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Tissue Residues and Urinary Excretion of Zilpaterol in Sheep Treated for 10 Days with Dietary Zilpaterol

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Zilpaterol is a β-adrenergic growth promoter approved in Mexico and South Africa for use in cattle. Understanding the rates of zilpaterol depletion from tissues and urine is of interest for the development of strategies to detect the off-label use of zilpaterol. Eight sheep were fed 0.15 mg/kg/day dietary zilpaterol hydrochloride (Zilmax) for 10 consecutive days; two sheep each were slaughtered 0, 2, 5, and 9 days after discontinuation of exposure to the zilpaterol-containing diet. Tissue zilpaterol levels rapidly decreased during the withdrawal period. On the basis of LC-MS/MS-ES (external standard) measurements, liver zilpaterol residues in sheep were 29.3, 1.5, 0.13, and 0.10 ng/g after 0, 2, 5, and 9 day withdrawal periods, respectively; kidney residues were 29.6, 1.10, and 0.09 ng/g and below the detection limit; and muscle residues were 13.3, 0.86, 0.12, and 0.08 ng/g at the same respective withdrawal periods. Between-animal variation in urinary zilpaterol concentrations during the feeding period was considerable, although zilpaterol concentrations converged somewhat as steady state was reached. During the first 3 days of the withdrawal period, zilpaterol elimination followed a first-order excretion pattern, having an average elimination half-life of 15.3 $\pm$ 1.8 h. Urinary zilpaterol concentrations during the withdrawal period were determined using ELISA, HPLC−fluorescence, LC-MS/MS-ES (external standard), and LC-MS/MS-IS (internal standard). Comparison of these methods showed a high correlation with each other. With the exception of LC-MS/MS-IS, the regression coefficients of the linear equations with a zero intercept were between 0.90 and 1.25, indicating the near equivalence of the methods. Because of its simplicity, ELISA is a convenient assay for determining zilpaterol levels in urine giving similar results to HPLC−fluorescence and LC-MS/MS-ES without requiring the extensive cleanup of the latter methods.

KEYWORDS: Analysis; ELISA; HPLC; LC-MS/MS; zilpaterol; residue

INTRODUCTION

Zilpaterol (**Figure 1**) is a β-adrenergic repartition agent demonstrated to enhance carcass leanness, improve growth rates, and decrease feed consumption in cattle and sheep (*1*, *2*). Although zilpaterol has been approved for use in feedlot cattle in both Mexico and South Africa, it has not been approved for use in the European Union, the United States, or most other countries. Indeed, use of β-agonists for growth-promoting purposes has been banned in the European Union (*3*). The history of illegal or off-label use of β-agonists and cases of human intoxication from the consumption of animal tissue containing β-agonist residues have been thoroughly documented (*3*−*11*).

Because the legal use of zilpaterol in food animals is limited to cattle in Mexico and South Africa, the detection of zilpaterol in live animals or in food animal carcasses is of interest to regulatory officials, importers, and exporters. Because a potentially large number of samples might need to be analyzed for zilpaterol content, a convenient and rapid screening assay for zilpaterol would be useful. Instrumental analyses were reported for zilpaterol determination using GC-MS (*12*, *13*) and LC-MS/MS (*14*−*16*). Although these multiresidue methods are capable of zilpaterol determination, they are expensive and time-consuming and require dedicated laboratory space and operators. In addition, these methods require stringent sample cleanup

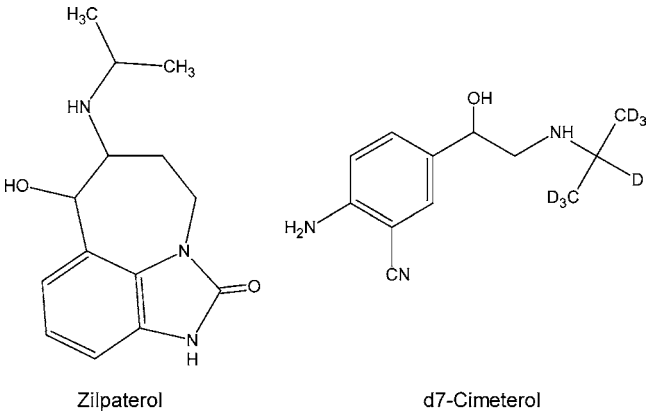

Figure 1. Structures of zilpaterol and d7-cimeterol.

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procedures with the possibility of concomitant loss in sensitivity and unacceptable variability. Shelver et al. (*17*−*19*) have developed polyclonal and monoclonal antibody-based enzyme-linked immunosorbant assays (ELISA) as well as an immunobiosensor assay toward zilpaterol. The ELISA has wide potential as a high-throughput method for the detection of zilpaterol residues in different matrices with minimal cleanup.

