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Development of a Monoclonal Antibody-Based Enzyme-Linked Immuosorbent Assay for the β-Adrenergic Agonist Zilpaterol

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Zilpaterol is a β-adrenergic agonist approved for use as a growth promoter in cattle in South Africa and Mexico but not in the European Union, United States, or Asia. Here, we report the development of a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for zilpaterol. Mice immunized with zilpaterol−butyrate−keyhole limpet hemocyanin were utilized for monoclonal antibody generation whereas zilpaterol−butyrate−bovine serum albumin was used as a coating antigen for ELISA. Thirteen clones were isolated, and after the initial sensitivity and isotyping experiments, three clones were selected for further ELISA optimization. Studies indicated that the optimum pH was near 7.4. Clone 3H5 had the highest sensitivity to zilpaterol and some interaction with clenbuterol and terbutaline at high concentrations but not other N-alkyl [bamethane, (−)-isoproterenol, (+)-isoproterenol, metaproterenol, or salbutamol] or N-arylalkyl (fenoterol, isoxsuprine, ractopamine, or salmeterol) β-agonists tested. However, clone 3H5 was not functional at high salt concentrations, which precluded further development for urine analysis. Clone 2E10 showed increased sensitivity as salt concentrations were increased and did not cross-react with any of the structural analogues tested. However, its sensitivity to salt and urine concentration changes could cause high variability. Clone 7A8 showed good sensitivity and only a modest change with the salt concentration changes. Clone 7A8 also demonstrated smaller changes in IC_{50} and B_0 with increasing sheep urine or cattle urine concentrations as compared to clones 2E10 or 3H5 and, thus, was selected for further development. The IC_{50} for all of the antibodies showed exponential increases with increasing organic solvents concentrations, making it desirable to minimize solvent levels. In conclusion, a sensitive, specific zilpaterol monoclonal antibody-based ELISA has been developed that can serve as a rapid screening assay.

KEYWORDS: Antibody; analysis; ELISA; assay; zilpaterol; β-agonist; growth promoter

INTRODUCTION

β-Adrenergic agonist repartition agents are utilized to increase feeding efficiency, increase carcass leanness, and promote animal growth, thus offering substantial economic advantages to producers. Zilpaterol (Zilmax), a β-adrenergic agonist repartition agent, can be legally used in Mexico and South Africa as a cattle feed additive but not in other countries. The Government of South Africa has set zilpaterol maximum residue limits for fat, kidney, liver, and muscle at 0.3, 14, 22, and 1.2 ng/g, respectively (*1*). Illegal usages of β-agonists have been reported in the United States as well as other countries (*2*, *3*). When an agent is not legal, there is zero residue tolerance, necessitating a sensitive and specific method to detect the analyte.

A gas chromatography−mass spectrometry method requires extensive sample cleanup and chemical derivatization in order to determine zilpaterol levels in bovine retina (*4*) (62−250 ng/g) and in feeds (30−120 ng/g) (*5*). A more sensitive liquid chromatography−tandem mass spectrometry (LC-MS-MS) method (*6*) was developed for analyzing zilpaterol in tissue and urine samples after heifers or pigs were fed zilmax. This method was able to determine the residues as low as 0.023 ng/g. A slightly different LC-MS-MS method was developed for multiresidue β-adrenergic agonist measurement in calf urine and zilpaterol detection in calf feces (*7*). These methods require stringent cleanup procedures and sophisticated instrumentation, making the methods impractical for the high throughput needed for routine residue monitoring.

Immunologically based rapid assays have been utilized to screen large quantities of samples for the presence of illegal β-agonist residues by regulatory agencies (*2*) as well as producers. Samples that test positive from screening assays are usually subjected to more rigorous confirmatory assays. For

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zilpaterol

2-hydroxybenimidazole (keto form)

Beta adrenergic agonists

Compound	R	R_1	R_2	R_3	R_4
N-alkyl β-adrenergic agonists					
bamethane	n-Butyl	H	H	OH	H
clenbuterol	t-butyl	H	Cl	NH_2	Cl
isoproterenol	i-propyl	H	OH	OH	H
metaproterenol	i-propyl	H	OH	H	OH
salbutamol	t-butyl	H	CH_2OH	OH	H
terbutaline	t-butyl	H	OH	H	OH
N-arylalkyl β–adrenergic agonists					
fenoterol	$-CH(CH_3)CH_2$-(4-hydroxyphenyl)	OH	H	OH	H
ractopamine	$-CH(CH_3)(CH_2)_3$-(4-hydroxyphenyl)	H	H	OH	H
salmeterol	$-(CH_2)_6O(CH_2)_4$-(phenyl)	H	CH_2OH	OH	H

Figure 1. Structure of zilpaterol and other chemicals used in this study.

screening purposes, enzyme-linked immunosorbent assay (ELISA) offers the advantages of simplicity, high throughput, rapid turnaround time, and portability. The utility of immunoassays for analysis of β-adrenergic agonists has been demonstrated for a wide range of compounds i.e., clenbuterol (*8*), albuterol (*9*), fenoterol (*10*), and ractopamine (*10−14*). Because of the great structural differences between zilpaterol and the other β-agonists (**Figure 1**), cross-reactivity between the antibodies utilized in the above assays and zilpaterol likely does not exist and were not examined. Consequently, new antibodies need to be produced for use in zilpaterol assays. Previously, we reported the development of a polyclonal antibody based ELISA for zilpaterol with a sensitivity of low ppb (IC_{50} = 3.94 ng/mL) (*15*). Here, we report the development of a monoclonal antibody based ELISA that has an IC_{50} of less than 0.5 ng/mL. The improved sensitivity will enable us to develop assays for tissue and urine residue analysis.

