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Enzyme-Linked Immunosorbent Assay Development for the β-Adrenergic Agonist Zilpaterol

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Zilpaterol is an β-adrenergic agonist approved for use in cattle in South Africa and Mexico as a growth promoter. It is not currently approved for use in the EU, USA, or Asia. Here, we report the development of an ELISA for zilpaterol. Zilpaterol was reacted with ethyl 4-bromobutyrate followed by refluxing in 0.1 M potassium hydroxide. The resulting hapten was reacted with two carrier proteins, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as an activating agent. Immunization of goats with the zilpaterol-butyrate-KLH resulted in an antibody useful for an ELISA. We utilized zilpaterol-butyrate-BSA as a coating antigen for ELISA development. The average IC_{50} derived from the developed zilpaterol immunoassay was 3.94 ± 0.48 ng/mL ($n = 25$). The antibody did not cross react with *N*-alkyl [bamethane, clenbuterol, (−)-isoproterenol, (+)-isoproterenol, metaproterenol, or salbutamol] or *N*-arylalkyl (dobutamine, fenoterol, isoxsuprine, ractopamine, or salmeterol) β-agonists. The assay was tolerant of up to 10% (v/v) of acetone, ethanol, or methanol, and 15% (v/v) of acetonitrile or DMSO. Salt concentrations ranging from 0.05 to 1.0 M minimally affected B_0 or IC_{50} values. When buffer pH was <7 or >8.8, the IC_{50} values increased in comparison to those measured at pH 7.4. In conclusion, a sensitive, specific zilpaterol ELISA has been developed that can serve as a rapid screening assay.

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

INTRODUCTION

Zilpaterol is an β-adrenergic agonist that has been approved for use in cattle as a growth promoter in South Africa and Mexico, but is proscribed from use in the EU, USA, and Asia. β-Adrenergic agonists are repartitioning agents used to increase feeding efficiency, increase carcass leanness, and promote animal growth. Illegal usages of β-agonists have been reported in the USA as well as other countries (*1*−*3*). In addition, β-agonist tainted meat products have poisoned consumers in several countries. Due to economic advantages incurred by the use of β-agonists, meat products containing illegal zilpaterol residues might represent a risk to consumers. To ensure the safety of consumers in countries for which zilpaterol approvals exist, residues of zilpaterol in meat products must be within tolerances established for zilpaterol. In countries where zilpaterol approval has not been granted, or for species not approved to receive zilpaterol, marketed animal tissues must be free of zilpaterol. Regulatory agencies generally have relied on immunologically based rapid screening assays to screen large quantities of samples for the presence of illegal β-agonist residues (*2*); samples which test positive from the screening assays are usually subjected to more rigorous confirmatory assays. For zilpaterol, no screening assay has been reported.

Currently, the only zilpaterol residue analysis reported in the open literature is a GC-MS method (*4*, *5*), which requires extensive sample cleanup and a chemical derivatization step. Zilpaterol concentrations measured by this method were in the high ppb range (62.5−250 ng/g); the method was adequate for measuring the zilpaterol in feed (∼6 mg/kg), but was not sensitive enough for use in determining zilpaterol residues in animal tissues. For screening purposes, ELISA is advantageous to chromatographic methods because of high sensitivities, high throughput, and rapid turnaround time. In addition, certain ELISA formats are portable and most are user friendly. The utility of immunoassays for applications involving β-adrenergic agonists has been demonstrated by the development assays for clenbuterol (*6*), albuterol (salbutamol; *7*), fenoterol (*8*), and ractopamine (*9*−*12*). Although these assays have been widely reported, and some of them widely used, none have been investigated for interaction with zilpaterol. Because of the relatively great structural differences between zilpaterol and other β-agonists, antibodies directed toward β-agonists other than zilpaterol are unlikely to be useful for zilpaterol analysis. Herein, we report the development of an ELISA specific to zilpaterol.