Data on zilpaterol residues in either target or nontarget animals are scant. Van Hoof et al. (*16*) reported zilpaterol residues in a single cow fed zilpaterol at 0.15 mg/kg/day for 14 days; zilpaterol residues in urine and feces were analyzed using ion trapping with LC-MS^n, but no withdrawal data or tissue residue data were reported. Stachel et al. (*15*) reported data from feeding studies on 2 heifers and 16 boars that were fed Zilmax for 14 and 25 days, respectively. The heifers were slaughtered on days 1 and 10 of withdrawal and the boars on days 1, 2, 4, and 5 of withdrawal. Urine as well as tissue samples (muscle, liver, kidney, and eyes) were analyzed for zilpaterol using LC-MS/MS. Because zilpaterol has been demonstrated to enhance meat production in sheep (*2*) and because of the need to understand zilpaterol kinetics in species other than cattle, we decided to determine residue levels of zilpaterol in the tissues and urine of sheep after dietary administration of 0.15 mg/kg/day zilpaterol hydrochloride (Zilmax) for 10 consecutive days. Incurred residues in tissues and urine were also used to validate the usefulness of the ELISA developed by Shelver et al. (*18*) in comparison to HPLC with fluorescence or mass spectral detection. To our knowledge, zilpaterol residues in sheep have not been reported.

MATERIALS AND METHODS

Animal Feeding and Sample Collection. Ten mature ewes were purchased from a livestock auction center and were adapted to the Biosciences Research Laboratory animal facilities for 1 week. Sheep were housed in three separate concrete-floored stalls (10.0 m^2) covered with wood shavings in accordance with the animal care protocol approved by the Institutional Animal Care and Use Committee. Sheep had ad libitum access to hay and water; in addition, each sheep was provided with 0.68 kg of a grain ration daily (14% crude protein; J & S Farmer's Mill, Barnesville, MN). During the adaptation period prior to the zilpaterol dosing, the sheep were trained to eat the grain supplement from head gates. Feeding through head gates allowed for the evaluation of grain consumption as well as facile urine collection.

Zilpaterol-containing supplements were prepared by sequentially placing four 10.25-g aliquots of Zilmax (4.8% zilpaterol hydrochloride premix; Intervet; Millsboro, DE) into 150 kg of a grain-based sheep supplement (14% crude protein) with a ribbon mixer. Feed was mixed for 10−20 min after the addition of each Zilmax aliquot. The sheep supplement contained corn, oats, soybean meal, cane molasses, vegetable oil, calcium carbonate, ammonium chloride, salt, vitamin A acetate, D-activated animal sterol, DL-α-tocopheryl acetate, and sodium selenite, having an analysis of >14% crude protein, >3% crude fat, <6% crude fiber, 0.4−0.8% calcium, >0.3% phosphorus, 0.8−1.2% NaCl, >3700 IU/kg vitamin A, >1300 IU/kg vitamin D, and >22 IU/kg vitamin E.

Sheep were fed 0.68 kg of the zilpaterol-containing grain supplement for 10 consecutive days in morning and evening portions of ≈0.34 kg; thereafter, the zilpaterol-containing supplement was replaced with unfortified grain supplement. Two control animals were fed in a similar manner except that their daily grain supplement contained no zilpaterol. Two zilptaterol-treated ewes each were euthanized by captive bolt stunning followed by exsanguination on days 0, 2, 5, and 9 of the withdrawal period; control animals were euthanized on day 0 of the withdrawal period. During the treatment and withdrawal periods, sheep had ad libitum access to hay and water.

Urine collected from each sheep during the adaptation period was used as control urine. In addition, urine was collected from sheep on treatment days 1, 3, 5, and 8 and on withdrawal days 0, 1, 2, 5, 7, and 9 (or until slaughter). Collected urine was stored at −20 °C. For the purposes of this study, treatment day 10 and withdrawal day 0 were the same day (i.e., the last zilpaterol feeding on day 10 was the beginning—day 0—of the withrawal period). At slaughter, skeletal muscle, kidney, and liver were collected. At processing, tissues were minced, mixed with an approximately equal amount of dry ice, and ground in a blender (*20*). The ground tissues were stored at −20 °C in plastic bags until the dry ice had sublimed. The ground tissues were then placed into 50 mL centrifuge tubes (Fisher Scientific, Pittsburgh, PA) and stored at −20 °C until analyzed.

