agonist immunoassay kits demonstrated no cross-reactivity with ractopamine (*15*) indicating that they would not be useful for ractopamine analysis.

We have previously generated both ractopamine polyclonal (*16*) and ractopamine monoclonal (*17*) antibody-based ELISAs. Both assays were used to determine ractopamine at low ppb levels in preliminary tests. In addition, both the monoclonal and the polyclonal antibodies were characterized for their binding ability toward ractopamine metabolites and other β-adrenergic agonists. We found that both the monoclonal and the polyclonal ractopamine antibodies were able to bind the major metabolites of ractopamine, ractopamine−glucuronides, and did not recognize the most commonly used illicit β-adrenergic agonist clenbuterol. The monoclonal antibody was also stereoselective toward the physiologically active *RR* isomer (*18*, *19*) of ractopamine.

For this paper, food animals for which no ractopamine approval exists were dosed with 20 ppm dietary ractopamine for a period consistent to bring tissue residues to steady state. Tissues and excreta were collected for the measurement of ractopamine and/or ractopamine metabolites in incurred matrices. Here, we report the performance of the monoclonal-based ractopamine ELISA, relative to HPLC analyses, for measuring ractopamine in incurred tissues and urine from cattle and sheep.

MATERIALS AND METHODS

Chemicals and Reagents. Ractopamine HCl {(1*R**,3*R**),(1*R**,3*S**)-4-hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]methyl]-benzenemethanol hydrochloride} was a gift from Elanco Animal Health, Greenfield, IN. TMB substrate was obtained from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Sodium octanesulfonate was obtained from Regis, Morton Grove, IL. Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were reagent grade or higher. Glucuronidase/sulfatase from *Patella vulgata* was obtained from Sigma Chemical Co.

Instrumentation and Supplies. 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 enzyme immunoassay/radioimmunoassay plates were obtained from Corning Incorporated (Corning, NY). ELISA assays were analyzed using a BIO-RAD model 550 (Bio-Rad Laboratories, Hercules, CA) ELISA plate reader with Microplate Manager 4.0. SPE cartridges (C18, 500 mg, Bakerbond) were obtained from J. T. Baker (Phillipsburg, NJ). SPE cartridges (acidic alumina) were obtained from Waters (Milford, MA). A Visiprep vacuum manifold was purchased from Supelco (Bellefonte, PA). Solvents were evaporated using a centrifugal evaporator (Savant, Holbrook, NY).

Ractopamine Feeding Study. A detailed description of a ractopamine feeding study is available in a companion paper (*20*). Briefly, dietary ractopamine (20 ppm) was fed to cattle ($n = 6$) and sheep ($n = 6$) for 7−8 days and animals were euthanized with 0, 3, or 7 day withdrawal (2 animals/withdrawal period). Urine samples were collected from sheep and cattle daily during feeding and withdrawal periods. Tissues were collected at slaughter. Samples were stored at −10 °C until used.

Ractopamine Isolation from Tissue Samples. Tissues were analyzed for parent ractopamine using Elanco Method B03766. This validated regulatory method is available from the U. S. FDA (Center for Veterinary Medicine's Document Control Unit (HFV-199), FDA, 7500 Standish Place, Rockville, MD 20855). Briefly, ractopamine was extracted from 10 g of tissue samples with methanol, an aliquot of the extract was evaporated, and ractopamine was converted to the free base with borate buffer (pH 10.3) and extracted into ethyl acetate. The ethyl acetate layer was then passed through an acidic alumina SPE tube, and ractopamine was eluted with methanol. The methanol fraction was evaporated, and the residue was dissolved in diluted acetic acid. Ractopamine was analyzed using HPLC with fluorescence detection (excitation wavelength 226 nm, emission wavelength 305 nm). The HPLC mobile phase consists of 280 mL of acetonitrile, 720 mL of water, and 20 mL of acetic acid, containing 1.08 g of 1-octanesulfonic acid.

Analysis of Urine Samples. Cow and sheep urine samples were used for three separate competitive ELISA analyses: (i) analysis of raw urine samples after simple dilution, (ii) analysis of urine samples after SPE cleanup, without hydrolysis of ractopamine conjugates, and (iii) analysis of urine samples after hydrolysis of ractopamine conjugates and subsequent cleanup by liquid/liquid extraction and SPE. Smith and Shelver (*20*) detailed procedures for sample preparations used for analyses ii and iii.

ELISA of Diluted Urine. Cow and sheep urine samples were diluted 100−2500-fold with 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL of BSA. The degree of dilution was dependent on whether the urine was collected during ractopamine treatment or during the withdrawal period. Final dilutions were always such that ractopamine responses of the unknown samples fell within the dynamic range of the established calibration curve.

