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Depletion of Urinary Zilpaterol Residues in Horses As Measured by ELISA and UPLC-MS/MS

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Three horses were dosed with dietary zilpaterol and the urine concentrations measured from withdrawal day 0 to withdrawal day 21. The analyses were carried out using both enzyme-linked immunosorbent assay (ELISA) and an ultraperformance liquid chromatography with triple-quadrupole−tandem mass spectrometric detection (UPLC-MS/MS). The UPLC-MS/MS method was developed to provide rapid analysis with positive analyte identification by following three product ions and computing the two independent ion ratios. When urinary zilpaterol concentrations were between 0.2 and 2 ng/mL, the ELISA had interday recoveries of 114−120% with coefficients of variation (CV) of <22%; intraday recoveries were 79−111% with CVs of <13%. For urinary zilpaterol concentrations of 0.4−40 ng/mL the UPLC-MS/MS method had interday recoveries of 94−104% with CVs of <8%; intraday recoveries were 97−102% with CVs of ≤7.5%. Correlation analysis demonstrated that the ELISA and UPLC-MS/MS methods returned essentially the same results, especially at urinary zilpaterol concentrations below 2000 ng/mL. Urinary excretion peaked rapidly after dosing between 5300 and 10800 ng/mL (UPLC-MS/MS) or between 5900 and 17900 ng/mL (ELISA) for the different horses, much higher than observed in other species. Urinary zilpaterol concentrations declined rapidly to below 3000 ng/mL within 24 h of study day 1. After about 5 days, zilpaterol elimination slowed markedly, taking nearly 10 days for an order of magnitude decrease. The analytical methods were able to detect zilpaterol in the urine even at withdrawal day 21, demonstrating the sensitivity of each analytical method and the slow rate of zilpaterol depuration from horses.

KEYWORDS: Analysis; ELISA; horses; UPLC-MS/MS; zilpaterol

INTRODUCTION

β-Adrenergic agonists have a multitude of physiological effects in mammalian systems, a fact that has allowed their use in humans (*1*−*3*), predominately to improve lung function, and in veterinary applications (*4*−*6*), most often as a food additive to improve growth characteristics. Important species differences in the action of β-agonists occur due to the variation of β-adrenergic receptor populations in various tissues in different species as well as differences in action of the particular β-agonist including relative β_1 and β_2 activity. Pharmacokinetic differences between species may also cause differences in the observed effects, making extrapolation of action between species unreliable.

The use of β-agonists in food animals (*7*, *8*) has led to multiple instances of food poisoning to consumers of meat products from treated animals (*9*, *10*). Consequently, β-agonist use in food animals has come under intense regulatory scrutiny (*11*), and many countries have adopted a zero tolerance for β-agonist residues in food animal products (*12*). In addition, chronic treatment of horses with the β-agonist clenbuterol at therapeutic and greater doses resulted in deposition of significant muscle mass (*13*, *14*). As far as we could determine, clenbuterol is the only β-adrenergic agonist that has been shown to increase muscle deposition in horses, but such a finding may cause exploration of other β-agonists for this purpose. Although the Federation Equestre Internationale has included sympathomimetic cardiac stimulants such as clenbuterol and other bronchodilators in their prohibited list previously, zilpaterol is specifically mentioned in their 2010 update, which will come into effect in April 2010 (*15*).

Zilpaterol (**Figure 1**) is a β-adrenergic agonist that has been approved for use as a feed additive for cattle (*16*) in Mexico, South Africa, the Dominican Republic, and a number of Central American countries for a number of years. Recently, zilpaterol was also approved by the United States in 2006 (*17*) and in Canada in 2009 (*18*), making it readily available for use. Although the elimination and some pharmacokinetic parameters have been obtained for zilpaterol in food animals, to our knowledge there are no elimination data from horses, particularly race horses. Consequently, a method to measure zilpaterol in horse urine