MATERIALS AND METHODS

Reagents. Hoechst-Roussel (Clinton, NJ) provided a gift of zilpaterol HCl [(±)-*trans*-4,5,6,7-tetrahydro-7-hydroxy-6-(isopropylamino)imidazo[4,5,1-j−k][1]benzazepin-2(1H)-one; CAS-117827-79-9. Lilly Research Laboratories (Greenfield, IN) provided a gift of ractopamine hydrochloride. Bamethane sulfate, clenbuterol HCl, fenoterol HCl, (+)-

isoproterenol HCl, (−)-isoproterenol HCl, isoxuprine HCl, ritodrine HCl, salmeterol 1-hydroxy-2-naphthoate, salbutamol hemisulfate, terbutaline, benzimidazole, 2-hydroxy-benzimidazole, DMSO, pristine (2,6,10,14-tetramethylpentadecane), horseradish peroxidase (HRP) labeled goat anti-mouse IgG, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) were obtained from Sigma-Aldrich Chemical Co., (St. Louis, MO). D-Salt Excellulose GF-5 Desalting Column, ImmunoPure Plus Immobilized Protein A AffinityPak Columns, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), monoclonal antibody isotyping kit 1, and Imject Alum were purchased from Pierce Biotechnology, Inc. (Rockford, IL). The HRP substrate was purchased from KPL (Gaithersburg, MD) as the proprietary SureBlue 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate referred to as TMB. RPMI 1640, hypoxanthine-thymidine (HT), hypoxanthine-aminopterine-thymidine (HAT), fetal bovine serum, penicillin-streptomycin-amphotericin were obtained from Life Technologies (Grand Island, NY). The fetal bovine serum was heat inactivated at 56 °C for 30 min prior to use. Hybridoma cloning factor was purchased from Fischer Scientific (Pittsburgh, PA). Murine myeloma cell line Sp2/0Ag14 (ATCC CRL-1581) was obtained from American Type Culture Collection (Manassas, VA). Polymethyl methacrylate (PMMA) beads were purchased from Sapidyne Instruments (Boise, ID). Goat anti-mouse antibody conjugated with Cy5 was obtained from Amersham Biosciences (Piscataway, NJ).

Mouse Immunization. Animal handling complied with institutional guidelines. Zilpaterol−butyrate−KLH (*15*) (100 μg in 0.25 mL of phosphate-buffered saline (PBS), mixed with an equal volume of complete Freund's Adjuvant to form an emulsion) was injected intraperitoneally into five female BALB/c mice. For subsequent boosters, incomplete Freund's Adjuvant was substituted for the complete Freund Adjuvant as an emulsifier. Mice received 100 μg of immunogen in booster injections every 3 weeks. After the third booster immunization, blood was obtained from the coccygeal vein section. The sera were checked for their titer and ability to compete with zilpaterol. Titer check was done in an indirect ELISA format, using zilpaterol−butyrate−BSA (*15*) as the coating antigen (5 μg/mL) and mice sera (1:2000−1:16000) as the primary antibody. The specificity test was performed in an indirect competitive ELISA format using zilpaterol as a competitor with zilpaterol−butyrate−BSA (*15*) (5 μg/mL) in bicarbonate coating buffer (14 mM, pH = 10) as the coating antigen in 96 well plates. The mouse whose antiserum showed strongest competition toward zilapterol was selected for the fusion experiment. Four days prior to splenocyte harvest, the mouse was injected with 100 μg of immunogen divided equally in intravenous and intraperitoneal injections.

Monoclonal Antibody Generation. The standard procedure for monoclonal antibody generation was used and is only briefly described. Murine myeloma cells Sp2/0Ag14 were maintained in an exponential growth stage in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. The spleen from the mouse with the strongest competition toward zilpaterol and highest titer was aseptically harvested and the splenocytes fused with Sp2/0Ag14 cells. The hybridomas were selected by adding HAT (10 μM sodium hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine). Cell culture supernatants were screened for hybridomas' ability to produce an antibody that recognized zilpaterol (10ng/mL) by indirect competitive ELISA (icELISA; see below). Hybridomas showing greater than 30% inhibition were subjected to further cloning (*16*). After cloning, the cells were expanded and cryopreserved in liquid nitrogen. The IgG from ascites were isolated using a protein G column followed by a size column, according to the manufacturer's instruction. The protein concentrations were quantitated by the method of Bradford and the sample was stored at −80 °C until used.

Zilpaterol ELISA Development. An icELISA format was utilized to measure zilpaterol inhibition and cross-reactivity to related compounds. A checkerboard design (*17*) was used to determine the optimal amounts of coating antigen, primary antibody and secondary antibody needed for icELISA. After optimization, the ELISAs were processed as follows: Zilpaterol−butyrate−BSA (150 ng/mL in bicarbonate buffer) was pipetted into 96 well flat-bottom ELISA plates (100 μL/well) and incubated at 37 °C for 2 h, the plate washed with phosphate-buffered saline plus 0.05% Tween 20 (PBST) three times and blotted dry. Competitor concentrations from 0 to 1000 ng/mL (12 points) in PBST containing 0.1% BSA were pipetted into the corresponding wells at 100 μL/well followed by the addition of 50 μL/well of primary antibody. Where the compounds do not produce a complete competition curve, a shift of the standard concentrations to 100 times higher was utilized. The mixtures were allowed to sit at 37 °C for 1.5 h. After washing the plate three times with PBST, 100 μL of rabbit anti-mouse IgG-HRP 1: 25,000 (2° Ab) was added and incubated at 37 °C for 1 h. After washing the plate 3 times with PBST, a HRP substrate solution (TMB) was added to the plate (100 μL/well) and incubated at 37 °C for 30 min. The color development was stopped by adding 50 μL/well of 2 N sulfuric acid. The plates were read at 450 nm with a Bio-Rad model 550 ELISA plate reader (Bio-Rad Laboratories, Hercules, CA) and the data fitted with a four-parameter logistic equation to determine the IC_{50}. The absorbance reading with no inhibitor was B_0. The initial clone sensitivity screenings have essentially the same ELISA procedure except coating antigen was fixed at 150 ng/mL and cell culture supernatants were utilized instead of purified IgG.