Analysis of Ractopamine after SPE without Hydrolysis of Ractopamine Conjugates. Briefly, C-18 SPE cartridges were preconditioned with 5 mL of methanol followed by a 10 mL water rinse before sample application. Sheep or cattle urine (1 mL) and an additional 1 mL of vial rinse were loaded onto the column. Columns were washed with 5 mL each of water, 50% MeOH/H_2O, and ractopamine was eluted with 50% MeOH/ammonium acetate buffer (0.05 M, pH 4.5).

Ractopamine Analysis after Enzyme Hydrolysis. Urine samples (1 mL) were mixed with 1 mL of 1 M ammonium acetate buffer, pH 5.0, and 50 μL of glucuronidase/arylsulfatase (5000 Fishman units) from *P. vulgata* was added. The mixture was incubated overnight using a shaking water bath (50 rpm, 37 °C). The reaction was terminated by the addition of 2 mL of 2 M sodium carbonate. The free base of ractopamine was extracted with ethyl acetate, the solvent was evaporated, and the residue was reconstituted in dilute HCl to form the HCl salt of ractopamine. The resulting solution was then applied to C-18 SPE tubes followed by rinses of water, 50/50 water methanol, and 100% methanol. Ractopamine was eluted with 50/50 methanol 0.05 M ammonium acetate buffer (pH 4.5).

Ractopamine cELISA. Ractopamine hapten synthesis, monoclonal antibody generation, and characterization were described previously (*16*, *17*). An indirect competitive ELISA (cELISA) format was used for the present study. The following modifications were made in order to further decrease interferences described in our previous report (*17*). The 96 well ELISA plates were coated with 500 ng/well of ractopamine-hemi-glutarate-BSA. Excess binding sites were blocked with 3% BSA instead of 1% Teleostean gelatin as described previously. Competitors were dissolved in 100 mM phosphate buffer (pH 7.4) containing 0.05% Tween 20, instead of PBST, and 10 mg/mL BSA and then were coincubated with primary antibody. Concentrations of unknowns were computed from a calibration curve consisting of ractopamine concentrations of 0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, and 500 ng/mL. A typical calibration curve is shown in **Figure 2**. Results obtained from the cELISA analyses were compared with those obtained from the HPLC analyses using regression analysis, with the regression equation forced through the origin.

RESULTS AND DISCUSSION

SPE of unhydrolyzed sheep and cattle urine samples allowed the isolation of parent ractopamine of sufficient purity to allow baseline separation (**Figure 3**) of ractopamine and impurities.

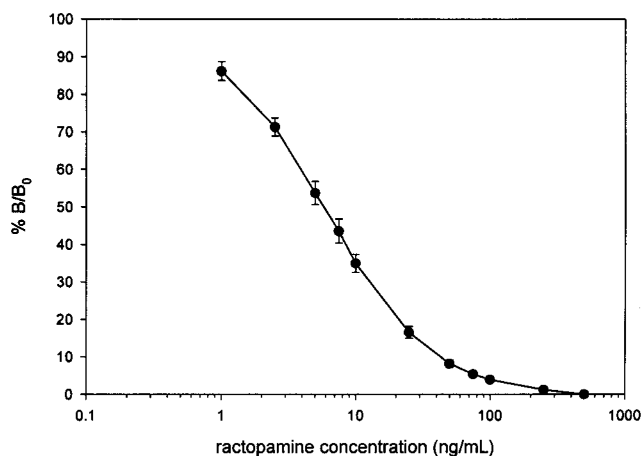

Figure 2. Mean of calibration curve over a 4 month period ($n = 34$). The average IC_{50} was 5.1 ng/mL, with the linear range (based on $\%B/B_0$ 20–80%) 1.5–20 ng/mL. The LOD (based on $\%B/B_0$ of 90%) was 0.76 ng/mL and the LOQ (based on $\%B/B_0$ of 80%) was 1.5 ng/mL. B is the average absorbance at the concentration indicated, and B_0 is the average absorption at a zero concentration. The IC_{50} is the concentration producing 50% of the absorbance of B_0 determined from a four parameter logit fit of the data points.