MATERIALS AND METHODS

Reagents. Zilpaterol HCl [(±)-*trans*-4,5,6,7-tetrahydro-7-hydroxy-6-(isopropylamino)imidazo[4,5,1-*j*−*k*][1]benzazepin-2(1*H*)-one; CAS Registry No. 117827-79-9], was a gift from

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Houchest-Rousell, Clinton, NJ. Ractopamine hydrochloride was a gift from Lilly Research Laboratories (Greenfield, IN). Clenbuterol HCl, dobutamine HCl, fenoterol HCl, (+)-isoproterenol HCl, (−)-isoproterenol HCl, isoxuprine HCl, ritodrine HCl, bamethane sulfate, salmeterol 1-hydroxy-2-naphthoate, salbutamol hemisulfate, benzimidazole, 2-hydroxybenzimidazole, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and Imject Alum were purchased from Pierce Biotechnology, Inc. Rockford, IL. The substrate was obtained from KPL (Gaithersburg, MD) as the proprietary SureBlue TMB microwell peroxidase substrate referred to as TMB.

Preparation of Zilpaterol-Butyrate. The synthesis of the zilpaterol hapten was similar to the procedure reported for the synthesis of zeranol-butyrate by Carter et al. (*13*). Briefly, 100 mg of zilpaterol HCl was added to 10 mL of 1 N NaOH (pH >10) and the zilpaterol free base was extracted with ethyl acetate (5 × 5 mL). The ethyl acetate layer was dried with anhydrous sodium sulfate and the solvent evaporated with a rotary evaporator. To a 50-mL three-necked, round-bottom flask equipped with a mechanical stirrer, a condenser, and an addition funnel was added 32 mg (0.12 mmol) of zilpaterol freebase (oil) dissolved in 10 mL of acetone. Ethyl 4-bromobutyrate (17.5 μL, 0.12 mmol) and 66 mg (0.48 mmol) of potassium carbonate were added and the reaction was refluxed with stirring overnight. The potassium carbonate was then removed by filtration and acetone was evaporated with use of a rotary evaporator to give 58 mg of yellow oil, which solidified on standing at room temperature. Thin-layer chromatography of the reaction product on silica plates (30% methanol/70% dichloromethane; v/v) showed two spots, having R_f values of 0.53 and 0.3 (zilpaterol). The ester was converted to the free acid by refluxing with 10 mL of 66% (v/v) ethanol and 34% (v/v) 0.1 M aqueous potassium hydroxide for 1 h. Unhydrolyzed zilpaterol-butyrate ester was removed by extraction with ethyl acetate and the zilpaterol-butyric acid (**Figure 2**) was characterized in the aqueous layer. Mass spectral analysis of zilpaterol-butyric acid (Q-TOF; Micromass; Manchester, England; MS-MS fragmentation with collision energy of 25 V) indicated masses of *m*/*z* 330, 312, 270, 242, and 214 compatible with the structure proposed in **Figure 2**.

Conjugation of Zilpaterol-Butyrate with Carrier Proteins. Zilpaterol-butyric acid (20 mg) was dissolved in 2 mL of 0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES) and the pH adjusted to 5.0, using 0.1 M NaOH. The resulting solution was divided into 2 parts; one portion was added to 100 mg of bovine serum albumin dissolved in 2 mL of 0.1 M MES (pH 5.0) and the other portion was added to 20 mg of keyhole limpet hemocyanin (KLH) dissolved in 2 mL of 0.1 M MES (pH 5.0). While the solutions were being stirred, 50 mg of EDC was added to the BSA solution and 20 mg of EDC was added to the KLH solution. Each solution was reacted at room temperature for 2 h and then dialyzed into PBS (2 × 4 L). After dialysis, protein concentration was measured, using the Bradford method with BSA as a standard. Protein conjugates were aliquoted and stored at −80 °C until used. The hapten load determination followed the method of Erlanger et al. (*14*). Zilpaterol's ϵ was determined to be 7.68×10^3 L cm^{-1} mol^{-1} at $\lambda_{max} = 287$ nm, and using a molecular weight of 66 000 for BSA and 1 000 000 for KLH, the moles of hapten per mole of carrier protein was computed. In addition, the success of conjugation was evaluated by using 10% SDS-PAGE to compare BSA and zilpaterol-BSA.

Production of Zilpaterol Antibody. Zilpaterol-butyrate-KLH (100 μg in 1.6 mL of PBS) was mixed with an equal volume of Alum Imject until an emulsion formed. The emulsion was initially subcutaneously injected at 6 sites, in each of two goats (nos. 133 and 134). Goats received monthly booster injections containing a total of 100 μg of immunogen. After the third booster immunization, serum from goat 134 was utilized to develop the zilpaterol ELISA because titers from goat 134 were higher compared to those of the other goat.