Determination of Zilpaterol in Urine. *HPLC−Fluorescence Analysis.* Urinary zilpaterol was quantified by HPLC with fluorescence detection after solid-phase extraction (SPE) cleanup. Isolute N-capped C18 cartridges (500 mg; Argonaut Technologies, Inc., Foster City, CA) were preconditioned with 3 mL of methanol followed by 3 mL of water on a vacuum manifold (Supelco VISIPREP, Bellefonte, PA). Urine samples (5 mL) were mixed with 5 mL of 150 mM sodium acetate, pH 7.4, and passed through the SPE cartridges followed by a 1 mL H_2O rinse. Columns were then washed sequentially with 6 mL of H_2O and 6 mL of 30% methanol, and zilpaterol was eluted with 4 mL of 1% diethylamine in methanol. The eluent was evaporated to dryness using a centrifugal evaporator (Savant, Holbrook, NY) and reconstituted with 5 mL of 1 N HCl. Each sample set contained a matrix blank and a zilpaterol-fortified (100 ng/mL) matrix blank for the determination of recovery.

High-performance liquid chromatography was carried out using a Waters model 600E HPLC pump equipped with a Symmetry C18 column (5 μm, 4.6 × 250 mm; Waters, Milford, MA). The effluent was monitored with a Jasco (Tokyo, Japan) model FP-920 fluorescence detector with excitation and emission wavelengths set at 230 and 635 nm, respectively. A binary mobile phase consisting of components A (10% acetonitrile in 50 mM ammonium acetate buffer, pH 4.5) and B (acetonitrile) was prepared for use as a gradient. The gradient program was as follows: from time 0 to 20 min, a linear change from 100% A to 100% B; from 21 to 25 min, 100% B; from 26 to 30 min, linear change from 100% B to 100% A; from time 31 to 45 min, 100% A using a flow rate of 1.0 mL/min. The mean retention time of zilpaterol under these conditions was 6.3 min. Zilpaterol concentrations were calculated using regression equations generated from standard curves consisting of standards of 0, 20, 40, 100, 200, 400, and 1000 ng/mL prepared in methanol and corrected for recovery. Samples and standards were injected at 25 μL/injection.

LC-MS/MS Analysis. A Varian Bond Elut Certify SPE cartridge (200 mg of sorbent, a mixed-mode SPE of nonpolar C8, and a strong cation exchange) (Varian Sample Preparation Products, Harbor City, CA) was preconditioned with 3 mL of MeOH, 3 mL of H_2O, and 3 mL of 100 mM phosphate buffer, pH 6.0. Five milliliters of sheep urine, 5 mL of 100 mM phosphate buffer, pH 6.0, and 100 μL of an aqueous solution containing 5 ng of d7-cimeterol, as an internal standard, were mixed and loaded onto the cartridge. After sample loading, each cartridge was sequentially washed with 1 mL of 100 mM phosphate buffer, pH 6.0, 3 mL of 1 M glacial acetic acid, and 3 mL of MeOH; zilpaterol was eluted with 3 mL of 1% diethylamine in ethyl acetate (v/v). The eluent was dried under a stream of nitrogen and dissolved in 1 mL of MeOH/10 mM ammonium acetate buffer (5:95, v/v). For each sample set, a matrix blank and a blank fortified with 10 ng of zilpaterol were included as a negative control and as an external recovery standard, respectively.

The LC-MS/MS consisted of a Waters Alliance 2695 LC pump equipped with an Atlantis dC18 column (3 μm, 2.1 × 100 mm; Waters) for peak separation. The gradient system was 100% A (2% acetonitrile in 5 mM ammonium acetate, pH 4.5, and 0.01% formic acid) to 100% B (pure acetonitrile) in 10 min with a flow rate of 0.2 mL/min. The approximate retention time of zilpaterol was 7 min with the internal standard eluting at 7.2 min. Samples and standards were injected at 10 μL/injection. A Q-TOF API-US (Waters) mass spectrometer was run in electrospray positive mode with a collision energy of 15 eV and a cone voltage of 25 V. Initial runs utilized acquisition from *m*/*z* 100 to 350 from 4 to 9 min of the run to search for metabolites. For analytical runs multiple-reaction-monitoring mode was used for signal acquisition; for zilpaterol *m*/*z* 244.14, 202.09, and 185.07 were monitored, and peak

area was calculated as the sum of each ion peak area. For d7-cimaterol, *m*/*z* 227.18, 209.17, and 161.09 were monitored and summed. Data were acquired and processed using MassLynx SP 4.0. Zilpaterol concentrations were calculated using regression equations generated from standard curves consisting of standards of 0.25, 0.5, 1, 2.5, 5, 10, 25, 100, and 200 ng/mL using either 10 ng/mL of zilpaterol as the external standard or 5 ng/mL d7-cimeterol for each calibration point as the internal standard. The sample concentrations were calculated from the standard curve computed as a quadratic function (typical $R^2 = 0.9989$). The data were nearly linear, but the quadratic function showed an improved fit and was incorporated into the instrument's software for computation of unknown concentrations. All sample concentrations fell between the high and low concentrations of the standards used to construct the standard curve, thus avoiding extrapolation errors.