Measurement of Binding Kinetics. Kinetic exclusion fluoroimmunoassay (KinExA) was used to determine the dissociation constants K_d and on and off rates (k_{on}, k_{off}) for zilpaterol binding to three different MAbs. For these measurements, the KinExA 3000 instrument (Sapidyne Instruments, Boise, ID) was used. The principles and details of the assay procedures have been reported elsewhere (*18*). Our procedure was essentially as described previously (*19*). Briefly, the free antibody and zilpaterol−antibody mixture is pumped through a flow cell in which zilpaterol−BSA, immobilized on PMMA beads, captures the unbound antibody. The antibody is then measured using the secondary antibody conjugated with fluorescent dye. Repetition of the measurement for fluorescent signals as a function of time at various analyte concentrations allows the measurement of K_d. The measurement of $K_{on/off}$ rates is carried out by simultaneous injection at a known flow rate of antibody and various concentrations of zilpaterol. The procedure and data processing are described in the literature and on the instrument manufacture's web site (www.sapidyne.com).

pH, Salt, Solvent, and Matrix (Urine) Effects. The effect of these variables were examined by running a standard curve in media of various pH, salt concentrations, urine dilutions, and percentage of organic solvent. The experiments included multiple levels of the variables and were repeated on three different days. The effect of the variable on B_0 (the absorbance of zero concentration of zilpaterol) and the IC_{50} values (from the parameter determined by the least-squares fit of the four parameter equation) were evaluated. To determine the effect of pH, zilpaterol was diluted in 0.1% BSA/PBST having pH values of 4.6, 5.3, 6, 6.7, 7.4, 8.1, and 8.8.

To determine the effect of salt on the assay performance, zilpaterol was diluted in 0.1% BSA/10 mM phosphate buffer (pH 7.4) and NaCl was added to give concentrations of 0, 0.05, 0.1, 0.15, 0.3, 0.5, and 1 M.

To evaluate the effect of solvent on assay performance, methanol, ethanol, acetonitrile, acetone, or DMSO were diluted in 0.1% BSA/PBST to yield final solvent compositions of 0, 2.5, 5, 10, 15, 20, and 30% (v/v).

The effect of urine dilution was determined using pooled urine from 10 ewes which had never been exposed to zilpaterol. Urine was diluted with 0.1% BSA in PBST (1:2, 1:3, 1:5, 1:10, 1:20, and 1:40) and the resulting solutions were utilized for subsequent analyses.

Determination of Inter- and Intraassay Variation. Zilpaterol HCl solution with concentrations of 0.1, 0.25, 0.5, and 1 ng/mL were diluted in 1:10 of sheep or cattle urine in 0.1% BSA/PBST. Zilpaterol concentration in urine samples was computed by interpolation of a zilpaterol HCl calibration standard curve of 10, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0 ng/mL with clone 7A8 as the primary antibody. Intraassay (within day) variation was measured for 12 replicates of each concentration of the zilpaterol-spiked urine. To measure inter-assay (between day) variation, each concentration of zilpaterol was determined on each of five different days with mean of triplicate measurements per day.

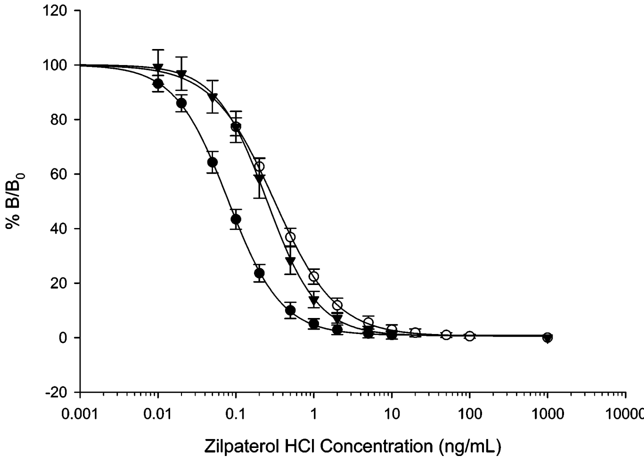


Figure 2. Zilpaterol standard curves for 3H5 (●), 2E10 (○), and 7A8 (▲) in assay buffer.

RESULTS AND DISCUSSION

The fusion results showed the following stable clones [mean IC_{50} in ng/mL (n = 2), isotype] that had IC_{50} below 100 ng/mL: 1G12 (28.3; IgG1 κ), 2A1 (36.7; IgG1 κ), 2E10 (11.8; IgG1 κ), 3G2 (16.1; IgG1 κ), 3H5 (5.7; IgG1 κ), 3H7 (42; IgA κ), 3E1 (33.1; IgG1 κ), 5C5 (46.2; IgG1 κ), 5G5 (36.5; IgG1 κ), 7A8 (18; IgG1 κ), 7B10 (65.6; IgG1 κ), 8F1 (30.3; IgG1 κ), and 8H11 (28; IgA κ). We selected the most sensitive clone from the plate and chose three clones (3H5, 2E10, and 7A8) for ascites generation and further ELISA optimization.