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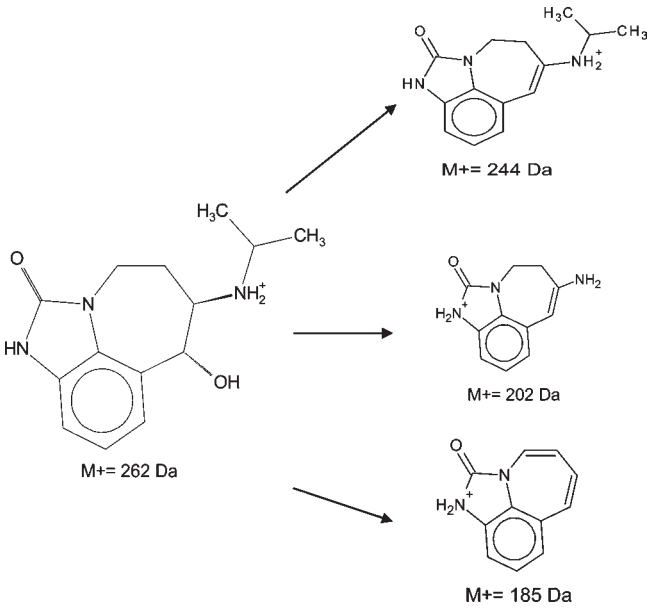

Figure 1. Structures of the zilpaterol precursor ion and three product ions used for LC-MS/MS detection. The collision energy (V) for 262 > 244 was 12, that for 262 > 202 was 20, and that for 262 > 185 was 26. The cone voltage (V) was 25.

would be useful to measure the elimination from horses, allowing more accurate monitoring to prevent illegal use.

Several β-agonists, including zilpaterol, have been detected with triple-quadrupole mass spectrometry subsequent to HPLC in matrices such as cattle urine and liver (*19*), and a multiresidue HPLC-MS/MS method measured 22 β-agonists in bovine and porcine urine (*20*). Another group (*21*) analyzed 16 β-agonists, not including zilpaterol, in pig liver, kidney, and muscle using UPLC-MS/MS. A biosensor approach, using a solublized β_2-adrenoreceptor, was also used to analyze a number of β-agonists in animal feed, but zilpaterol showed very low sensitivity (*22*). Although these multiresidue analyses are valuable for screening, they are often more complex than is needed for a single analyte. In addition, the analyses are often expensive and time-consuming and require stringent sample cleanup procedures and a highly trained operator working in a laboratory environment. We have previously developed monoclonal (*23*) and polyclonal (*24*) ELISA assays toward zilpaterol for potential use for on-site screening purposes. In addition, the monoclonal antibody has also been used in a biosensor format (*25*).

Because zilpaterol is widely available as a feed additive and because precedence has been established for illicit use of β-agonists in sporting animals, including horses, we undertook a study to determine the depletion pattern of zilpaterol in horses. The availability and convenience of the ELISA assay was particularly attractive for a study of this nature; however, we thought it was advantageous to validate results obtained from the ELISA using UPLC-MS/MS so that the ELISA's performance might be evaluated. Therefore, our specific objectives were to (1) measure the urinary excretion of zilpaterol from orally treated horses using ELISA and UPLC-MS/MS formats and (2) determine the usefulness of the ELISA format for detecting the off-label use of zilpaterol in a competitive animal species. Knowledge of the elimination pattern of zilpaterol in horses would allow regulators to rationally evaluate methods of detection.

EXPERIMENTAL PROCEDURES

Monoclonal Antibody Generation. Monoclonal antibodies were generated using standard procedures as reported previously (*23*). Antibodies were screened for their ability to bind zilpaterol using an indirect competitive (ic) ELISA format, and the most sensitive clones that showed minimum effects from salt or other potential matrix effects were selected for further ELISA development. The assay conditions were optimized using an icELISA format to determine the most appropriate amounts of coating antigen, primary antibody, and secondary antibody (*23*).

ELISA Procedure. Control horse urine ($n = 3$) was pooled across several collection days to form a composite control urine; aliquots of pooled urine were buffered (1 to 1 portion, 1:2 final dilution) with 100 mM phosphate buffer and mixed. Zilpaterol was a gift from Houchest-Rousell, Clinton, NJ. Zilpaterol standards were added to buffer diluted control horse urine (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL) to account for matrix effects. The standards were prepared from a zilpaterol stock solution (100 μg/mL) stored at −20 °C that was periodically checked by UPLC-TQ-MS/MS to verify no decomposition had occurred. To provide consistent matrix, incurred horse urine samples were diluted with buffered control urine rather than water prior to the ELISA being conducted. The ELISA procedure was adapted from that of Shelver et al. (*23*). Briefly, 100 μL of zilpaterol-butyrate−BSA (150 ng/mL in bicarbonate buffer) was pipetted into a 96-well plate, incubated, washed, and blotted dry. Aliquots (100 μL) of the sample or standard were added, followed by 50 μL of primary antibody (3H5 at 125 ng/mL). After incubation at 37 °C for 1.5 h and three washings with PBST, rabbit anti-mouse IgG−HRP, 1:25000, was added and incubated for 1 h. Color development was carried out by adding TMB and incubated at 37 °C for 0.5 h, after which the reaction was stopped by adding 50 μL per well of 2 N sulfuric acid, and the plate was read at 450 nm with a Bio-Rad model 550 ELISA plate reader (Bio-Rad Laboratories, Hercules, CA). Calibration data were fitted with a four-parameter logistic equation, and unknown concentrations were computed from the linear portion of the calibration curve. In instances when the zilpaterol concentration present in incurred samples was > 20% B/B_0 from the calibration curve, the sample was further diluted with buffered control horse urine and reassayed.