ELISA Analysis. The ELISA procedures for urine analysis were similar to those previously reported (*18*). Briefly, a 96-well ELISA plate was coated by adding 100 μL/well of a solution containing 150 ng/mL of 4-carboxybutyl-zilpaterol BSA in 15 mM bicarbonate buffer, pH 9.8, followed by shaking at 37 °C on an orbital shaker for 2 h. After the plate had been washed three times with phosphate-buffered saline−0.05% Tween 20 (PBST), zilpaterol standards or urine samples were co-incubated with the zilpaterol antibody for 90 min at 37 °C. The plate was washed, and 100 μL/well of rabbit anti-mouse IgG-HRP 1:25000 (Sigma-Aldrich Corp., St. Louis, MO) was added and incubated at 37 °C for 60 min. Following a plate washing, the substrate tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) was added, and the color was allowed to develop for 30 min prior to the addition of 50 μL/well of 2 N sulfuric acid. The plate was read at 450 nM using a Bio-Rad model 550 ELISA plate reader (Bio-Rad Laboratories, Hercules, CA). Zilpaterol concentrations in incurred samples (raw urine diluted 1:100−1:10000 to maintain a reading on scale) were computed to a calibration standard curve containing zilpaterol at concentrations of 0, 0.1,0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, and 1000 ng/mL and adjusted for appropriate dilution factors.

Data Processing. The instrument responses for the samples were converted to concentrations utilizing standard curves appropriate for each instrumental method utilizing software supplied with the instrument. The data from each individual animal was processed separately because interanimal variation is less than intra-animal variation and such processing preserves the interanimal consistency while more clearly demonstrating the extent of intra-animal variation. The half-life determination was carried out for each individual animal using the standard assumption for first-order elimination; plots of log concentration versus time should be linear with the negative slope giving the elimination constant. Statistical computations were made using Excel, and all correlations were examined utilizing plots as well as the statistical support package of Excel.

Determination of Zilpaterol in Tissue Samples. *HPLC−Fluorescence.* Tissue samples (5 g each) were weighed, and 5 mL of 150 mM sodium acetate, pH 7.4, was added. The samples were homogenized using a tissuemizer (Tekmar Co., Cincinnati, OH) followed by centrifugation at 15000*g*, and the supernatant was collected. The process of homogenizing the pellet in the presence of 5 mL of buffer, centrifuging, and collecting the supernatant was repeated twice more. The combined supernatants were filtered through a 1.2 μm filter prior to SPE cleanup. The SPE procedure was the same as described for the HPLC−fluoresence analysis of urine.

LC-MS/MS. Liver, kidney, and muscle samples were prepared for LC-MS/MS analyses using a single process. Samples (5 g) were weighed, and 5 ng of internal standard d7-cimeterol in 100 μL of H_2O was added followed by the addition of 10 mL of 100 mM borate buffer, pH 9. The mixture was homogenized with a tissuemizer for 30 s and centrifuged at 10000*g* for 10 min. The supernatant was removed with a pipet and placed in a new container; the pellet was resuspended in 10 mL of borate buffer, and the homogenization and centrifugation steps were repeated. The combined supernatant was confirmed to have a pH >8.5 and filtered through a 1.2 μm syringe filter. The filtrates were added to Bond Elut Certify 200 mg SPE cartridges that were preconditioned with 4 mL of MeOH and 4 mL of water. After sample loading, the cartridges were washed with 2 mL of water, 2 mL of 100 mM acetate buffer, pH 4.0, and 4 mL of MeOH followed by vacuum-drying of the cartridge for 5 min. Samples were eluted with 4 mL of eluant solution [80:20:2.5 (v/v/v) methylene chloride/isopropyl alcohol/30% aqueous ammonium hydroxide]. After the evaporation of the elution solvent, the residue was reconstituted with 1 mL of MeOH/10 mM ammonium acetate, pH 4.5 (5:95, v/v), and subjected to LC-MS/MS analysis as described above for urine.

ELISA. An aliquot of the supernatant obtained after the tissue homogenization step described for the LC-MS/MS analysis was diluted 1:10 with ELISA buffer and used directly for the zilpaterol analysis by ELISA. The tissue ELISA procedure was the same as that described for the analysis of urinary zilpaterol by ELISA.

RESULTS AND DISCUSSION

A detailed study of ion scans derived from the LC-MS/MS analysis of urine samples containing substantial amounts of zilpaterol provided no evidence for the presence of glucuronide or sulfate conjugates of zilpaterol; consequently, hydrolytic procedures were not utilized in the subsequent analysis of urine samples for parent zilpaterol. Although *n*-desisopropylzilpaterol has been reported to be excreted by cattle (≈5% of the zilpaterol level), no ions corresponding to *n*-desisopropylzilpaterol were found in this study. Consequently, our analysis focused on parent zilpaterol.