The checkerboard optimization established 150 ng/mL as the optimum coating antigen concentration for all three clones. The optimum primary antibody (IgG fraction) concentration (ng/mL) was 31.2 for 2E10 or 3H5 and 78 for 7A8. The optimum secondary antibody dilution was 1:25,000 for 2E10 or 3H5 and 1:50000 for 7A8. After checkerboard optimization, the competitive ELISA in 0.1% BSA/PBST had an IC_{50} of 0.079 ± 0.008 ng/mL (n = 37) for 3H5, 0.310 ± 0.033 ng/mL (n = 40) for 2E10, and 0.249 ± 0.039 ng/mL (n = 32) for 7A8. The calibration curves for all three clones are shown in **Figure 2** where the greater sensitivity of 3H5 and the nearly identical sensitivity of 2E10 and 7A8 are clearly depicted. The working range (20−80% B/B_0) for the assay was 0.25−0.02 ng/mL for 3H5, 1.01−0.09 ng/mL for 2E10, and 0.71−0.09 ng/mL for 7A8. The limit of detection, based on 80% B/B_0, was 0.02 ng/mL for 3H5 and 0.09 ng/mL for 2E10 and 7A8.

Antibody Specificity. The hapten was originally designed to generate a zilpaterol specific antibody. To test this design we evaluated a number of chemicals structurally resembling portions of zilpaterol's structure. We divided the β-agonists into two separate classes: those with an N-alkyl substituent and those with N-arylalkyl substituents (**Figure 1**). No cross-reactivity of the antibody to any of the tested β-agonists for concentrations up to 1 μg/mL was observed, indicating excellent antibody−analyte specificity for clones 2E10 and 7A8. Of particular importance was the fact that the zilpaterol antibody did not cross-react with clenbuterol (the most often found illicitly used β-agonist) or ractopamine (a β-agonist approved for use in finishing swine and cattle). Cross-reactivity with either of these compounds would diminish the value of the assay as false positives could be encountered. Clone 3H5 has cross-reacted with clenbuterol·HCl with IC_{50} of 88.6 ± 9.4 ng/mL (0.09%) (n = 3) and terbutaline sulfate with IC_{50} of 2860 ± 105 ng/mL

Table 1. Effects of pH, Salt, and Urine on the B_0[a] and IC_{50}[b] of ELISA Assays for the 3H5, 2E10, and 7A8 Clones

	B_0 (OD_{450nm})			IC_{50} (ng/mL)		
var.	3H5	2E10	7A8	3H5	2E10	7A8
			pH of assay buffer			
4.6	1.18 ± 0.05	0.92 ± 0.03	1.07 ± 0.19	0.105 ± 0.007	0.392 ± 0.035	0.220 ± 0.007
5.3	1.19 ± 0.06	0.93 ± 0.02	1.07 ± 0.18	0.088 ± 0.001	0.400 ± 0.029	0.210 ± 0.013
6.0	1.19 ± 0.05	1.00 ± 0.02	1.10 ± 0.16	0.083 ± 0.005	0.356 ± 0.049	0.197 ± 0.006
6.7	1.19 ± 0.02	1.05 ± 0.03	1.14 ± 0.18	0.078 ± 0.002	0.341 ± 0.053	0.199 ± 0.010
7.4	1.09 ± 0.03	1.03 ± 0.02	1.07 ± 0.09	0.071 ± 0.026	0.304 ± 0.014	0.179 ± 0.018
8.1	1.08 ± 0.02	0.98 ± 0.03	1.08 ± 0.19	0.075 ± 0.012	0.349 ± 0.066	0.195 ± 0.015
8.8	1.14 ± 0.04	0.98 ± 0.03	1.16 ± 0.19	0.073 ± 0.005	0.375 ± 0.093	0.200 ± 0.014
			sodium chloride concentration (M)			
0	1.03 ± 0.14	1.14 ± 0.06	1.44 ± 0.05	0.083 ± 0.012	0.766 ± 0.058	0.311 ± 0.014
0.05	1.07 ± 0.15	1.10 ± 0.07	1.38 ± 0.05	0.078 ± 0.008	0.524 ± 0.047	0.256 ± 0.009
0.1	1.08 ± 0.15	1.13 ± 0.08	1.34 ± 0.05	0.073 ± 0.006	0.405 ± 0.032	0.234 ± 0.006
0.15	1.09 ± 0.17	1.09 ± 0.10	1.31 ± 0.05	0.073 ± 0.010	0.339 ± 0.018	0.220 ± 0.018
0.3	0.69 ± 0.10	1.10 ± 0.08	1.26 ± 0.06	0.085 ± 0.005	0.269 ± 0.011	0.189 ± 0.002
0.5	0.46 ± 0.07	1.07 ± 0.08	1.23 ± 0.06	0.136 ± 0.054	0.218 ± 0.021	0.173 ± 0.019
1	0.05 ± 0.01	1.04 ± 0.09	1.19 ± 0.05	ND[c]	0.173 ± 0.015	0.169 ± 0.007
			percentage of sheep urine in assay buffer			
0	0.98 ± 0.04	0.92 ± 0.08	1.26 ± 0.14	0.077 ± 0.008	0.271 ± 0.047	0.207 ± 0.020
5	0.89 ± 0.04	0.90 ± 0.08	1.25 ± 0.17	0.085 ± 0.008	0.311 ± 0.035	0.216 ± 0.015
10	0.79 ± 0.04	0.89 ± 0.08	1.19 ± 0.16	0.099 ± 0.006	0.327 ± 0.052	0.224 ± 0.012
20	0.61 ± 0.03	0.81 ± 0.09	1.07 ± 0.16	0.130 ± 0.014	0.365 ± 0.032	0.235 ± 0.010
33	0.44 ± 0.04	0.72 ± 0.10	0.98 ± 0.18	0.200 ± 0.027	0.463 ± 0.035	0.258 ± 0.016
50	0.36 ± 0.03	0.68 ± 0.11	0.90 ± 0.15	0.276 ± 0.018	0.555 ± 0.057	0.270 ± 0.012
			percentage of cattle urine in assay buffer			
0	1.00 ± 0.02	0.94 ± 0.05	1.32 ± 0.09	0.074 ± 0.004	0.314 ± 0.004	0.212 ± 0.016
5	0.59 ± 0.04	0.81 ± 0.05	1.10 ± 0.09	0.083 ± 0.006	0.379 ± 0.047	0.213 ± 0.030
10	0.46 ± 0.01	0.73 ± 0.05	1.01 ± 0.09	0.119 ± 0.021	0.443 ± 0.032	0.213 ± 0.032
20	0.32 ± 0.01	0.66 ± 0.03	0.86 ± 0.06	0.210 ± 0.059	0.536 ± 0.012	0.239 ± 0.025
33	0.26 ± 0.01	0.57 ± 0.04	0.77 ± 0.05	0.212 ± 0.065	0.656 ± 0.048	0.263 ± 0.058
50	0.20 ± 0.02	0.47 ± 0.02	0.65 ± 0.06	0.395 ± 0.232	0.801 ± 0.080	0.280 ± 0.066