SPE Cleanup for UPLC-MS/MS. The solid phase extraction (SPE) cleanup procedure was similar to that reported previously (*26*); briefly, 1 mL of horse urine was passed through a preconditioned Varian Bond Elut Certify SPE cartridge (200 mg sorbent) (Varian Sample Preparation Products, Harbor City, CA). Conditioning of SPE tubes consisted of sequential rinsing with 3 mL each of MeOH, H_2O, and 100 mM phosphate buffer (pH 6). Once samples were loaded, cartridges were sequentially washed with 1 mL of 100 mM phosphate buffer (pH 6), 3 mL of 1 M acetic acid, and 3 mL of MeOH. Zilpaterol was eluted with methylene chloride/isopropyl alcohol/30% aqueous ammonium hydroxide (80:20:2.5 v/v/v). Solvent was removed under a stream of nitrogen, and the residue was reconstituted in 200 μL of 50% MeOH/H_2O containing 0.2% formic acid. Samples were centrifuged at 10000*g* for 10 min, filtered through a 0.45 μm syringe filter, and stored in amber LC glass vials at −20 °C until analyzed.

UPLC-TQ-MS/MS Procedure. A Waters Acquity UPLC system in conjunction with a Waters triple-quadrupole mass spectrometer made up the LC-MS/MS system. Data were acquired, processed, and quantified using MassLynx 4.1 with TargetLynx systems. The use of TargetLynx allowed the analysis of three ion transitions, permitting the quantification of two ion ratios for the analyte. Sample aliquots (5 μL) were injected onto an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 50 mm; Waters) and VanGuard precolumns (1.7 μm, 2.1 × 5 mm). The autosampler was maintained at 4 °C and the column at 40 °C. The binary gradient system consisted of A, 5% MeOH/H_2O + 0.2% formic acid, and B, 100% MeOH + 0.2% formic acid. The solvent program was time 0−1.5 min, 10% B → 100% B; 1.5−2.25 min, isocratic at 100% B; 2.25−2.26 min, 100% B → 10% B; 2.26−4.5 min, 10% B; solvent flow for the entire program was 0.5 mL/min.

To optimize mass spectrometric conditions, zilpaterol was directly infused into the mass spectrometer and ionized using electrospray ionization in the positive mode. The precursor ion, product ions, optimum collision energies, and cone voltage were determined by the AutoTune Wizard within the MassLynx 4.1. Product ions used for quantitative purposes are shown in **Figure 1**. The desolvation temperature was set at 500 °C, and the source temperature was set at 150 °C. The flow of nitrogen as cone gas was set at 50 L/h and desolvation gas flow was set at 800 L/h, whereas the collision gas flow of argon was set at 0.16 mL/min. Because the UPLC-MS/MS procedure was developed as a confirmatory method for

the ELISA, the sensitivity was not optimized by applying larger urine samples to the SPE or injecting a larger fraction of the sample (by decreasing the reconstitution volume or increasing the injection volume). This minimized the amount of sample background, allowing better MS properties.

Incurred Urine Samples. The details of the live-phase portion of the study have been reported previously (*27*), and only the essentials will be reported here. The study was conducted at the Animal Metabolism−Agricultural Chemicals Research Unit (USDA-ARS, Fargo, ND) under a protocol approved by the Institutional Animal Care and Use Committee. Horses (a 469 kg, 4-year-old gelding; a 479 kg, 3-year-old filly; and a 462 kg, 5-year-old mare) were under the daily supervision of a veterinarian during the first week. The horses were orally dosed on study day 0 with 0.17 mg/kg of body weight of zilpaterol mixed into a cracked-corn supplement as the commercial feed additive Zilmax. Because of adverse effects such as increased heart rate, tremor, and profuse sweating (*27*), the dose was reduced to approximately 0.04 mg/kg the second day (study day 1) for two of the horses (the gelding and mare); the 3-year-old filly did not fully consume the second dose. Dosing of zilpaterol was terminated after the study day 1 dose was provided. Urine samples were collected daily for 21 days. The samples were frozen and stored at −20 °C until analysis.

