Immunochemical-based zilpaterol measurement and validation in urine and tissues

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Because of abuse potential of the feed-additive zilpaterol, a need exists for rapid, sensitive and specific analyses. Polyclonal and monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs) were developed and their usefulness for agricultural applications explored. Immunobiosensor formats were developed for both monoclonal and polyclonal zilpaterol antibodies. Zilpaterol ELISA and immunobiosensor were tested by measuring tissue and urinary concentrations from sheep treated with zilpaterol for 10 days. The study demonstrated that sheep eliminated zilpaterol rapidly. A zilpaterol study in horses demonstrated that urinary zilpaterol in horses was initially much higher than in other species and that urinary zilpaterol depleted in a biphasic manner. Zilpaterol was detectable using either ELISA or ultra-high performance liquid chromatography-triple quadrupole-tandem mass spectrometry (UHPLC-TQ-MS/MS) after 21 days of withdrawal in horses. These studies demonstrated that the ELISA procedure was rapid and was in good agreement with instrumental methods while the biosensor method provided greater precision than the ELISA procedure.

Keywords: analysis; β-agonist; ELISA; LC-MS/MS; zilpaterol

Introduction

Zilpaterol hydrochloride [(+/-)trans-4,5,6,7-tetrahydro-7-hydroxy-6-(isopropylamino)imidazo[4,5,1-jk]-[1]benzazepin-2(1H)-one hydrochloride, Figure 1] is unique among commercial β-adrenergic agonists because of its tricyclic ring system that “locks” the free rotation of the β-hydroxyl group and the alkylamino group into a single conformer. The fixed conformation is unlike the free rotation that occurs between the β-hydroxyl group and secondary amine found in most other β-adrenergic agonists. As reviewed by Ruffolo (1991) and discussed by Smith (1998), the relationship between the orientation of the β-hydroxyl group, the phenyl group, and the secondary amine is critical to β-adrenergic agonists’ specificity and potency. Zilpaterol has modest specificity toward the β2-adrenergic receptor, approximately 10 fold greater than the β1-adrenergic receptor (Verhoecks, Doornbos, Van Der Greef, Witkamp, & Rodenburg, 2005). The no observable effect level of zilpaterol in humans (after oral intake) is about 19 times greater than that of the...
highly potent β-agonist clenbuterol and about 125 times less than that of the β-agonist feed additive ractopamine (Smith et al., 2002).

Beta-agonists such as zilpaterol and ractopamine are useful as feed additives to improve growth rates, the efficiency of feed utilisation, and body composition of livestock species (Moody, Hancock, & Anderson, 2000). While both zilpaterol and ractopamine have been approved for use in food animals in Mexico, South Africa and the USA (Montgomery et al., 2009), zilpaterol has not been approved in other countries, and animals or animal products containing zilpaterol residues are considered to be adulterated in those countries where no approval exists.

Unlike other β-agonists such as clenbuterol and ractopamine, there are few analytical methods published for zilpaterol. The first instrumental analysis used gas chromatography-mass spectrometry (GC-MS) to measure zilpaterol in feeds (Bocca et al., 2003) and tissues (Bocca, Fiori, Cartoni, & Brambilla, 2003). Liquid chromatography-mass spectrometric (LC-MS) methods were later reported for zilpaterol in urine, feces and tissues (Stachel, Radeck, & Gowik, 2003; Van Hoof et al., 2005) without derivatisation. However, these methods required intensive, time-consuming cleanup procedures.

Because of the potential for zilpaterol’s abuse, there is a need for sensitive, specific and rapid analytical methods that are robust and capable of adaptation to high throughput formats. Although instrumentation methods such as GC-MS and LC-MS/MS are very sensitive and specific, these methods are not typically suitable for high throughput systems. We have developed polyclonal and monoclonal antibody-based immunoassay and immunobiosensor analyses towards zilpaterol that are amenable to urinary analyses of swine, cattle and sheep (Shelver, Keum, Li, Fodey, & Elliott, 2005; Shelver, Kim, & Li, 2005; Shelver & Smith 2004, 2006) and in tissues from sheep (Shelver & Smith 2006). In this review, development of the zilpaterol immunoassay formats are described, and the performance of the monoclonal-based enzyme-linked immunosorbent assay (ELISA) in horse urine is presented. The horse urine data are compared with data obtained from sheep. The ELISA performance from incurred urine samples is validated by correlating results with results from instrumental analysis.