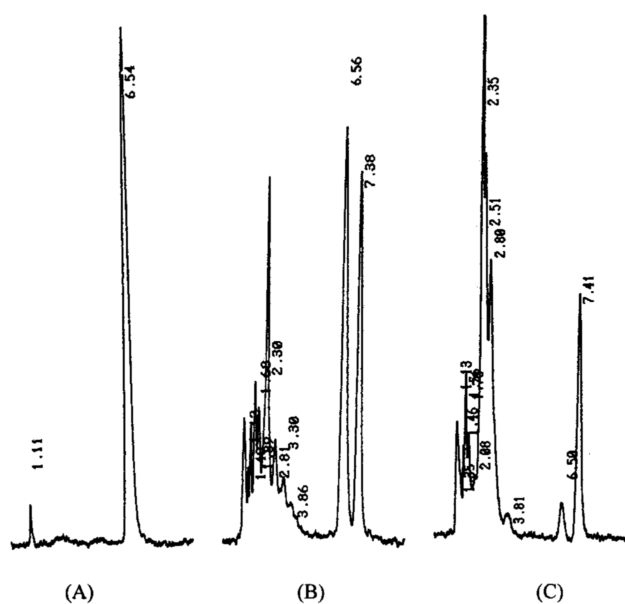

Figure 3. (**A**) HPLC chromatogram of ractopamine fortified into H_2O, and processed using the SPE procedure used for the analysis of unhydrolyzed ractopamine (retention time 6.54 min). (**B**) HPLC chromatogram of ractopamine (retention time 6.56 min) isolated from urine of a ractopamine-treated cow and processed using the SPE procedures; no urine hydrolysis was done. (**C**) HPLC chromatogram of ractopamine (retention time 6.50 min) isolated from urine of a ractopamine-treated sheep and processed using the SPE procedures; no urine hydrolysis was done.

Recoveries of [^{14}C]ractopamine fortified (1.2−1.6 μg/mL) into blank urine samples from the C-18 SPE were 90.5 ± 9.0% for cattle samples ($n = 15$) and were 99.4 ± 3.4% ($n = 14$) for sheep urine samples. This simple isolation procedure allowed us to prepare samples that could be analyzed for parent ractopamine by HPLC or cELISA with minimal interference from various metabolites or endogenous components of urine.

Elliott et al. (*14*) used an ELISA and LC−MS−MS to determine ractopamine in urine of cattle after enzymatic hydrolysis. They found that ractopamine levels varied quite widely among animals, similar to our findings (range 498−3700 ng/mL by HPLC, first day feeding, our results). Elliott et al. (*14*) also observed differences between results obtained from their ELISA and results obtained from the LC−MS−MS procedures. Although they incorporated a longer ractopamine feeding period (17 vs 7 days) in their study, they reported lower urinary ractopamine concentrations at the end of the feeding period (280 ng/mL from LC−MS−MS and 487 from ELISA) as compared to our results (5000 ng/mL from HPLC and 5200 ng/mL from cELISA, $n = 6$). This is not surprising because we used a larger ractopamine dose than Elliot et al. (∼100 vs ∼432 μg/kg BW). Both studies found that ractopamine concentrations dropped rapidly during the withdrawal period. After a 3 day withdrawal, Elliott et al. (*14*) measured ractopamine concentrations of 387 ng/mL by ELISA and 18 ng/mL by LC−MS−MS. In our study, after a 3 day withdrawal period, ractopamine concentrations in cattle urine were less than 20 ng/mL using either cELISA or HPLC, although with enzymatic hydrolysis ractopamine concentrations were 252 and 434 ng/mL as measured by cELISA and HPLC, respectively.

The large increase in free ractopamine after enzymatic hydrolysis of urine samples provides evidence that ractopamine is extensively metabolized, consistent with previous reports (*21*−*24*). The differences before and after the hydrolysis can be used to provide an estimate of the extent of metabolism. For example, in sheep urine, unmetabolized ractopamine represented less than 1% (often <0.5%) of the total ractopamine excreted throughout both the feeding and the withdrawal periods. Similarly, parent ractopamine present in cattle urine represented less than 2% of the total ractopamine residue excreted.

Our observations are not entirely consistent with the results of Elliott et al. (*14*) who observed, on average, only a 2.5-fold increase in ractopamine concentration after hydrolysis of conjugates with enzyme from *Escherischia coli* or *Helix pomatia.* Using synthetic [^{14}C]ractopamine glucuronides as standards fortified into control urine, we found that enzymes from *E. coli*, *H. pomatia*, and *P. vulgata* had highly variable hydrolytic efficiencies depending upon pH and duration of the incubation. Specifically, low hydrolytic efficiencies were obtained when the pH of urine samples was not stabilized with strong buffers (we used 1 M ammonium acetate, pH 5.0). Urine from ruminants on forage and cereal diets is normally alkaline (*25*); preliminary experiments in our lab indicated that a 1:1 dilution of urine with 0.5 M ammonium acetate (pH 5.0) was not sufficient to bring the urine pH to optimal for the enzyme. Although enzyme from *H. pomatia* was effective at hydrolyzing ractopamine−glucuronides, we chose to use enzyme from *P. vulgata* in all incurred urine hydrolyses because the hydrolysis product did not contain chromatographic interferences. Apparent differences of this study and of Elliott et al. (*14*) are probably due to differences in hydrolytic efficiencies between the studies, caused by differences in incubation conditions. For their HPLC-MS-MS and the ELISA analsyses, Elliott et al. used a 2 h incubation period, whereas we incubated urine with enzyme for greater than 18 h, we adjusted the pH of the urine samples with concentrated buffers, and we validated the efficiencies of hydrolysis using synthetic ractopamine−glucuronides. It was not clear how many units of enzyme were added to the incubations of Elliott et al. (*14*) because they reported a volume of enzyme added rather than units.