[a] B_0, absorbance reading with no competitors. [b] IC_{50}, concentration of zilpaterol required to inhibit color development by 50% as compared to control wells containing no competitors. [c] ND, not detectable.

Table 2. Effects of Various Solvents on the B_0 and IC_{50} of ELISA Assays for the 3H5, 2E10, and 7A8 Clones

	B_0 (OD_{450nm})			IC_{50} (ng/mL)		
var.	3H5	2E10	7A8	3H5	2E10	7A8
percentage acetone in assay buffer						
0	1.01 ± 0.06	1.00 ± 0.14	1.29 ± 0.25	0.075 ± 0.001	0.305 ± 0.028	0.220 ± 0.041
2.5	0.94 ± 0.04	0.96 ± 0.11	1.29 ± 0.24	0.091 ± 0.005	0.389 ± 0.033	0.278 ± 0.013
5	0.87 ± 0.05	0.89 ± 0.11	1.23 ± 0.24	0.105 ± 0.006	0.487 ± 0.021	0.302 ± 0.012
10	0.77 ± 0.07	0.81 ± 0.10	1.12 ± 0.18	0.140 ± 0.004	0.770 ± 0.062	0.398 ± 0.034
15	0.68 ± 0.05	0.71 ± 0.09	1.04 ± 0.16	0.221 ± 0.014	1.337 ± 0.138	0.564 ± 0.006
20	0.61 ± 0.06	0.61 ± 0.08	0.91 ± 0.14	0.313 ± 0.023	2.557 ± 0.386	0.871 ± 0.027
30	0.51 ± 0.04	0.48 ± 0.06	0.76 ± 0.10	0.611 ± 0.023	8.789 ± 2.063	2.119 ± 0.023
percentage acetonitrile in assay buffer						
0	0.96 ± 0.13	1.09 ± 0.14	1.45 ± 0.17	0.083 ± 0.008	0.325 ± 0.023	0.240 ± 0.008
2.5	0.98 ± 0.08	1.01 ± 0.06	1.47 ± 0.16	0.090 ± 0.003	0.446 ± 0.059	0.282 ± 0.009
5	0.99 ± 0.06	1.00 ± 0.07	1.41 ± 0.14	0.094 ± 0.001	0.570 ± 0.095	0.316 ± 0.005
10	0.97 ± 0.06	0.96 ± 0.07	1.35 ± 0.15	0.122 ± 0.007	1.021 ± 0.118	0.463 ± 0.000
15	0.95 ± 0.08	0.89 ± 0.04	1.24 ± 0.13	0.182 ± 0.027	1.900 ± 0.171	0.785 ± 0.054
20	0.85 ± 0.08	0.78 ± 0.04	1.12 ± 0.12	0.305 ± 0.078	4.973 ± 0.579	1.633 ± 0.235
30	0.62 ± 0.10	0.49 ± 0.14	0.92 ± 0.15	0.771 ± 0.190	25.61 ± 7.764	6.339 ± 0.949
percentage dimethyl sulfoxide in assay buffer						
0	0.98 ± 0.05	1.02 ± 0.03	1.30 ± 0.21	0.079 ± 0.014	0.298 ± 0.012	0.272 ± 0.058
2.5	0.90 ± 0.05	0.97 ± 0.03	1.31 ± 0.19	0.093 ± 0.014	0.380 ± 0.051	0.299 ± 0.059
5	0.85 ± 0.05	0.89 ± 0.04	1.25 ± 0.19	0.101 ± 0.016	0.474 ± 0.054	0.335 ± 0.069
10	0.72 ± 0.04	0.80 ± 0.04	1.13 ± 0.13	0.121 ± 0.020	0.730 ± 0.063	0.416 ± 0.107
15	0.63 ± 0.04	0.70 ± 0.03	1.03 ± 0.13	0.165 ± 0.026	1.230 ± 0.116	0.563 ± 0.143
20	0.53 ± 0.03	0.62 ± 0.03	0.90 ± 0.09	0.215 ± 0.038	1.937 ± 0.312	0.800 ± 0.156
30	0.39 ± 0.06	0.45 ± 0.03	0.70 ± 0.06	0.400 ± 0.051	6.303 ± 0.820	1.909 ± 0.567
percentage ethanol in assay buffer						
0	0.94 ± 0.06	1.10 ± 0.10	1.23 ± 0.19	0.074 ± 0.009	0.358 ± 0.020	0.241 ± 0.009
2.5	0.94 ± 0.06	1.08 ± 0.06	1.23 ± 0.18	0.082 ± 0.008	0.444 ± 0.026	0.310 ± 0.020
5	0.94 ± 0.05	1.04 ± 0.08	1.18 ± 0.21	0.089 ± 0.011	0.588 ± 0.021	0.349 ± 0.040
10	0.92 ± 0.05	1.00 ± 0.09	1.12 ± 0.20	0.108 ± 0.013	0.939 ± 0.036	0.555 ± 0.068
15	0.86 ± 0.04	0.95 ± 0.08	1.05 ± 0.17	0.143 ± 0.025	1.644 ± 0.168	0.789 ± 0.086
20	0.84 ± 0.06	0.88 ± 0.07	0.97 ± 0.15	0.182 ± 0.029	2.596 ± 0.488	1.310 ± 0.149
30	0.74 ± 0.07	0.76 ± 0.10	0.85 ± 0.12	0.294 ± 0.047	8.554 ± 0.245	3.604 ± 0.248
percentage methanol in assay buffer						
0	0.86 ± 0.08	1.04 ± 0.11	1.29 ± 0.25	0.075 ± 0.001	0.330 ± 0.036	0.238 ± 0.016
2.5	0.86 ± 0.06	1.02 ± 0.09	1.29 ± 0.24	0.091 ± 0.005	0.467 ± 0.106	0.309 ± 0.108
5	0.84 ± 0.06	1.02 ± 0.09	1.23 ± 0.24	0.105 ± 0.006	0.558 ± 0.121	0.315 ± 0.028
10	0.85 ± 0.06	1.01 ± 0.08	1.12 ± 0.18	0.140 ± 0.005	0.860 ± 0.200	0.459 ± 0.033
15	0.84 ± 0.05	1.02 ± 0.09	1.04 ± 0.16	0.221 ± 0.014	1.520 ± 0.301	0.611 ± 0.048
20	0.82 ± 0.05	0.99 ± 0.09	0.91 ± 0.14	0.313 ± 0.023	2.396 ± 0.537	1.121 ± 0.415
30	0.77 ± 0.05	0.96 ± 0.10	0.76 ± 0.10	0.611 ± 0.023	5.886 ± 1.178	2.432 ± 0.255