**Materials and methods**

**Hapten synthesis and antigen conjugation**

The hapten was prepared by the reaction of zilpaterol with ethyl 4-bromobutyrate in the presence of potassium carbonate in acetone. The ester was converted to the free acid by hydrolyzing with 1M potassium hydroxide/ethanol. The hapten’s (zilpaterol-butyric acid) structure was deduced by molecular ion and fragmentation using

![Figure 1. The structure of zilpaterol.HCl.](image-url)
Q-ToF-MS/MS (Micromass, Manchester, England). The zilpaterol hapten was conjugated to either bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 0.1 M 2-[N-morpholino]ethane sulfonic acid] buffer, pH 5 in an overnight reaction in the dark. The free and conjugated haptens were separated by dialysis.

**Antibody generation**

Polyclonal antibodies against zilpaterol were prepared by injection of 100 µg zilpaterol-butyrate-KLH into 2 goats at monthly intervals. After the third booster injection, sera from the goat with the higher titer and better sensitivity were used to develop the ELISA. Monoclonal antibodies were generated by immunizing five female BALB/c mice with zilapaterol-butyrate-KLH. The mouse with the highest titer was selected for the fusion experiments. After spleen harvest, standard procedures were followed for monoclonal antibody generation. Three antibodies were selected for further study based on their sensitivity towards zilpaterol.

**ELISA procedure**

An indirect competition ELISA format was developed for monoclonal and polyclonal antibody-based immunoassays. Zilpaterol-butyrate-BSA, in bicarbonate coating buffer, was allowed to coat the plate overnight followed by plate washing and the addition of either zilpaterol standard or sample, mixed with primary antibody. After incubation, the plate was washed, and either rabbit anti-goat IgG-HRP (1:6000) for the polyclonal assay or rabbit anti-mouse IgG-HRP (1:25,000) for the monoclonal assay was added. Colour was developed by adding 3,3',5,5'-tetramethylbenzidine, and after 30 minutes the reaction was stopped with 2 N sulfuric acid. The ELISA plates were read with a BioRad model 550 ELISA plate reader (Hercules, CA, USA), and curves were fitted with a 4 parameter logistic equation. Concentrations of the unknowns were computed from the linear portion of the curve.

**Immunobiosensor method**

The development of an immunobiosensor assay utilised standard procedures for surface plasmon resonance (SPR) techniques, as described by Shelver, Keum, Li, Fodey and Elliott (2005). A total of four monoclonal antibodies (three originated from the zilpaterol hapten, one originated from a clenbuterol hapten) and five polyclonal antibodies were evaluated. Zilpaterol concentration determinations via SPR were carried out using a Biacore Q instrument equipped with the appropriate control software. Sensor chips (CM-5) were prepared by reacting 4-carboxybutyl zilpaterol, activated with EDC and N-hydroxysuccinimide, with the ethylenediamine activated sensor chip surface. Maximum binding capacity was determined by injecting zilpaterol antibodies and measuring the instrument response. The working dilutions were determined by beginning with concentrated solutions and diluting until a response in the range of 250–500 units was produced. Sensitivity was optimised by varying flow rate, contact time, and the ratio of antibody to analyte. Monoclonal antibody 2E10 was selected for assay development on the basis of low nonspecific binding, reasonable sensitivity, and stability.
**Specificity determination**

Numerous β-adrenergic agonists (bamethane, clenbuterol, isoproterenol, isoxyurine, metaproterenol, fenoterol, ractopamine, ritodrine, salbutamol, salmeterol, terbutaline) and some structurally related compounds including 1-hydroxy-2-naphthoate and 2-hydroxy-benzimidazole were used in place of zilpaterol to generate standard curves (concentration range of 0–1 μg/mL). Resulting 50% inhibition concentrations (IC$_{50}$) were then compared with the IC$_{50}$ of zilpaterol to calculate cross-reactivity of each compound.