Statistical Analyses. Urinary half-lives of zilpaterol in horses were estimated by regressing urinary zilpaterol concentrations ($n = 3$) on time using a two-phase exponential decay curve (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA; www.graphpad.com) described by

$$C(t) = A\,e^{-\alpha t} + B\,e^{-\beta t}$$

where C is the concentration of zilpaterol at a given time (t), α and β are elimination rate constants, and A and B are intercepts associated with rate constants α and β, respectively. Depletion data were fit exclusive of study days 0 and 1 because each horse was exposed to zilpaterol on each of those dates. Sums of squares for Y were calculated after weighing using the $1/y$ transformation. The half-lives were computed from the rate constants by the equations

$$t_{1/2} = 0.693/\alpha \qquad t_{1/2} = 0.693/\beta$$

RESULTS AND DISCUSSION

Initially, the zilpaterol immunoassay matrix interferences were evaluated by generating calibration curves in PBST−BSA and with composite control urine diluted with PBST−BSA (final dilutions of 1:2, 1:5, 1:10, and 1:20). With zilpaterol concentrations below about 1 ng/mL, curves obtained with 1:5, 1:10, and 1:20 buffer dilutions were similar to the curve obtained with zilpaterol standards dissolved in PBST−BSA. At zilpaterol concentrations greater than about 1 ng/mL in urine/buffer dilutions of 1:5, 1:10, and 1:20, none of the dilution curves corresponded closely to the PBST−BSA curve (**Figure 2A**), indicating that significant matrix effects occurred at higher zilpaterol concentrations. However, at the 1:2 dilution the zilpaterol standard curve and the PBST−BSA standard curves were indistinguishable. Thus, the PBST−BSA curve could be used for quantitation of zilpaterol only if urine samples containing <1 ng/mL zilpaterol were diluted 1:5 or greater. To maximize our ability to measure dilute urinary zilpaterol concentrations expected after a prolonged withdrawal period, we elected to utilize a 1:2 dilution and computed zilpaterol concentrations from standard curves prepared in 1:2 dilutions of blank urine. Calibration curves over a 6 month period had a mean ± SEM IC_{50} value of 0.28 ± 0.08 ng/mL ($n = 20$; **Figure 2B**). The limits of detection at 90% B/B_0 were 0.09 ng/mL (or 9 pg/well); the assay range, based on 80% B/B_0 and 20% B/B_0, was from 0.14 ng/mL (or 14 pg/well) to 1.53 ng/mL. The four-parameter logistic fitted curves had $R^2 > 0.99$.