Our results indicated that data obtained from the monoclonal-based cELISA and HPLC assays were highly correlated after parent ractopamine was isolated from bovine urine with SPE

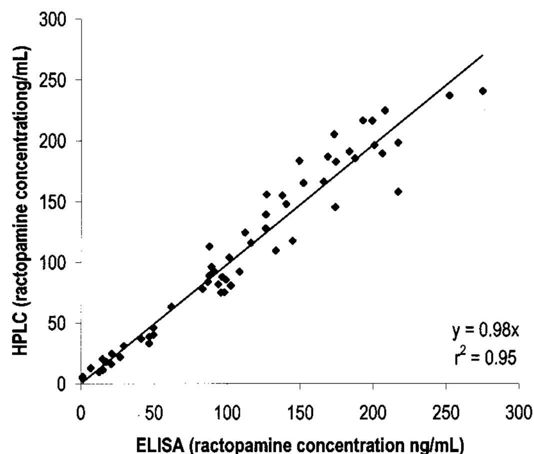

Figure 4. Correlation between results from cELISA and HPLC analyses of parent ractopamine in urine of beef cattle. Samples were prepared for analysis by SPE as described in the Materials and Methods.

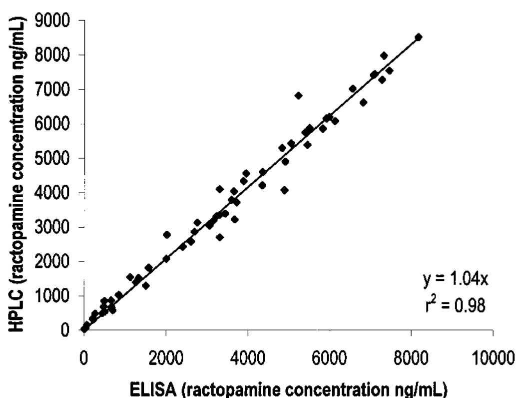

Figure 5. Correlation between results from cELISA and HPLC analyses of ractopamine in urine of cattle after the hydrolysis of ractopamine conjugates with glucuronidase/sulfatase from *P. vulgata.* After hydrolysis, samples were prepared for analysis with liquid and SPE steps. Note that most of the residue in urine was present as conjugates.

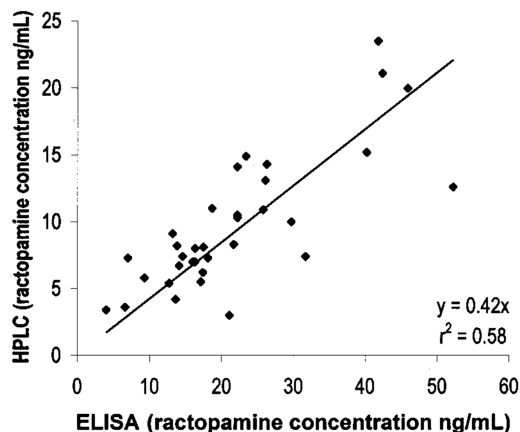

Figure 6. Correlation between results from cELISA and HPLC analyses of parent ractopamine in urine of sheep. Samples were prepared for analysis by SPE as described in the Materials and Methods. Note that urine samples of sheep contained fairly low concentrations of parent ractopamine relative to those measured in cattle urine (**Figure 4**).

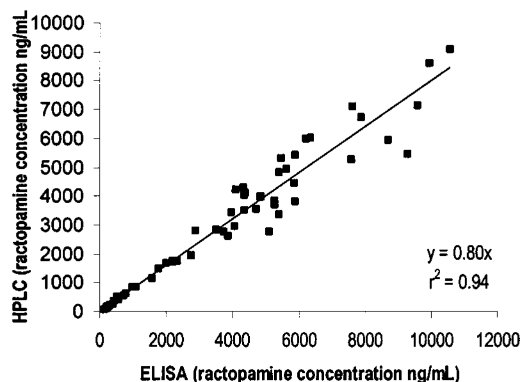

Figure 7. Correlation between results from cELISA and HPLC analyses of ractopamine in urine of sheep after the hydrolysis of ractopamine conjugates with glucuronidase/sulafatase from *P. vulgata.* After hydrolysis, samples were prepared for analysis with liquid and SPE steps. Note that most of the residue in urine was present as conjugates and that the correlation between the methods for hydrolyzed sheep urine improved substantially relative to that for unhydrolyzed samples.