**Animal feeding studies**

Eight mature ewes were fed zilpaterol HCl at 0.15 mg/kg contained within a grain supplement for 10 consecutive days. Two animals were fed a grain supplement containing no zilpaterol and were used as controls. Urine was collected from the animals until they were euthanised. Two animals each were euthanised on days 0, 2, 5 and 9 of the withdrawal period and tissue samples were collected. Similarly, a gelding, a filly and a mare were initially dosed with 0.17 mg/kg zilpaterol HCl contained within a grain supplement for 1 day. The dose was cut to approximately 0.04 mg/kg, the second day because of adverse effects (Wagner, Mostrom, Hammer, Thorson, & Smith, 2008) and only the gelding and mare consumed the reduced dose. No further doses were given. Urine samples were collected each afternoon until withdrawal day 21. Urine was frozen and stored at –20° C until used.

**Sample treatment for ELISA**

Incurred sheep urine samples were diluted 1:100–1:10,000 and incurred horse urine samples were diluted 1:2–1:10,000 to maintain a reading on scale. Five grams of sheep tissue (liver, kidney, or muscle) were homogenised in acetate buffer and the supernatant obtained after centrifugation was diluted to 1:10. Diluted urine or tissue homogenates were then analysed by ELISA and zilpaterol concentrations were computed with a calibration curve and adjusted for the appropriate dilution factor.

**Sample treatment for LC-MS/MS**

Five grams of sheep tissue were homogenised in acetate buffer and centrifuged. The resulting supernatant, 1 mL of horse urine or 5 mL of sheep urine was loaded on a Varian Bond Elut Certify Solid phase extraction (SPE) cartridge (200 mg sorbent) (Varian Sample Preparation Products, Harbor City, CA) that was preconditioned with MeOH, H$_2$O, and either acetate or phosphate buffer. The cartridges were washed with buffer, H$_2$O, and MeOH, followed by elution with methylene chloride/isopropyl alcohol/30% aqueous ammonium hydroxide 80:20:2.5 (v/v/v) for sheep tissue and horse urine samples or diethylamine/ethyl acetate 1:99 (v/v) for sheep urine. A stream of nitrogen gas was used to remove the solvent and the sample was reconstituted with either MeOH/10 mM ammonium acetate (5:95 v/v; for Q-ToF-LC-MS/MS) or 50% MeOH/H$_2$O containing 0.2% formic acid (for UHPLC-MS/MS).
**qTOF-LC-MS/MS procedure**

The LC-MS/MS analysis used a Waters Alliance 2695 LC pump, an Atlantis dC18 column, and a Q-ToF API-US mass spectrometer (Waters Corporation, Milford, MA). The gradient consisted of 100% solvent “A” (2% acetonitrile in 5 mM ammonium acetate, pH 4.5 and 0.01% formic acid) to 100% solvent “B” (pure acetonitrile) in 10 min at a flow rate of 0.2 mL/min. The Q-ToF API-US mass spectrometer employed electrospray ionisation in the positive mode with a collision energy of 15 eV and a cone voltage of 25 V. A multiple reaction monitoring mode with the sum of m/z 244.14, 202.09 and 185.07 was used for analysis.