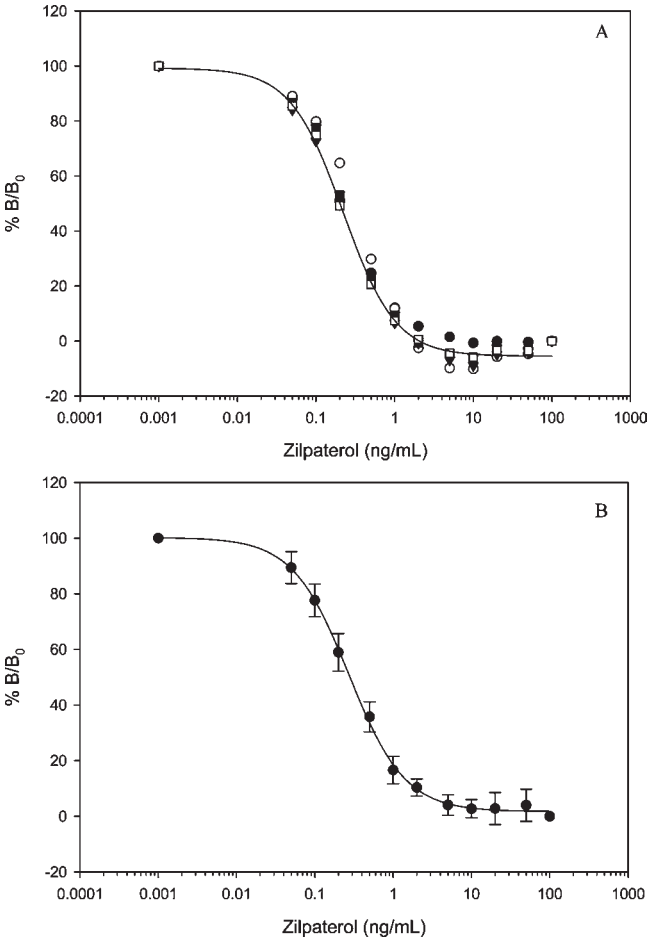

Figure 2. Calibration curves for zilpaterol ELISA: (**A**) evaluation of the matrix effect (●, buffer; ○, urine 1:2; ▼, urine 1:5; ▽, urine 1:10; ■, urine 1:20; and □, urine 1:50); (**B**) calibration curve in urine 1:2, $n = 20$, with standard error lines. The calibration curves were fitted with a four-parameter logistic equation.

Zilpaterol has a unique structure relative to other β agonists with the important 2-alkylamino-1-hydroxy side chain constrained by a fused ring system rather than freely rotating (or energy constrained) side chains common to other β-agonists. For the UPLC-MS/MS analyses of zilpaterol, three ion transitions were simultaneously measured (m/z 262 → 244; m/z 262 → 202; m/z 262 → 185; **Figure 1**). Panels **A**, **B**, and **C** of **Figure 3** show total ion chromatograms of zilpaterol in mobile phase, in fortified urine, and in incurred urine samples, respectively, as well as selected ion chromatograms of the three ion transitions for each sample (panels **D**, **E**, and **F**). The total ion chromatograph results demonstrated that the SPE cleanup efficiently eliminates any interferences because the chromatograph is similar to that of zilpaterol prepared in mobile phase. The three transitions used for quantitation from the urine samples were also demonstrated to be free of interference. Composite control urine samples were fortified with zilpaterol to form standard curves (0.1, 0.2, 1, 2, 10, 20, 100 ng/mL), and the fortified standard curve was processed identically to incurred samples. The matrix-fortified standard curve used for UPLC-MS/MS was linear, $R^2 > 0.99$, with a limit of detection of 0.58 ng/mL (or 14.6 pg on column) based on S/N > 3; the limit of quantitation was 1.77 ng/mL (or 44.4 pg on column) based on S/N > 10. Although the limit of detection can differ by definition, matrices, instrumentation, and cleanup methods, our urine sample results are comparable with those reported by Hoof et al. with their limit of detection defined