(0.003%) ($n = 3$). This was somewhat surprising because the structures are quite unrelated to zilpaterol. Both clenbuterol (chlorine) and terbutaline (hydroxyl) contains electron donor groups in 3,5 phenyl substitution. In addition, clenbuterol contains an additional electron donor (amino) aromatic substitution that improves the binding 30-fold relative to terbutaline. Despite this recognition pattern, this represents less than 0.1% cross-reactivity, and although it would be better to have undetectable cross-reactivity, it is unlikely to cause false positives. While it is technically a phenethanolamine β-agonist, zilpaterol is structurally distinct relative to other phenethanolamine β-agonists, because of a unique benzimidazole nucleus, which is very likely the cause of the antibodies' high selectivity. We also determined cross-reactivity to two simple chemicals containing the benzimidazole nucleus, namely benzimidazole and 2-hydroxy benzimidazole (**Figure 1**); however, neither compound at concentrations up to 1 μg/mL showed binding with the antibody. The specificity of this antibody is quite unique when compared to the antibodies developed for the analysis of other β-agonists because nearly all other antibodies cross-react to some extent with other β-agonists. The major metabolite of zilpaterol, purported to be deisopropyl zilpaterol (*20*), could cross-react with the antibody; to date the lack of availability of the metabolite has precluded cross-reactivity testing.

Media Effects. The salt effects on clone 3H5 were dramatic, although the B_0 and IC_{50} remained essentially constant at low salt concentrations 0, 0.05, 0.1, and 0.15 M (**Table 1**). The IC_{50} increased 2-fold and the B_0 decreased more than 50% when the salt concentration increased from 0.15 to 0.5 M. At 1 M NaCl, the antibody binding was completely eliminated with B_0 close to zero and no measurable IC_{50}. For clone 2E10, the IC_{50} decreased rapidly at low concentrations and then more slowly with a 4-fold change from zero to 1 M NaCl. The B_0 decreased as salt concentrations increased, although the change was small (<10%). For clone 7A8, the IC_{50} change was less than the other two, and there was an approximately 40% decrease in going from 0 to 1 M sodium chloride (**Table 1**). The decrease in B_0 was nearly linear with about an 18% decrease in going from 0 to 1 M sodium chloride. Clone 7A8 is least affected among the three clones by ionic strength changes and has small effects (26% IC_{50} decrease) from 0.05 to 0.3 M NaCl. Clone 3H5 had the highest sensitivity (lowest IC_{50}) but can be useful only in applications where ionic strength is low, such as in water analysis.