Very few data are available on residue levels of zilpaterol in Zilmax-treated food animals. The Zilmax technical brochure (*21*) indicates zilpaterol levels of 1.7−15 ppb in edible tissue (liver, kidney, skeletal muscle, and adipose tissue) of cattle fed Zilmax for 10 days and slaughtered with a 0 day withdrawal period. The technical brochure (*21*) also reported cattle fed with Zilmax for 50 days and slaughtered with a 0 day withdrawal period had residue concentrations ranging from 0.5 to 53.7 ppb; however, after 24 h of withdrawal, zilpaterol concentrations had dropped to 0.3−21 ppb. Stachel et al. (*15*) reported similar ranges of zilpaterol residues in cattle and swine fed 0.15 mg of zilpaterol/kg of body weight over 14 days. For their study, animals were slaughtered with either a 1 or 10 day withdrawal period (cattle) or 1, 2, 4, and 5 day withdrawal periods (swine). These authors found zilpaterol residues on withdrawal day 2 averaged only ≈6% of those found in liver and skeletal muscle on withdrawal day 1; in kidney, zilpaterol residues were not detectable on withdrawal day 2. These results are in excellent agreement with our data, in which more extensive urinary data were obtained.

The ELISA procedure was extensively examined in our previous paper for susceptibility to environmental variables, and the accuracy and precision in both cattle and sheep urine were reported there (*18*). Because of the previous work we elected to generate our standard curves using buffer rather than biological matrices, because our procedure was demonstrated to eliminate a matrix effect utilizing our conditions with sheep or cattle urine. The standards were prepared in a similar fashion for HPLC−fluorescence and for HPLC-MS/MS because HPLC−fluorescence was demonstrated to not show matrix interference and HPLC-MS/MS using multiple-reaction monitoring would be very unlikely to show matrix interference.

Urinary concentrations of zilpaterol as determined by ELISA showed considerable variation in individual sheep as illustrated in **Figure 2**. Differences among sheep would be expected under commercial feeding conditions, and the fact that substantial variation occurred is not unusual. Animal variation does, however, represent a potential problem in the use of feed additives, because some animals might be exposed to greater levels of additives than other animals. Interestingly, differences in urinary zilpaterol concentrations narrowed somewhat as time

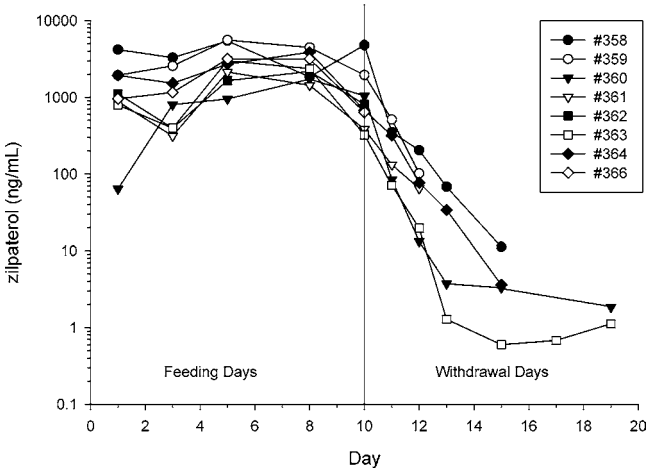

Figure 2. Urine concentrations determined using ELISA during feeding and withdrawal days for all zilpaterol-treated sheep.

went on, probably a function of reaching steady state by day 4. Urinary zilpaterol elimination appeared to be a first-order process with a half-life of 15.3 ± 1.8 h (**Table 1**) using the data from withdrawal day 0 through day 5. The agreement between the half-lives for the individual sheep, as well as the high R^2 for the first-order elimination model for each animal, indicates the elimination process was reasonably consistent among the sheep. Analyses of the analogous data obtained from the HPLC−fluoresence or LC-MS/MS method gave essentially the same results (**Table 1**). Although our data cannot rule out a biphasic elimination with the second phase much slower than the initial elimination, clearly the majority of zilpaterol is eliminated quickly, assuming urinary concentrations are proportional to plasma concentrations in the animal.