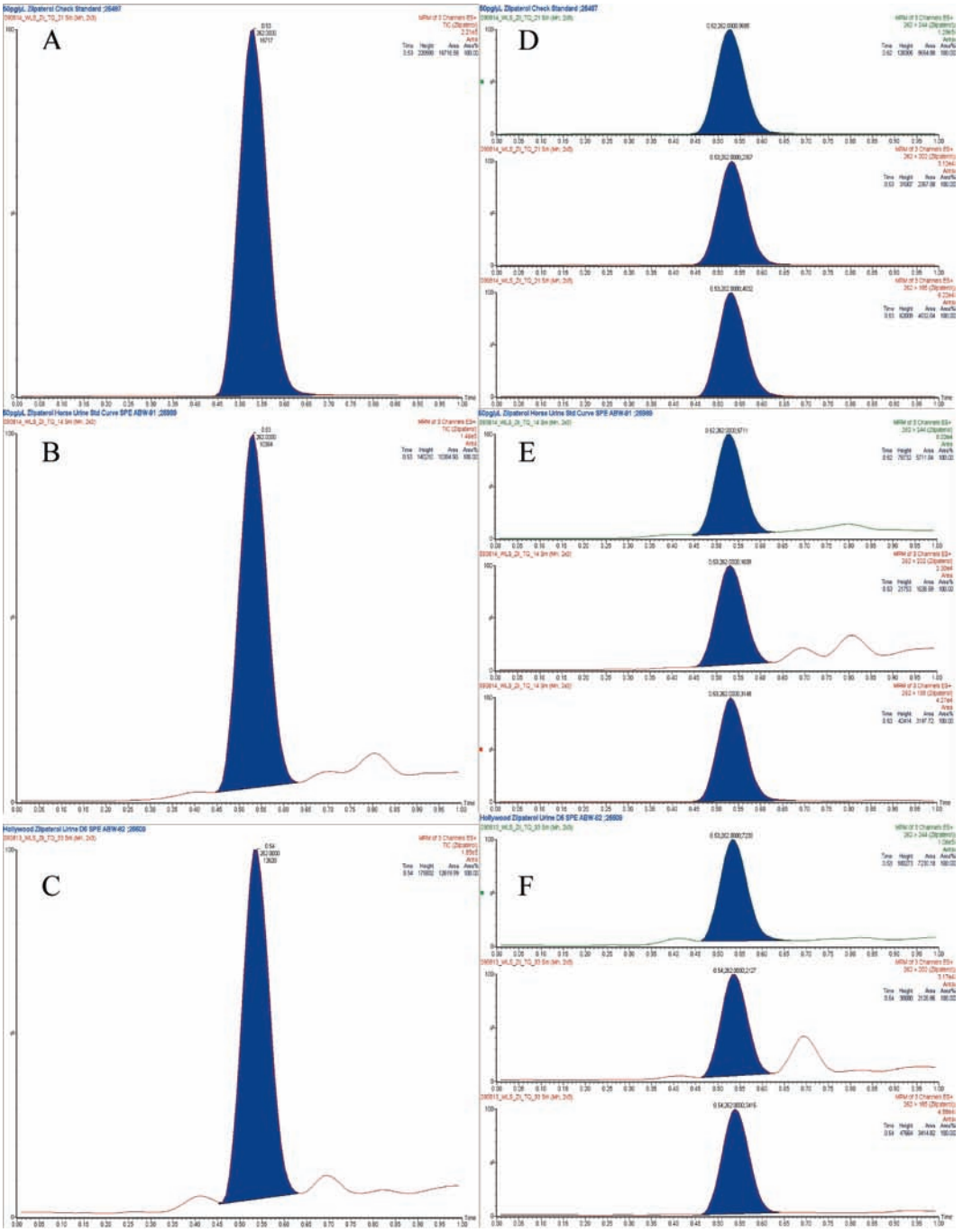

Figure 3. Ion chromatograms from the LC-MS/MS results: total ion chromatogram of zilpaterol in (**A**) buffer, (**B**) spiked urine sample, (**C**) incurred urine sample; multiple reaction monitoring of zilpaterol in (**D**) buffer, (**E**) spiked urine sample, and (**F**) incurred urine sample.

at ≤1 ng/g (*29*) and by Blanca et al. with their limit of detection reported at 0.26 ng/mL (*19*). The UPLC-MS/MS can be further optimized to improve the assay sensitivity by utilizing an increased urine volume for the SPE cleanup, by increasing the injected volume, or by reducing the reconstitution volume or combination of the above approaches. However, these steps could also potentially increase matrix background. Because our sensitivity was adequate for the purposes of the experiment, no opimization of this type was carried out.

Both the ELISA and LC-MS/MS analytical methods had acceptable intra- and interday variation and recoveries (**Table 1**). The UPLC method showed excellent recovery and variability as expected from the use of a matrix-matched standard curve. Recovery was generally 100 ± 6% with the coefficient of variation (CV) of <8% for UPLC-MS/MS. For the ELISA procedure, the recovery was 100 ± 20% with the CV generally <20%. The ELISA method has a much narrower dynamic range compared with the UPLC-MS/MS method. The simplicity and potential portability of the ELISA method compared with the identification information of the UPLC-MS/MS method make the two methods complementary.

Application of the ELISA technique to the measurement of zilpaterol in urine samples of zilpaterol-dosed horses is shown in **Figure 4**. High concentrations (μg/mL) of zilpaterol were measured in the urine of horses the afternoon after the initial zilpaterol dose (study day 0). From study days 3 to 5 urinary zilpaterol concentrations fell fairly rapidly until about study day 6 when urinary zilpaterol concentrations began to fall more slowly.

Table 1. Percent Recovery, Interassay Variation, and Intra-assay Variation of the ELISA and LC-MS/MS Methods[a]

ELISA				
	interassay variation (n = 4)		intra-assay variation (n = 12)	
fortification (ng/mL)	recovery (%)	CV (%)	recovery (%)	CV (%)
0.2	118	22	111	13
0.5	116	15	79	11
1.0	114	20	108	10
2.0	120	9.8	83	11
UPLC-MS/MS				
	interassay variation (n = 3)		intra-assay variation (n = 3)	
fortification (ng/mL)	recovery (%)	CV (%)	recovery (%)	CV (%)
0.4	94	7.4	97	7.5
4.0	97	7.0	102	5.8
40.0	104	0.9	102	3.9

[a] Both ELISA ahd LC-MS/MS standard curves were generated from control horse urine mixed with an equal volume of 100 mM phosphate buffer. The LC-MS/MS samples and standard solution went through solid phase extraction prior to analysis.