**UHPLC-TQ-MS/MS procedure**

The UHPLC-TQ-MS/MS system consisted of a Waters Acquity UPLC™ coupled to a Waters triple quadrupole mass spectrometer. A VanGuard pre-column and an ACQUITY UPLC™ BEH C18 column (Waters corporation, Milford, MA) were maintained at 40°C with a solvent flow of 0.5 mL/min. Sample aliquots of 5 μL were injected using a Waters autosampler maintained at 4°C. The binary gradient system consisted of solvent “A” (5% MeOH/H2O containing 0.2% formic acid) and solvent “B” (100% MeOH containing 0.2% formic acid). The solvent programme was: 0–1.5 min, 10% B→100% B; 1.5–2.25 min, 100% B; 2.25–2.26 min, 100% B→10% B; 2.26 min–4.5 min 10% B. Zilpaterol was directly infused using electro-spray ionisation in the positive mode to identify the precursor ion, product ions, and to find the optimum collision energies and cone voltage using the AutoTune wizard with the MassLynx™ 4.1 software. Quantitation was based on the simultaneous measurement of three ion transitions (m/z 262→244; m/z 262→202; m/z 262→185).

**Results and discussion**

Hapten synthesis was unremarkable with mass spectrometric evidence demonstrating the presence of the ethyl 4-bromobutyrate moiety attached to the imidazole nitrogen of zilpaterol. After hydrolysis, the free carboxyl group was readily coupled with protein to provide a suitable immunogen. The binding ratio of 3.2 mol of zilpaterol per mol of BSA and 274 mol of ziplaterol per mol of KLH was obtained based on Erlanger’s method (Erlanger, Borek, Beiser, & Lieberman, 1957). In addition, zilpaterol-BSA conjugate’s apparent molecular weight was higher than BSA determined by SDS-PAGE.

Polyclonal antibodies generated by this immunogen showed reasonable sensitivity (IC₅₀ of 3.94 ± 0.48 ng/ml, n = 25), but were quite sensitive to high concentrations of salt or moderate concentrations of solvent. The polyclonal antibodies did not cross-react with any of the β-agonists tested. Recovery values obtained from zilpaterol-fortified swine urine samples ranged from 94 to 106%, with a coefficient of variation of less than 14% at zilpaterol concentrations of 0.5–10 ng/mL. Similarly, zilpaterol-fortified cattle urine samples had recoveries ranging from 98 to 100% and coefficients of variation that were less than 13%. These results demonstrated that the polyclonal/antibody-based zilpaterol ELISA had satisfactory performance, but was limited by salt and (or) solvent effects.
To improve the analytical properties of the ELISA and to ensure a reliable supply of antibodies with reproducible properties, we decided to generate monoclonal antibodies against zilpaterol. Three monoclonal antibodies (3H5, 2E10, and 7A8) with sufficient sensitivities for further evaluation were obtained. Standard curves were generated (Figure 2) and the IC$_{50}$s for 3H5, 2E10, and 7A8 were calculated to be $0.079 \pm 0.008$ ($n = 37$), $0.310 \pm 0.033$ ($n = 40$) and $0.249 \pm 0.039$ ($n = 32$) ng/mL, respectively. The sensitivities of the monoclonal antibodies were greater than the sensitivity of the polyclonal antibodies.

Because of zilpaterol’s unique structure among β-adrenergic agonists, no appreciable cross-reactivity towards other β-adrenergic agonists was expected. Indeed, measurement of competitive binding among the β-agonists tested showed that no measurable cross-reactivity occurred up to the maximum concentration tested, 1 µg/mL for the polyclonal antibodies. These results demonstrate an unusual degree of within-class specificity for the antibody we tested. Among the three zilpaterol monoclonal antibodies tested, 3H5 had a minute amount of cross-reactivity with clenbuterol (0.09%). Interestingly, monoclonal antibody F140 which was generated towards clenbuterol recognised zilpaterol, but was about 10 times less sensitive than monoclonal antibody 3H5 (Shelver, Keum, Li, Fodey, & Elliott, 2005).

Monoclonal antibody 7A8 was more resilient to salt effects than the other clones so it was selected for further study. An ELISA utilizing clone 7A8 gave excellent recoveries and both inter- and intra-day reproducibility using zilpaterol-fortified sheep or cattle urine (0.25, 0.5, and 1.0 ng/ml; data not shown). As expected, recovery and reproducibility were less satisfactory at the lower concentration of 0.1 ng/ml in cattle urine although the analytical properties were satisfactory in sheep urine. Such results underscore the necessity of evaluating each species individually before applying the assay to a specific application.