Zilpaterol ELISA Development. An indirect competition ELISA format was utilized to measure zilpaterol binding and cross reactivity to related compounds. A checkerboard method (*15*) was used to determine the optimal amounts of coating antigen (zilpaterol-butyrate-BSA, 10 μg/mL−10 ng/mL in bicarbonate buffer, 11 steps with 1:2 dilutions) and primary antibody (3rd boost serum 1:2000 to 1:128000 in 0.1% BSA/PBST, 7 steps, using 1:2 dilutions). A second checkerboard experiment was performed using optimum coating antigen with primary antibody (3rd boost serum 1:2000 to 1:2048000 in 0.1% BSA/PBST, 11 steps of 1:2 dilutions) and secondary antibody (rabbit anti-goat-IgG conjugated with horseradish peroxidase (HRP) 1:750 to 1:48000 in 0.1% BSA/PBST, 7 steps of 1:2 dilutions). 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, or at 37 °C for 2 h then at 4 °C overnight. The plate was washed with PBST three times and blotted dry. Competitors (zilpaterol or bamethane, clenbuterol, dobutamine, fenoterol, *R*-isoproterenol, *S*-isoproterenol, isoxsuprine, metaproterenol, salmeterol, ractopamine, salbutamol, benzimidazole, and 2-hydroxy-benzimidazole at 1,000, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0 ng/mL in PBST containing 1% BSA) were pipetted into the corresponding wells at 100 μL/well followed by the addition of 50 μL/well of primary antibody (1:16000). The mixtures were allowed to interact at 37 °C for 1.5 h. After the plate was washed 3 times with PBST, 100 μL of rabbit anti-goat IgG-HRP (1:6000) was added followed by incubation at 37 °C for 1 h. After the plate was washed 3 times with PBST, a HRP substrate solution (TMB) was added to the plate (100 μL/well) followed by incubation at 37 °C for 30 min. 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 resulting curves fitted with a four-parameter logistic equation to determine the IC_{50}. The IC_{50} was defined as the concentration of inhibitor required to inhibit color development by 50% compared to control wells containing no competitors.

Data Processing. Curves were generated as described in sections describing the effects of salts, solvents, and matrix and the resulting competition curves were analyzed by using a standard four-parameter logistic equation to determine the IC_{50} values. B_0 values were determined experimentally. Within a set of experiment data examining an experimental factor (matrix, pH, salt, or solvent) the effects of various levels of the factor on IC_{50} values or B_0 were initially analyzed by a single factor ANOVA; when differences ($P < 0.05$) existed, the Dunnett's t test (*16*) was utilized to determine at what point the experimental data differed statistically from control values.

pH, Salt, Solvent, and Matrix Effects. 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. IC_{50} determinations were made at each pH on three different days. To determine the effect of salt on the assay performance, zilpaterol was diluted in 0.1% BSA/10 mM