Table 1. Computation of Half-Lives and R^2 for the Urinary Elimination of Zilpaterol in Individual Sheep Using a First-Order Elimination Model

	ELISA			HPLC			MS/MS-ES		
sheep	half-life (h)	R^2	n^a	half-life (h)	R^2	n^a	half-life (h)	R^2	n^a
358	14.8	0.936	5	13.4	0.973	5	11.5	0.977	5
359	14.5	0.997	3	13.0	0.999	3	10.6	0.999	3
360	14.7	0.805	5	9.7	0.987	4	13.7	0.747	5
361	18.7	0.987	3	19.4	1	3	12.3	0.940	3
363	13.5	0.927	5	10.5	0.998	3	9.9	0.991	5
364	15.7	0.995	5	12.5	0.987	4	13.1	0.993	5
av	15.3			13.1			11.8		
SD	1.8			3.4			1.5		

[a] Number of points used to fit the equation.

Representative HPLC−fluorescence chromatograms of zilpaterol levels of kidney, liver, muscle, and urine samples are

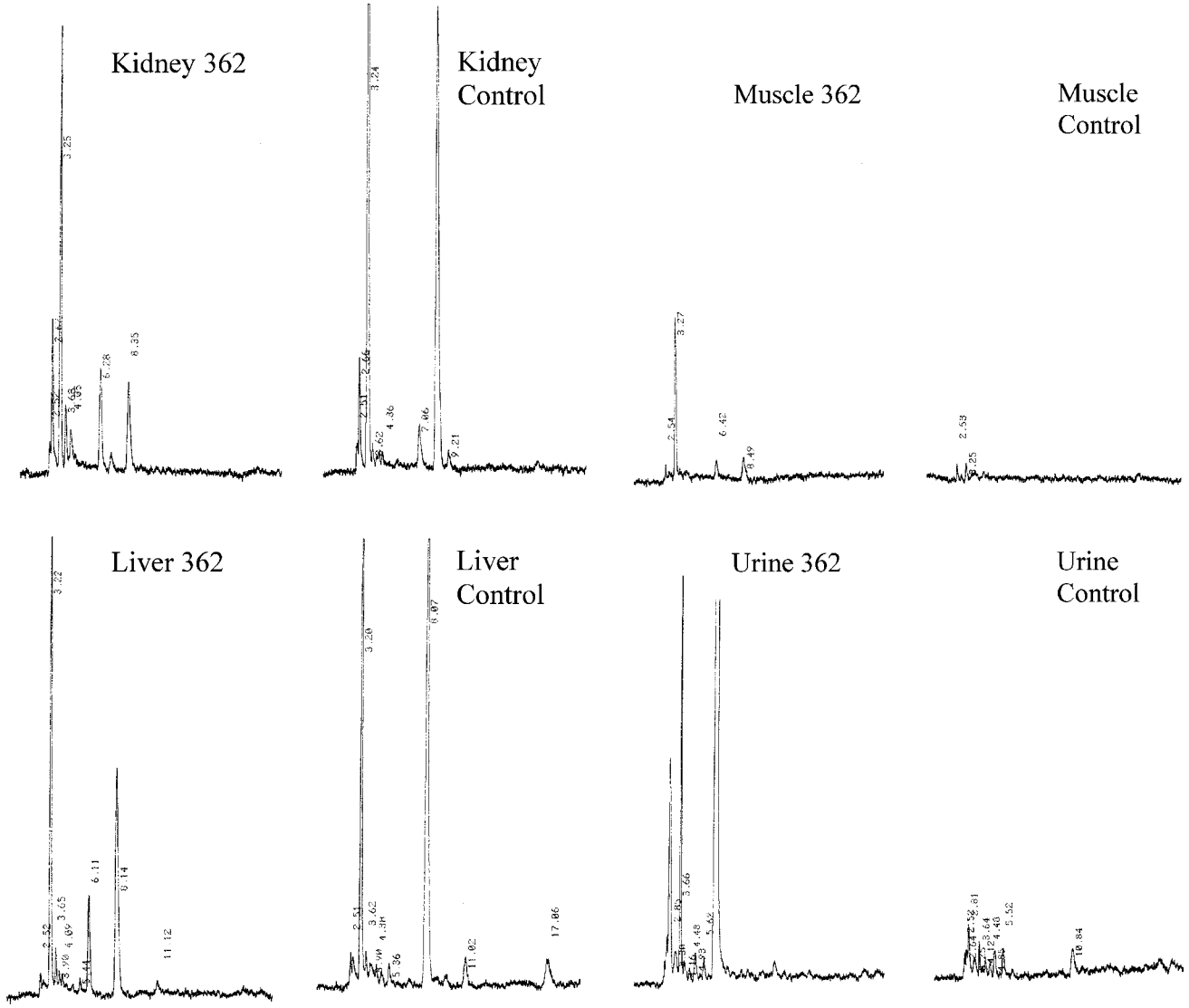

Figure 3. HPLC–fluorescence chromatograms of control and zilpaterol (eluting at 6.3 min) incurred tissue and urine samples.