($r^2 = 0.95$; concentration range of cELISA, 1−275 ng/mL). The slope of the line, forced through the origin, was 0.98 indicating good agreement between the two assays (**Figure 4**). In addition, an excellent correlation between the cELISA and the HPLC results was obtained with enzyme-hydrolyzed cattle urine samples, yielding a slope of 1.04 with an r^2 of 0.98 (range of cELISA, 14−8159 ng/mL, $n = 62$; **Figure 5**). With sheep urine, the slope of the line was only 0.42 and the correlation with HPLC results was $r^2 = 0.58$ (range of cELISA, 0.85−51 ng/mL) (**Figure 6**). These data demonstrate that the cELISA analyses of parent ractopamine in sheep urine overestimated the amount of ractopamine in comparison to the HPLC analyses. In addition, the poor correlation indicated either the techniques measured different analytes or there was a sample-dependent interference. When the sheep urine samples were hydrolyzed with glucuronidase/sulfatase, the cELISA and HPLC results were very consistent with a slope of 0.80 and $r^2 = 0.94$ (range of cELISA, 123−10 554 ng/mL, $n = 60$; **Figure 7**). Two explanations exist for the low correlation between the ELISA and the HPLC results from unhydrolyzed sheep urine. First, sheep urine contained very low concentrations of parent ractopamine during the feeding and withdrawal periods. As a result, the error surrounding the HPLC analysis of the sheep urine is greater than the error associated with analysis of sheep urine after hydrolysis of conjugates. Likewise, the excellent correlation between the cELISA results of the analysis of ractopamine in unhydrolyzed cattle urine is probably due to a 10 times greater concentration of ractopamine. The error associated with the HPLC assay decreased substantially as urinary concentrations of ractopamine increased. A second possibility is the presence of a cross-reacting metabolite in the sample extract from sheep urine. Such a metabolite would react with the antibody but not be quantified during the HPLC analysis.

The effect of the urine matrix on the immunoassay was minimal at a dilution of 1:20 for cattle urine and essentially nonexistent after dilutions of 1:100 or greater for both cattle and sheep urine (data not shown). Consequently, the large difference between cELISA results obtained after analysis of raw urine and HPLC analysis after hydrolysis and sample cleanup (shown in **Table 1**) was probably not due to matrix effects. Concentrations of ractopamine in raw urine quantified by cELISA were roughly 4-fold greater than the concentrations of total ractopamine measured by cELISA and HPLC after enzymatic hydrolysis (**Tables 1** and **2**). Differences between the cELISA of raw urine and the cELISA of hydrolyzed urine samples cannot be explained by incomplete hydrolysis of ractopamine conjugates, because recoveries of ractopamine from

Table 1. Comparisons of Analyses of Cattle Urine by HPLC and cELISA (ng/mL)[a]

	cow 158					mean ± SD of all animals				
	ELISA			HPLC		ELISA			HPLC[b]	
day[c]	urine (× 10³)[d]	parent[e]	total (× 10³)[f]	parent[e]	total (× 10³)[f]	urine (× 10³)[d]	parent[e]	total (×10³)[f]	parent[e]	total (× 10³)[f]
T1	5.1	47	1.5	39	1.3	7.3 ± 5.0	62 ± 41	1.9 ± 1.2	62 ± 41	1.8 ± 1.2
T2	8.6	50	2.0	46	2.1	8.2 ± 5.5	64 ± 41	2.1 ± 1.4	61 ± 41	2.1 ± 1.3
T3	13.0	98	2.0	75	2.8	13.8 ± 6.9	113 ± 46	3.6 ± 1.8	93 ± 40	3.8 ± 1.8
T4	17.9	99	4.4	86	4.6	23.5± 10.9	158 ± 67	5.8 ± 2.5	158 ± 76	6.1 ± 2.5
T5	12.6	112	3.6	125	3.8	22.5 ± 6.2	191 ± 57	6.0 ± 1.6	179 ± 40	6.3 ± 1.7
T6	15.0	88	3.3	113	3.4	17.5 ± 3.1	136 ± 28	4.5 ± 0.98	158 ± 33	4.9 ± 0.96
T7	10.6	62	3.3	63	2.7	19.1 ± 8.7	143 ± 52	5.2 ± 1.3	143 ± 50	5.0 ± 1.6
T8/W0	4.8	88	0.67	89	0.68	11.9 ± 6.2	153 ± 64	3.4 ± 1.9	164 ± 62	3.8 ± 2.4
W1	5.8	41	1.6	37	1.8	7.4 ± 4.1	63 ± 34	2.1 ± 1.1	57.7 ± 33	2.5 ± 1.4
W2	1.7	15	0.48	20	0.68	1.8 ± 0.98	18 ± 10	0.62 ± 0.38	22.3 ± 8	0.85 ± 0.52
W3	0.78	5	0.22	<LOQ[g]	0.34	0.88 ± 0.57	10 ± 8	0.25 ± 0.19	18.6[h]	0.43 ± 0.33
W4	0.40	3	0.07	<LOQ	0.16	0.24	3	0.04	<LOQ	0.1
W5	0.18	2	0.04	<LOQ	0.07	0.12	2	0.04	<LOQ	0.07[i]
W6	0.09	<LOQ[j]	0.01	<LOQ	<LOQ[k]	0.06	<LOQ	0.01	<LOQ	<LOQ
W7	0.09	<LOQ	0.01	<LOQ	<LOQ	0.03	<LOQ	0.01	<LOQ	<LOQ