An immunobiosensor analytical format was developed because presentation of numerous formats ultimately yields wider use and more applications for immuno-logically based assays. An examination of both monoclonal and polyclonal antibodies revealed that monoclonal antibody 2E10 had an optimum sensitivity,
stability, and non-specific binding relative to the other antibodies, thus a biosensor application was developed using antibody 2E10. The biosensor was less sensitive (IC$_{50}$ = 4.47 ng/mL) than the ELISA (IC$_{50}$ = 0.31 ng/mL), but it had good recoveries (87–108%) with inter- and intra-day coefficients of variation of <8%, a level of variation approximately 50% less than that obtained from the ELISA methods. Despite the sensitivity differences of the ELISA and biosensor formats, a good correlation was obtained using these methods for measurements of zilpaterol in incurred sheep urine (Figure 3). 

Tissue and urine concentrations in sheep were determined following the administration of dietary zilpaterol (0.15 mg/kg bw/day) to eight sheep. Zilpaterol was measured using ELISA, LC-MS/MS-IS (with an internal standard) and LC-MS/MS-EX (using an external standard). The LC-MS/MS-IS method was not particularly accurate because a deuterated zilpaterol standard was not available at the time and the internal standard, d7-cimeterol, was apparently not retained proportional to zilpaterol during sample cleanup. However, results from the zilpaterol ELISA and LC-MS/MS-EX analyses were well correlated (R$^2 = 0.9599$) giving essentially equivalent results. In sheep urine, zilpaterol concentrations fell rapidly after withdrawal; with zilpaterol levels after 24 hours of withdrawal ranging from about 10 to 50% of urinary zilpaterol concentrations measured during the dosing period. After 72 hours of withdrawal, urinary zilpaterol concentrations were very low being generally 1–2% of pre-withdrawal concentrations. Both the ELISA and the LC-MS/MS-EX assays could detect zilpaterol on day 9 of the withdrawal period, although zilpaterol concentrations were low at approximately 1 ng/mL. Because of the small sample size for each withdrawal day (n = 2 sheep) it was more difficult to draw extensive conclusions regarding tissue withdrawal kinetics. With this limitation, it was observed that zilpaterol concentrations in tissues at 2 days after withdrawal were about 20% of those 1 day after withdrawal, with relatively little changes on days 5 and 9. Urinary half-life of zilpaterol was estimated to be approximately 14 hours (Table 1).
We dosed horses with zilpaterol because β-agonist use has been banned from, and is monitored in, most sporting events including those involving animals. Thus, for detection of illicit β-agonists in competitive events, analytical methods should have maximum sensitivity. In our analysis of horse urine, we explored the use of minimal dilution, but in making minimal dilutions we had to ensure that matrix effects did not affect our results. In the study of dilution effects we observed that a zilpaterol standard curve prepared in a 1:5 dilution of horse urine produced essentially the same standard curve as that prepared in buffer (Shelver, Thorson, Hammer, & Smith, 2010). By using a standard curve prepared with a 1:2 dilution of blank horse urine, maximum sensitivity could be achieved and any matrix effect could still be compensated. Calibration curves ($n=20$) prepared in blank horse urine over a 6-month period showed excellent reproducibility with an IC$_{50}$ value of 0.28 ± 0.06 ng/mL.