Table 2. Comparison of ELISA (Using Crude Extracts), HPLC–Fluorescence (Extract Purified with C18 SPE Cartridge), and LC-MS/MS-ES (Extract Purified with Mixed-Mode SPE Column) Results of Different Zilpaterol Tissue Residues at Different Withdrawal Periods (in Nanograms of Zilpaterol per Gram of Tissue)

		liver				kidney				muscle			
		LC-MS/MS				LC-MS/MS				LC-MS/MS			
animal	day	IS	ES	HPLC	ELISA	IS	ES	HPLC	ELISA	IS	ES	HPLC	ELISA
362	0	32.50	33.41	52.45	19.55	13.33	30.98	49.49	56.1	13.38	12.06	14.04	15.38
366	0	28.27	25.15	35.18	47.80	49.67	28.19	53.28	83.2	13.69	14.52	19.18	11.92
359	2	2.09	1.26	nd[a]	8.68	1.63	1.26	nd	12.5	0.99	1.27	nd	4.09
361	2	1.66	1.71	nd	7.99	1.81	0.94	nd	6.22	0.59	0.45	nd	5.20
358	5	0.18	0.09	nd	7.09	0.1	0.03	nd	9.35	0.1	0.07	nd	4.78
363	5	0.18	0.18	nd	5.08	0.23	0.15	nd	5.14	0.21	0.16	nd	4.20
360	9	0.12	0.06	nd	5.21	0.02	nd	nd	6.81	0.25	0.16	nd	3.78
364	9	0.13	0.13	nd	3.21	nd	nd	nd	6.83	nd	nd	nd	4.68

[a] The assay did not detect any zilpaterol for these samples.

shown in **Figure 3**. Zilpaterol in kidney, liver, and muscle samples was successfully isolated using SPE cleanup procedures in all three tissues, and baseline separation of zilpaterol from other components was achieved. However, neither HPLC−fluorescence nor ELISA showed credible results for tissue samples from animals harvested after ≈2 days of withdrawal, indicating that further improvements are required before these methods can be used for tissue analysis of animals withdrawn from zilpaterol. In contrast to the other methods, HPLC−fluorescence data showed no detectible zilpaterol from day 2 withdrawal onward, demonstrating the method's inability to detect low zilpaterol concentrations. Although the ELISA and both of the MS methods showed measurable levels at withdrawal days 5 and 7, the methods gave very different results, making the measurements uninterpretable. Tissue residue data obtained by LC-MS/MS (**Table 2**) indicate that 95% of the initial zilpaterol residue had been eliminated by withdrawal day 2. Although direct computation of tissue half-life is not valid using only two points, a half-life of 15.3 h would predict that nearly 90% of the zilpaterol would have been excreted, which is in excellent agreement with our data. This demonstrates that these tissues are in rapid equilibrium with the plasma, and the results are similar to those of other investigators. The kidney and liver levels of zilpaterol were nearly twice that found in muscle, indicating some preferential accumulation in these tissues.

Table 3 shows urinary zilapaterol concentrations on withdrawal days 0−9 as measured by ELISA, HPLC−fluorescence, and LC-MS/MS methods. Results of regression analyses comparing the three basic methods and comparing the use of an internal or external standard for the LC-MS/MS method are shown in **Table 4**. There was generally a good correlation among the methods, but examination of the slopes demonstrated some interesting differences. Comparison of the slopes of ELISA versus MS/MS-IS (1.731) and HPLC−fluorescence versus MS/MS-IS (1.341) demonstrated that either MS/MS-IS was producing lower concentrations or the others were producing results that were too high. Comparison of MS/MS-IS with MS/MS-ES standard produced a slope of 0.649, indicating the internal standard method was producing lower results. The slopes of HPLC−fluorescence versus MS/MS-ES (0.9101), ELISA versus HPLC−fluorescence (1.253), and ELISA versus MS/MS-ES (1.154) indicate these methods gave essentially equivalent results. Taken together, this evidence indicates the MS/MS-IS method gives erroneous results.