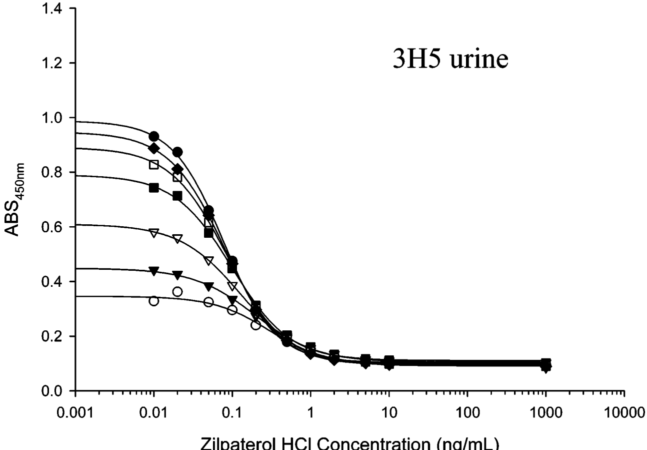
Overall, the antibodies can be used for assays in a broad range of pH (**Table 1**). Variation of assay pH caused little fluctuation in the IC_{50} and B_0 values. Clone 3H5 had the greatest variation of IC_{50} with pH. Clones 2E10 and 7A8 showed little change in

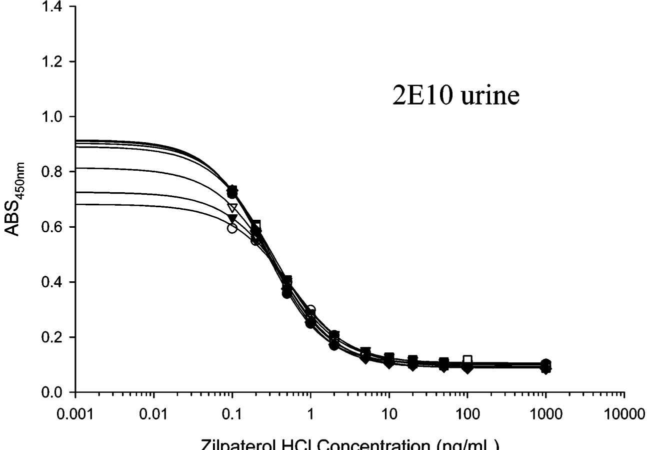
IC_{50} throughout the pH range, although all clones showed the highest sensitivity at pH 7.4. The clones showed little variability of B_0. Thus, pH does not cause much variation, particularly if the pH was kept close to 7.4.

When determining chemical residues from tissues or excreta, organic solvents are commonly used to extract the chemicals out of tissue or excreta and could be transferred in further cleanup (*21*). Acetone, methanol, ethanol, acetonitrile, and DMSO are commonly used solvents for extraction. Because of their utility in such extractions, we tested the effects of these solvents on the performance of the zilpaterol immunoassay. **Table 2** shows solvent effects on B_0 and IC_{50} for all three clones. Our previous examination demonstrated the zilpaterol polyclonal antibody-based ELISA was not significantly altered by 10% acetone, 10% methanol, 10% ethanol, 15% acetonitrile, or 15% DMSO (v/v) (*15*). However, the monoclonal antibodies reported in this study do not tolerate solvent. For all solvents the IC_{50} increased as the solvent percentage increased in an exponential fashion. At 2.5% methanol the IC_{50} increased by 15, 42, and 30%, whereas at 2.5% acetone the increases were 21, 27, and 26% for clones 3H5, 2E10, and 7A8, respectively. B_0 decreased in a linear fashion as the percentage of any given solvent increased, but the decrease in B_0 was small, less than 10% even for 10% (v/v) of solvent added. Similar effects for IC_{50} and B_0 are also observed for ethanol, acetonitrile, and DMSO. For maximum sensitivity, analytical procedures should minimize solvent presence in the ELISA. Alternatively, if higher solvent concentrations are necessary, consistent results could be obtained by maintaining constant solvent concentrations in both the standards and the samples and if the solvent concentrations were kept at 10% or less, where the loss in sensitivity would be approximately 2-fold.

Matrix Effects. Feeding studies of zilpaterol have shown that urine is the major excretion route for zilpaterol in both swine and cattle (*6*, *7*). Because the zilpaterol immunoassay could be easily adapted to applications involving urine (i.e., screening for zilpaterol in live animals), we elected to study the effect of sheep and cattle urine as a matrix. Sample matrices often adversely affect many assays, including immunoassay. To minimize matrix effects, two common routes are often taken, dilution or sample cleanup such as liquid or solid phase extraction. Obviously, the more the urine is diluted the smaller the matrix effect one would expect but with concomitant lowering of the assay sensitivity. Consequently, it is necessary to measure the maximum amount of urine that will minimally affect the assay sensitivity.

Figure 3 shows the calibration curve of all three clones with different sheep urine dilution, whereas **Table 1** contains the values of IC_{50} and B_o derived from calibration curves in various urine dilutions from sheep and cattle. Clearly, from the tabular data there is a systematic decrease in both the IC_{50} and B_0 as the urine concentration increases. **Figure 3** shows the greater interferences of sheep urine for of clone 3H5, but for the other two clones for concentrations up to 10% urine the standard curves are indistinguishable from the buffer. Clearly, higher urine concentrations produce a more marked change in the shape of the calibration curve, predominantly due to a decrease in B_0, although IC_{50}s also change substantially. Comparison between sheep and cattle urine indicate all three antibodies consistently show greater susceptibility to cattle urine although the differences are small (**Table 1**). As anticipated, even with the same matrix (urine), species differences are observed requiring thorough validation prior to application of the assay to a new species. The response of the different antibodies was similar in