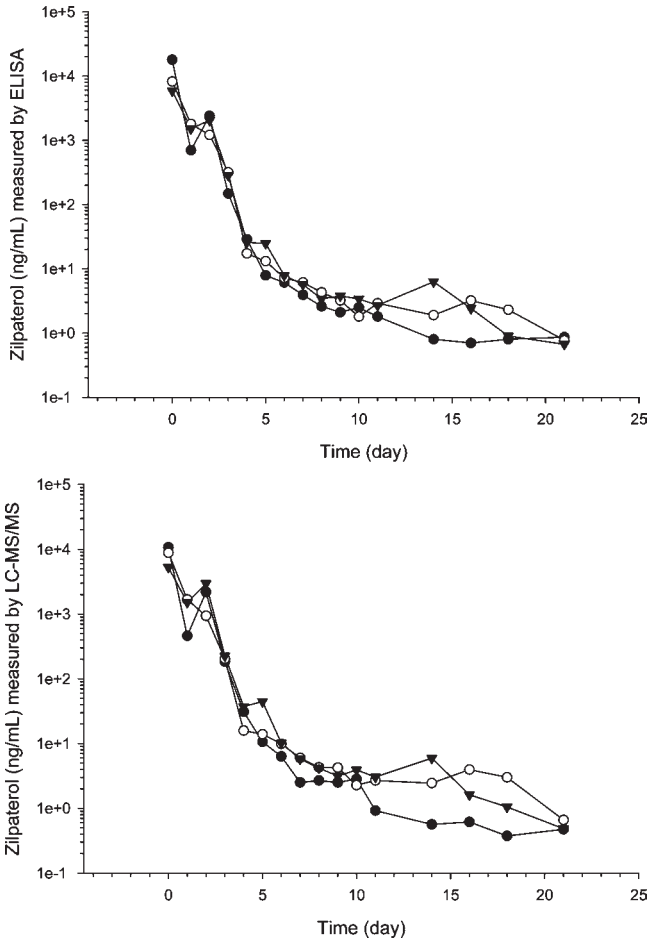

Figure 4. Urinary concentrations of zilpaterol in horses orally administered zilpaterol on study days 0−21 measured by ELISA and LC-MS/MS. Individual values for each horse are shown by solid circles (gelding), open circles (filly), and triangles (mare).

Urinary depuration data, modeled using a two-phase exponential decay curve, indicated that the zilpaterol had α- and β-half-lives of 0.33 and 3.1 days, respectively (weighted R^2 = 0.992).

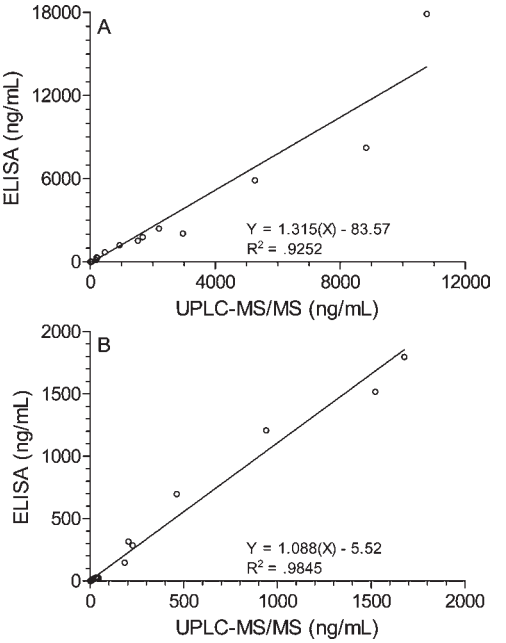

Figure 5. Regression of urinary zilpaterol concentrations derived from ELISA analysis on urinary zilpaterol concentrations derived from UPLC-MS/MS analysis: (**A**) regression analysis of all the data; (**B**) regression of all values under 2000 ng/mL.

Mean ± SE for the α and β rate constants were 2.1 ± 0.16 and 0.22 ± 0.39, respectively. Thus, the uncertainty surrounding the terminal half-life of zilpaterol in horse urine is great. Nevertheless, zilpaterol was detectable in the urine of each horse throughout the 21 day study period. We do not know of other data sets from zilpaterol-dosed animals that encompassed a 21 day depuration period. Because of the high sensitivity of both methods, there is a reasonable chance any use of zilpaterol to increase muscle mass can be detected probably even after this advantage is diminished. Because the muscle mass increase effect generally ceased after 2 weeks of withdrawal and our methods could detect zilpaterol in horse urine after a 3 week withdrawal, these methods would provide powerful tools to discourage the misuse of zilpaterol in horses.