[a] Results shown are from a representative animal (cow 158) as well as averages of all six treated animals. [b] This HPLC data was from ref 20. [c] T1 = treatment day 1, W0 = withdrawal day 0. [d] Raw urine samples analyzed by cELISA after dilution with variable amounts of 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL BSA. [e] Parent ractopamine was analyzed by cELISA and HPLC after sample cleanup with SPE. [f] Total ractopamine was measured by cELISA and HPLC after hydrolysis of conjugates and sample cleanup with liquid/liquid extraction and SPE. [g] LOQ for parent ractopamine analyzed by HPLC was 5 ng/mL. [h] Two samples were <LOQ. [i] One sample was <LOQ. [j] LOQ for parent ractopamine cELISA was 1.5 ng/mL. [k] LOQ for total ractopamine residues analyzed by HPLC after hydrolysis of conjugates was 50 ng/mL.

Table 2. Comparisons of Analyses of Sheep Urine by HPLC and cELISA (ng/mL)[a]

	sheep 351					mean ± SD of all animals				
	ELISA			HPLC		ELISA			HPLC[b]	
day[c]	urine (× 10³)[d]	parent[e]	total (× 10³)[f]	parent[e]	total (× 10³)[f]	urine (× 10³)[d]	parent[e]	total (× 10³)[f]	parent[e]	total (× 10³)[f]
T1	3.4	11.8	1.5	<LOQ[g]	1.8	4.7 ± 3.4	13 ± 7	1.7 ± 1.2	6 ± 3[h]	2.2 ± 1.6
T2	4.4	16.3	1.8	7.0	2.2	8.2 ± 5.8	22 ± 15	2.9 ± 1.7	8 ± 3[i]	3.8 ± 2.4
T3	4.6	16.3	2.0	8.0	2.7	8.8 ± 3.4	22 ± 15	3.7 ± 1.7	10 ± 6[i]	4.7 ± 1.8
T4	10.2	26.1	3.4	13.1	5.4	13.3± 2.7	29 ± 11	4.8 ± 1.7	13 ± 5	7.3 ± 2.1
T5	10.8	24.4	4.8	<LOQ	5.4	14.8 ± 8.0	22 ± 13	5.7 ± 3.0	22 ± 13[j]	4.3 ± 1.8
T6	11.4	26.3	5.0	14.3	5.6	10.8 ± 3.2	19 ± 5	4.8 ± 1.1	11 ± 3[k]	5.1 ± 1.1
T7/W0	10.2	22.2	4.0	14.1	4.8	12.4 ± 3.7	20 ± 5	4.6 ± 1.4	10 ± 3[l]	5.3 ± 1.4
W1	4.1	9.3	1.8	5.8	2.3	6.5 ± 4.5	9 ± 6	2.3 ± 1.3	5 ± 1[l]	2.9 ± 1.8
W2	1.0	4.0	0.4	3.4	0.54	1.1 ± 0.4	3 ± 1	0.43 ± 0.15	3[m]	0.52 ± 0.18
W3	1.1	3.2	0.4	< LOQ	0.56	0.65 ± 0.4	2 ± 1	0.23 ± 0.14	< LOQ	0.33 ± 0.2
W4	1.2	5.1	0.27	< LOQ	0.41	1.5	4	0.4	< LOQ	0.5
W5	0.26	1.6	0.09	< LOQ	0.13	0.54	2	0.14	< LOQ	0.19
W6	0.27	1.9	0.12	< LOQ	0.18	0.46	2	0.24	< LOQ	0.31
W7	0.22	<LOQ[n]	0.09	< LOQ	0.12	0.33	<LOQ	0.14	< LOQ	0.18