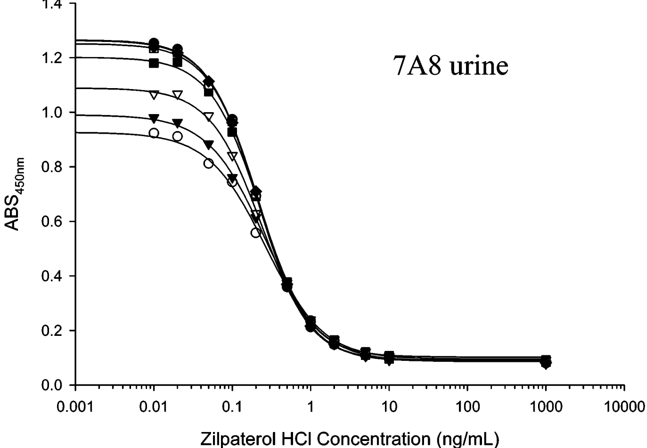


Figure 3. Comparison of sheep urine effects vs buffer calibration curves for 3H5, 2E10, and 7A8 [calibration curve in assay buffer (●), 50% urine (○), 33% urine (▲), 20% urine (△), 10% urine (■), 5% urine (□), and 2.5% urine (♦)].

both species with antibody 3H5 being most sensitive and 7A8 least sensitive to matrix effects. The IC_{50} of clone 7A8 showed a smaller increase than the other clones and this was a critical factor in selecting this clone for further study.

Inter- and intraassay validation studies for clone 7A8 are shown in **Table 3**. The performances for inter- and intra-assay variations are comparable. Recoveries ranged from 90 to 104% for interassay and 86−107% for intraday assay for sheep urine, and 88−99% for interassay and 75−98% for intraday assay for cattle urine. The coefficients of variation (CV) ranged from 4

Table 3. Inter- and Intraassay Variations of the Zilpaterol Immunoassays When Performed in Sheep and Cattle Urine at Various Zilpaterol Fortification Levels

	interassay variation				intraassay variation			
fortification level (ng/mL)	*n*	measured (ng/mL)	average recovery (%)	CV (%)	*n*	measured (ng/mL)	average recovery (%)	CV (%)
				sheep urine				
0.1	5	0.104 ± 0.007	104	6.7	12	0.107 ± 0.005	107	4.7
0.25	5	0.241 ± 0.008	96	3.3	12	0.239 ± 0.010	96	4.2
0.5	5	0.511 ± 0.024	102	4.7	12	0.466 ± 0.023	93	4.9
1.0	5	0.902 ± 0.036	90	4.0	12	0.863 ± 0.082	86	9.5
				cattle urine				
0.1	5	0.099 ± 0.016	99	16	12	0.075 ± 0.026	75	35
0.25	5	0.233 ± 0.016	93	6.9	12	0.216 ± 0.014	86	6.5
0.5	5	0.485 ± 0.014	97	2.9	12	0.488 ± 0.026	98	5.3
1.0	5	0.877 ± 0.028	88	3.2	12	0.867 ± 0.059	92	6.8

Table 4. Binding Characteristics of Zilpaterol to the Antibodies

	KinExA			ELISA
antibody	K_d (nM)	k_{on} ($M^{-1}s^{-1}$) × 10^6	k_{off} (s^{-1})	I_{50}[a] (nM)
3H5	0.04	16.4	0.0007	0.27
2E10	0.13	3.1	0.0004	1.03
7A8	0.12	3.6	0.0004	0.84

[a] The I_{50} is the IC_{50} converted from ng/mL to nM/L to facilitate comparison to the K_d.

to 6.7% for interassay and 4.2−9.5% for intraassay for sheep urine, and 3.2−16% for interassay and 5.3−35% for intraassay for cattle urine. The lowest level (0.1 ng/mL) showed low recoveries and a larger CV for cattle urine although the corresponding values for sheep urine were acceptable. Conversely, higher concentrations, 2 and 5 ng/mL (data not shown) recoveries were 25−49% below the expected value, indicating that antibody 7A8 has a somewhat limited linear working range. The 7A8 has nearly 10 times the sensitivity of the polyclonal antibody we previously reported (*15*), making it potentially very useful.

The kinetic experiments confirmed the characterization done by the ELISA (**Table 4**). Antibody 3H5 was clearly the most sensitive whereas 2E10 and 7A8 were of comparable sensitivity. The greater sensitivity of antibody 3H5 was indicated by a much larger k_{on} rate that more than compensated for a slightly larger k_{off}. The ELISA IC_{50}s were approximately 7−8 times the kinetic experiments K_d values. The reason for the smaller K_d values from the kinetic measurements compared with the ELISA IC_{50} measurements is the kinetic measurements represent true solution measurements for the interaction between the antibody and the ligand whereas the ELISA measurement represent equilibrium between the coating antigen and the antibody.

In conclusion, the three monoclonal antibodies differed considerably in their properties. Although all the three clones were quite specific, their sensitivities differ significantly. While they behaved similarly to some variables such as the presence of organic solvent and pH, other variables such as salt concentrations and urine dilution generated different behavior. Clone 7A8 was the most suitable because it gave the most stable performance in salt and urine, variables that might be hard to control in the analysis of various agricultural samples. The use of 7A8 in an optimized ELISA format to analyze various concentrations of zilpaterol in urine demonstrated excellent reproducibility even though the linear range was somewhat limited.

ABBREVIATIONS USED

BSA, bovine serum albumin; CV, coefficient of variation; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; HT, hypoxanthine−thymidine; HAT, hypoxanthine−aminopterine−thymidine; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.05% Tween 20; PMMA, polymethyl methacrylate; TMB, 3,3′,5,5′-tetramethylbenzidine.

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