We elected to use UHPLC-MS/MS as both an alternate and confirmatory method for zilpaterol determination in horse urine because LC-based MS/MS methods are clearly becoming the method of choice for confirmatory analysis (De Brabander et al., 2009). The high chromatographic resolution power of UHPLC methods permits separation of analytes and interferences. In addition, the fairly short run-times associated with UHPLC speeds an analytical method and increases overall throughput. Others (Blanca et al., 2005) have used HPLC methods for zilpaterol analyses; however, lower resolution and longer analysis times were associated with use of the HPLC. Still others (Shao et al., 2009) have used UHPLC-MS/MS for the analyses of β-adrenergic agonists, but failed to include zilpaterol as an analyte. Nielen analysed 22 beta-agonists using LC-MS/MS but because of a limitation on the number of multiple reactions that can be monitored

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<th>Urine ng/mL</th>
<th>Liver ng/g</th>
<th>Kidney ng/g</th>
<th>Muscle ng/g</th>
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(MRM) simultaneously, the method of Nielen et al. (2008) required two injections per analysis. Our results demonstrated that sample purification using SPE columns worked well with either total ion chromatography or with MRM when using zilpaterol-fortified buffer, fortified control urine, and with incurred urine. No traces of interferences were detected in any of the matrices. In addition, our UHPLC-MS/MS procedure was rapid with a chromatographic run-time of 4.5 minutes (including re-equilibration). Consequently, the chromatographic method is capable of medium throughput with the ability to run 50–100 samples in an 8-hour day. Although one could argue that the sensitivity of our ELISA was better than the UHPLC-MS/MS method, a counter argument in support of the UHPLC-MS/MS method was that its dynamic range was larger than that of the ELISA. The sensitivity of the UHPLC-MS/MS method could have possibly been enhanced by using a larger volume of sample applied to the SPE cartridges. Recoveries and inter- and intra-assay results using fortified horse urine are shown in Table 2. A comparison of results obtained after analyzing horse urine samples by both the ELISA and UHPLC methods indicated that the assays produced very similar results even at low zilpaterol concentrations.

Mean \((n=3)\) urinary zilpaterol concentrations of zilpaterol-dosed horses are shown in Table 1 along with urinary and tissue zilpaterol concentrations measured in dosed sheep (mean of at least 2 animals: animal numbers vary depending upon analysis; for example, sheep numbers for urine values are variable depending upon withdrawal time). Mean urinary zilpaterol concentrations of horses on withdrawal day 0 were greater than 10,600 ng/mL, a value much higher than that observed in sheep (858 ng/mL) or cattle (approximately 5000 ng/mL estimated from the graph of Stachel, Radeck, & Gowik, 2003). The variation in urine zilpaterol concentration among horses at withdrawal day 0 (5900–17800 ng/mL; \(n = 3\)) was much less than

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the relative urinary zilpaterol concentration in sheep at withdrawal day 0 (300–4800 ng/mL, \(n=8\)). Urinary zilpaterol elimination was rapid with nearly an order of magnitude drop per day corresponding to a urinary zilpaterol half-life of 7–8 hours, a half-life more rapid than that of 14 hours estimated in urine of sheep (Shelver & Smith, 2006). Urinary elimination of zilpaterol in horses was clearly multiphasic. A similar multiphase elimination of zilpaterol was also observed with sheep although the variability in sheep made it impossible to compute a meaningful half-life for the terminal portion of the elimination curve.

Results obtained from the analyses of horse urine samples analysed by ELISA and those analysed by UHPLC-TQ-MS/MS are correlated in Figure 4 (5 points in which zilpaterol concentrations were greater than 2000 ng/mL were not included in the graph). The correlation shows that excellent agreement between the two methods \((R^2 = 0.9845, \text{regression coefficient } 1.088)\) occurred. A similar correlation (Figure 5) obtained after analyses of zilpaterol in sheep urine by ELISA and Q-ToF-LC-MS/
MS was observed. Collectively, these results confirm that values obtained after ELISA analyses were in excellent agreement with results obtained from rigorous confirmatory methods.

**Conclusion**

We have developed a zilpaterol ELISA that has been demonstrated to be highly sensitive, selective, and reproducible. The ELISA method has been validated by comparison of the results from incurred samples with the results from sensitive and specific LC-MS/MS methods. The ELISA method has been utilised in measuring zilpaterol in horse and sheep urine. We have also shown that urinary zilpaterol concentrations are highly variable within species, appear to vary across species, and that urinary zilpaterol elimination is multiphasic in sheep and horses.

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**Disclaimer**

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

**References**