[a] Results shown are from a representative animal (sheep 351) as well as averages of all six treated animals. [b] This HPLC data was from ref 20. [c] T1 = treatment day 1, W0 = withdrawal day 0. [d] Raw urine samples analyzed by ELISA after dilution with variable amounts of 100 mM phosphate buffer containing 0.05% Tween 20 and 10 mg/mL BSA. [e] Parent ractopamine was analyzed by ELISA and HPLC after sample cleanup with SPE. [f] Total ractopamine was measured by ELISA and HPLC after hydrolysis of conjugates and sample cleanup with liquid/liquid extraction and SPE. [g] LOQ for parent ractopamine analyzed by HPLC was 5 ng/mL. [h] Mean of 3 measurements, 3 samples were <LOQ. [i] Mean of 5 measurements, 1 sample was <LOQ. [j] One measurement, the rest of samples were <LOQ. [k] Mean of 5 measurements, 1 sample was <LOQ. [l] Mean of 3 measurements, 1 sample was <LOQ. [m] One measurement, 3 samples were <LOQ. [n] LOQ for parent ractopamine cELISA was 1.5 ng/mL.

blank urine samples fortified with ractopamine−glucuronides averaged about 90% (*20*).

We believe that our previous observations regarding the cross reactivity of the monoclonal antibody toward specific ractopamine−glucuronides and ractopamine stereoisomers (*17*) will adequately explain quantitative differences shown in raw urine observed in **Tables 1** and **2**. The ractopamine monoclonal antibody is 5 times more sensitive for the *RR* stereoisomer than for racemic ractopamine and 4 times more sensitive toward the major ractopamine−glucuronide metabolite (ractopamine−glucuronide "C" conjugated to the ethanolamine phenol of ractopamine) than for racemic ractopamine (*17*). Raw urine used in this study contained large quantities of ractopamine conjugates of unknown composition (i.e., the proportion of ractopamine metabolites conjugated to either phenol of ractopamine was unknown). In addition, the standard curve used for the quantitative analysis of raw urine was constructed using racemic ractopamine. Quantitative analysis of urine samples containing mainly ractopamine conjugates may not be appropriate when the standard curve was comprised of racemic ractopamine. Quantitative analysis of urine samples after enzymatic hydrolysis of ractopamine conjugates was successful because conjugates were converted to parent ractopamine before the cELISA and because the standard curve was based on parent ractopamine. Because the hydrolyzed urine analysis by HPLC represents the maximum total ractopamine concentration in urine, the large

Table 3. Concentrations of Parent Ractopamine in Liver as Determined by the cELISA Assay and by HPLC with Fluorescence Detection

withdraw period (d)	animal	ELISA (ng/g)	HPLC (ng/g)
0	156	9.7	11.3
0	160	13.4	12.9
3	155	2.1	2.6
3	157	1.4	1.7
7	158	1.1	1.7
7	159	1.6	1.7

response of the cELISA toward raw urine must be due to the antibody's high sensitivity toward metabolites. Thus, analysis of raw urine by cELISA would not be appropriate for quantitative purposes unless proper validation of the standard curve was first performed, but it would be very useful for qualitative screening purposes.

Beef liver contained only minute amounts of free ractopamine (**Table 3**). Even on withdrawal day 0, liver concentrations of ractopamine were less than 15 ppb. At the 3 day withdrawal time point, ractopamine liver concentrations dropped to approximately 1 ppb. Comparison of cELISA and HPLC results indicated a good correlation when the matrix blank was subtracted from the cELISA results; comparison of the two methods indicated a slope of 1.06 with an $r^2 = 0.96$. When beef liver samples were treated with enzyme to hydrolyze ractopamine metabolites, a peak was produced that interfered with the HPLC analysis. As a result, estimates of total residues in liver were not obtained.

In conclusion, the ractopamine cELISA may be used for the quantitative analysis of cattle tissues or urine. Quantitative analysis may be performed before or after hydrolysis of ractopamine conjugates provided that samples are carried through a cleanup step to remove impurities. The cELISA showed excellent agreement with HPLC analysis. In addition, the cELISA is applicable to samples containing a wide concentration range of ractopamine or hydrolyzed ractopamine conjugates, although high concentrations need appropriate dilution. Application of the ractopamine cELISA to samples of sheep urine required that the urine be subjected to enzymatic hydrolysis prior to quantitative analysis. The cELISA provides a quick and easy screen for the presence of ractopamine and its metabolites in a wide concentration range without the need for expensive instrumentation (HPLC). The ability to detect ractopamine metabolites offers greater sensitivity because ractopamine is rapidly and extensively metabolized.

ABBREVIATIONS USED

PBST, phosphate-buffered saline-0.05% Tween 20; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; SPE, solid phase extraction; TMB, 3,3′,5,5′-tetramethyl benzidine; BSA, bovine serum albumin.