For the urine analysis the ELISA method required no sample cleanup and showed excellent correlation with fluorescence and mass spectral detection. The HPLC−fluorescence method utilized an external standard to correct for incomplete recovery during the cleanup process. Our recovery was 87.8%, with a coefficient of variation of 10.4% in agreement with other work (*12*). We utilized both internal and external standards for the LC-MS/MS method, and although they were highly correlated, the external standard method produced urinary zilpaterol

Table 3. Residues of Zilpaterol Measured in Sheep Urine Using ELISA, HPLC–Fluorescence, and LC-MS/MS Methods

		urine residue (ng/mL)			
withdrawal period (days)	animal	ELISA	HPLC–fluorescence	LC-MS/MS-IS	LC-MS/MS-ES
0	358	4783	3539	2422	3714
	359	1949	1579	1670	2219
	360	1042	827	403	972
	361	383	355	273	633
	362	808	899	211	591
	363	323	399	201	554
	364	674	954	541	902
	366	636	706	304	718
1	358	348	414	302	351
	359	511	467	328	446
	360	84.3	91.3	44	25.6
	361	132	151	107	90.1
	363	70.7	91.4	51.2	49.5
	364	316	390	335	397
2	358	203	200	154	193
	359	101	123	82.8	95.2
	360	13.2	13.3	34.2	6.13
	361	64.6	64	33.9	42.1
	363	19.6	16.6	18.4	9.72
	364	76.4	70.2	91.5	67.4
3	358	67.8	72.9	137	56.6
	360	3.7	5.1	45.7	1.42
	363	1.3	nd[a]	3.3	2.17
	364	34	19.8	25.7	24.8
5	358	11.2	8.4	31.1	1.7
	360	3.3	nd	1.1	1.5
	363	0.6	nd	0.1	0.1
	364	3.6	nd	1.1	1.8
7	360	nd	nd	0.4	0.8
	364	0.7	nd	1.5	1.2
9	360	1.8	nd	0.8	nd
	364	1.1	nd	1.0	0.8

[a] The assay did not detect any zilpaterol in these samples.

Table 4. Correlation and Regression (Zero Intercept) Analysis of the Three Analytical Methods

method	R^2	slope	standard error of slope
ELISA vs HPLC–fluorescence	0.971	1.253	0.034
ELISA vs MS/MS-IS	0.921	1.731	0.084
ELISA vs MS/MS-ES	0.954	1.154	0.042
HPLC–fluorescence vs MS/MS-IS	0.873	1.341	0.082
HPLC–fluorescence vs MS/MS-ES	0.925	0.901	0.041
MS/MS-IS vs MS/MS-ES	0.970	0.649	0.019

concentrations that were in closer agreement with the ELISA and HPLC−fluorescence than mass spectral analysis using the internal standard. The ideal internal standard is an isotopic species (usually deuterated) of the analyte, but deuterated zilpaterol is not commercially available. The use of deuterated analyte ensures the internal standard will demonstrate essentially the same properties as the analyte, avoiding any biases and providing recoveries identical to those of the analyte. Selecting an internal standard when the deuterated analyte is not available is more difficult. Such a standard should have extractive and analytical properties as close to those of the analyte as possible. Van Hoof et al. (*16*) reported the use of d5-ractopamine as an internal standard in an LC-MS^2 procedure for multiple β-agonists determination including zilpaterol in urine and the use of cimaterol as internal standard for zilpaterol determination in feces. Stachel et al. (*15*) reported on an LC-MS/MS method for determining zilpaterol using d7-cimaterol. Consequently, we elected to utilize cimaterol as an internal standard because cimaterol's lack of a phenolic group should result in better results than provided by ractopamine. The reasons for the failure of the internal standard are speculative and might include extractive differences or other analytical differences.

Use of Isolute SPE cartridges for the cleanup of urine samples analyzed by HPLC with fluorescence detection was not satisfactory for the LC-MS/MS detection. Consequently, a new method was developed using mixed-mode (nonpolar C8 and strong cation exchange sorbents) SPE for preparation of samples assayed by LC-MS/MS. Using the mixed-mode method, recoveries were significantly lower than for the HPLC−fluorescence method (~60 versus 88%), but the mixed-mode method demonstrated satisfactory reproducibility and cleaner extracts. The multiple ions selected for quantitation for zilpaterol as well as internal standard exceeded the four minimal identification points for zero-level tolerance compounds requirement set by European Union standard (*22*), thereby providing specificity for the analysis.

In conclusion, this feeding study demonstrated large, nearly 10-fold differences in urinary zilpaterol concentrations among sheep, which decreased as steady state was reached. Even after 10 days of zilpaterol feeding, considerable animal-to-animal variation existed. Estimates of urinary zilpaterol half-lives were roughly 15 h. Zilpaterol residues in liver and kidney were generally greater than zilpaterol residues in muscle, but all tissues showed ≈5% of the initial zilpaterol concentration remaining after a 2 day withdrawal period.

A comparison of the results generated using various analytical methods indicated a high correlation between the methods, but with slightly different results. For LC-MS/MS analyses, use of d7-cimeterol as an internal standard was markedly inferior to use of an external zilpaterol standard. Stringent cleanup was required for HPLC−fluorescence, and even more stringent methods were required for LC-MS/MS. Consequently, an ELISA in which simple dilution is used could be an excellent high-throughput method for zilpaterol analysis.

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