The initial (study day 0) urine zilpaterol levels measured in this study were nearly 10 times the levels measured in cattle (*28*, *29*) provided essentially the same dose. The rates at which urinary zilpaterol concentrations decreased appeared to be similar in cattle and horses (about an order of magnitude per day), although by withdrawal day 10 the levels in the urine of the heifer studied by Stachel et al. (*28*) was about 10 times below what we measured in horse urine at withdrawal day 10. Rate constants were not estimated by Stachel et el. (*28*).

The urinary zilpaterol concentrations in horses were nearly 100 times those observed in pigs (*28*), but again in pigs the initial decrease was rapid showing a 10-fold decrease from withdrawal day 1 to withdrawal day 2; by withdrawal day 4 to withdrawal day 5 the rate of elimination had dropped to an approximate 3-fold decrease in 24 h. The studies of Stachel et al. (*28*) in cattle and pigs were not of sufficient duration to unambiguously measure whether a slow elimination phase for zilpaterol was present in either species.

A comparison of results obtained by ELISA and LC-MS/MS methods was generally favorable (**Figure 5A**), although when all of the data were considered, the ELISA tended to overestimate zilpaterol concentrations, especially at urinary zilpaterol concentrations above 2000 ng/mL (slope, 1.315; intercept, −83.57 ng/mL;

$R^2 = 0.925$). The overall slope of the regression was disproportionately influenced by the high relative response of the ELISA at the greatest urinary zilpaterol concentration (**Figure 5A**); this datum clearly affected the estimation of the slope between the ELISA and UPLC-MS/MS analyses because minimizing the sum of squares for the regression with $1/Y^2$ weighing reduced the slope to 0.962 with an intercept of 0.269 ($R^2 = 0.993$; data not shown). For this reason, the regression analysis was repeated with zilpaterol concentrations of >2000 ng/mL (5 points of 48 total) excluded from the regression (**Figure 5B**). When zilpaterol concentrations were <2000 ng/mL, the ELISA and UPLC-MS/MS regression had a slope of 1.088 and an intercept of −5.5 ng/mL (R^2 = 0.985; **Figure 5B**), indicating that the two analytical methods returned essentially the same results.

There could be multiple causes for the small differences between the responses of the ELISA and UPLC-MS/MS methods, a major factor being possible cross-reactivity in the ELISA due to the presence of zilpaterol metabolites. Alternatively, ion suppression in the UPLC-MS/MS method (*30*) could bias the instrumental method. Our data indicate that the ELISA and UPLC-MS/MS methods returned relatively consistent results, suggesting that if metabolites were present in horse urine, they were present in low amounts or they did not cross-react with the antibody to a significant degree. To our knowledge, there are no commercially available zilpaterol metabolites to confirm whether cross-reactivity occurs. Our observations are supported indirectly from previous reports. For example, data from rats and cattle indicate that >90% of zilpaterol is excreted unchanged, indicating metabolites are relatively unimportant in eliminating zilpaterol (*17*). In cattle urine, deisopropyl zilpaterol represented 2.45%−5.66% of parent plus metabolite; although no glucuronide conjugates were detected, a sulfate conjugate was present. On the basis of urine hydrolysis versus nonhydrolysis peak area ratios, it was concluded that zilpaterol is excreted in the urine mainly unchanged (*29*). Confirmation of ELISA results using UPLC-MS/MS, particularly in samples with low zilpaterol concentrations, provided substantial evidence of the specificity and accuracy of the zilpaterol immunoassay.

We have demonstrated that an ELISA procedure developed toward zilpaterol works well for the analysis of horse urine samples. We believe that the assay could be utilized in a variety of applications involving the detection of zilpaterol in animals treated in an off-label manner. The ELISA assay is amenable to formulation into a kit format that would be useful for on-site analysis. In contrast, the UPLC-TQ-MS/MS analysis would be restricted to a modern analytical laboratory, but is rapid enough (4.5 min) to accommodate a large number of samples per day. The use of a rapid, mobile, and specific test kit on site at competitive animal sporting events could serve as a disincentive for those who might be tempted to use zilpaterol in a manner not approved by regulatory bodies. The UPLC-TQ-MS/MS would provide definitive confirmation of the ELISA results.

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