ACKNOWLEDGMENT

The authors thank Grant E. Harrington, John M. Giddings, and Stephanie Hoff for their excellent technical support.

LITERATURE CITED

(1) FDA. *Fed. Regis.* **2000**, *65*, 4111−4112.
(2) Muirhead, S. *Feedstuffs* **2000**, *72*, 1.
(3) Anonymous. *Feedstuffs* **2001**, *73*, 19.
(4) Moody, D. E.; Hancock, D. L.; Anderson, D. B. Phenethanolamine repartitioning agents. In *Farm Animal Metabolism and Nutrition*; D'Mello, J. P. F., Ed.; CAB International: New York, 2000; pp 65−96, 2000.
(5) Freedom of Information Summary Document 141−172, Paylean and Tylan. http://www.fda.gov/cvm/efoi/section 2/141172051401/html, 2001.
(6) Martinez-Navarro, J. F. *Lancet* **1990**, *336*, 1311.
(7) Pulce, C.; Lamaison, D.; Kock, G.; Bostvlronnols, C.; Nicolas, J.; Descotes, J. **1991**, *33*, 480−481.
(8) Brambilla, G.; Loisso, A.; Fontana, L.; Strozzi, M.; Guarino, A.; Soprano, V. *JAMA* **1997**, *278*, 635.
(9) Sporano, V.; Grasso, L.; Esposito, M.; Oliviero, G.; Brambilla, G.; Loizzo, A. *Vet. Hum. Toxicol.* **1998**, *40*, 141−143.
(10) Kuiper, H. A.; Noordam, M. Y.; van Dooren-Flipsen, M. M. H.; Schilt, R.; Roos, A. H. *J. Anim. Sci*. **1998**, *76*, 195−207.
(11) Mitchell, G. A.; Dunnavan, G. *J. Anim. Sci*. **1998**, *76*, 208−211.
(12) Haasnoot, W.; Stouten, P.; Lommen, A.; Cazemier, G.; Hooijerink, D.; Schilt, R. *Analyst* **1994**, *119*, 2675−2680.
(13) Haasnoot, W.; Cazemier, G.; Piet stouten; Kemmers-Voncken, A. Immunochemical approaches to the analysis of β-agonist drugs. In *Immunoassays for Residue Analysis*: *Food Safety*; Beier, R. C., Stanker, L. H., Eds.; American Chemical Society: Washington, DC, 1996; pp 60−73.
(14) Elliott, C. T.; Thompson, C. S.; Arts, C. J. M.; Crooks, S. R. H.; vanBaak, M. J.; Verheij, E. R.; Baxter, G. A. *Analyst* **1998**, *123*, 1103−1107.
(15) Wicker, A. L.; Turberg, M. P.; Coleman, M. R. *Analyst* **1995**, *120*, 2879−2881.
(16) Shelver, W. L.; Smith, D. J. *J. Immunoassay* **2000**, *21*, 1−23.
(17) Shelver, W. L.; Smith, D. J.; Berry, E. S. *J. Agric. Food Chem.* **2000**, *48*, 4020−4026.
(18) Ricke, E. A.; Smith, D. J.; Feil, V. J.; Larsen, G. L.; Caton, J. S. *J. Anim. Sci.* **1999**, *77,* 701−707.
(19) Shappell, N. W.; Feil, V. J.; Smith, D. J.; Larsen, G. L.; McFarland, D. C. *J. Anim. Sci.* **2000**, *78*, 699−708.
(20) Smith, D. J.; Shelver, W. L. *J. Anim. Sci.*, in press.
(21) Dalidowicz, J. E.; Thomson, T. D.; Babbitt, G. E. Ractopamine hydrochloride, a phenethanolamine repartitioning agent: Metabolism and residues. In *Xenobiotics and Food-Producing Animals*; Hutson, D. H., Hawkins, D. R., Paulson, G. D., Struble, C. B., Eds.; American Chemical Society Symposium Series 503; American Chemical Society: Washington, DC, 1992; pp 234−243.
(22) Smith, D. J.; Feil, V. J.; Huwe, J. K.; Paulson, G. D. *Drug Metab. Dispos.* **1993**, *21*, 624−633.
(23) Smith, D. J.; Giddings, J. M.; Feil, V. J.; Paulson, G. D. *Xenobiotica* **1995**, *25*, 511−520.
(24) Smith, D. J. *J. Anim. Sci.* **1998**, *76*, 173−194.
(25) Finco, D. R. Kidney function. In *Clinical Biochemistry of Domestic Animals*; Kaneko, J. J., Harvey, J. W., Bruss, M. L., Eds.; Academic Press: San Diego, CA, 1997; pp 441−484.

Received for review October 16, 2001. Revised manuscript received January 3, 2002. Accepted January 3, 2002. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

JF011372+