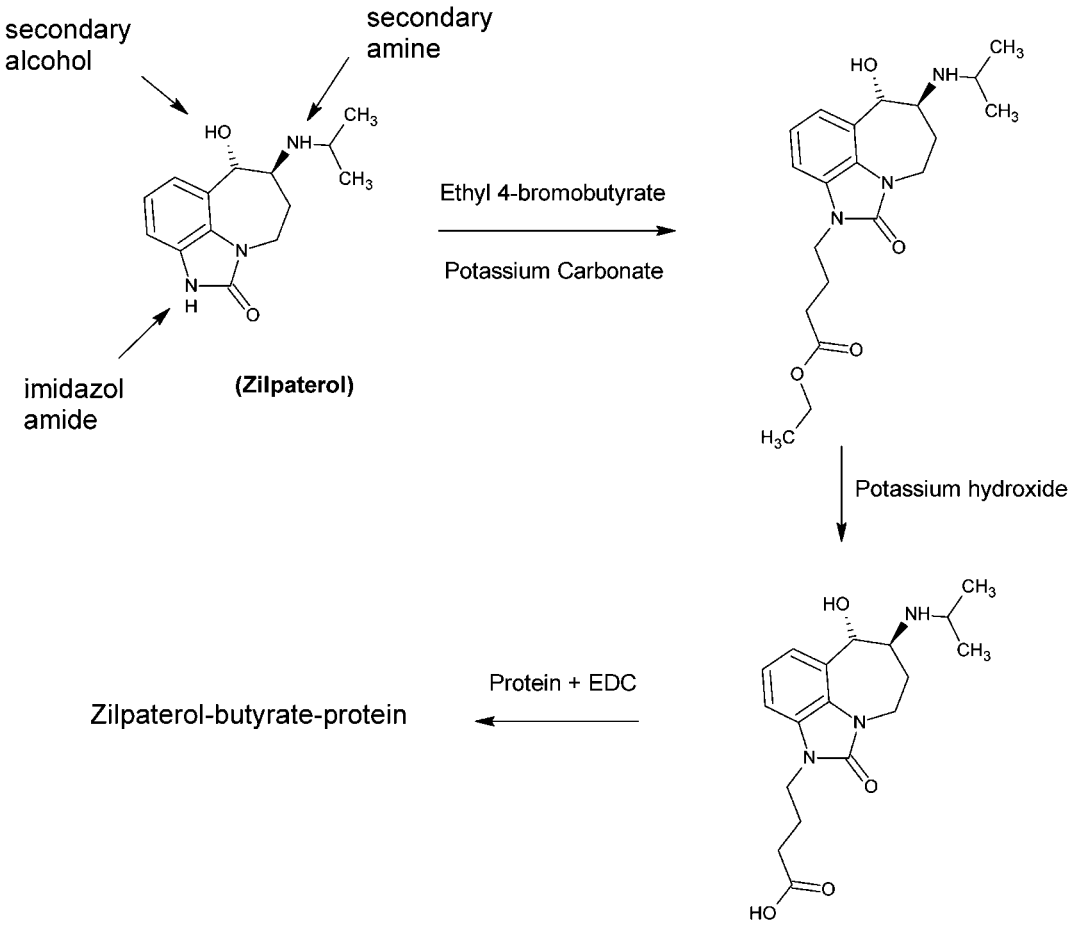


Figure 1. Structure of zilpaterol with potential nucleophilic sites and the synthetic scheme of zilpaterol immunogens.

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, and 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). Calibration curves were constructed for each solvent concentration and IC_{50} and B_0 values were determined.

Urine was collected and pooled from 10 Holstein cows which had never been exposed to zilpaterol. Urine was diluted with 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. Standard curves generated from zilpaterol, diluted in 0.1% BSA/PBST, were compared to standard curves prepared in diluted cow urine. Similar calibration curves were generated with swine urine (pooled from two domestic pigs).

Determination of Inter- and Intraassay Variation. Zilpaterol concentrations of 0.5, 1, 2, 4, 8, and 10 ng/mL were diluted in 1:10 0.1% BSA/PBST diluted cattle urine or 1:5 0.1% BSA/PBST diluted pig urine. Zilpaterol concentration in urine samples was computed by using a zilpaterol calibration standard curve of 1000, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, and 0 ng/mL. Intraassay (within day) variation was measured for 12 replicates of each concentration of the zilpaterol spiked urine. To measure interassay (between day) variation, each concentration of zilpaterol was determined on each of 5 different days.

RESULTS AND DISCUSSION

When developing an immunoassay the hapten must be carefully designed to achieve appropriate specificity. We elected to couple zilpaterol with ethyl 4-bromobutyrate under conditions which ultimately allow the free carboxyl group of the butyrate linker to be activated and coupled to the amino groups of the target protein. Zilpaterol (**Figure 1**) contains three nucleophilic groups (a secondary alcohol, a secondary amine, and the benzimidazole amide) that are potentially capable of displacing the bromine on ethyl 4-bromobutyrate. The secondary alcohol group of zilpaterol would be expected to be considerably less reactive than the aliphatic or benzimidazole nitrogens. The secondary amine would have considerable steric hindrance due to the freely rotating isopropyl group, so the most likely reaction site for zilpaterol and ethyl 4-bromobutyrate would be the secondary amide on the benzimidazole portion of zilpaterol. From a design viewpoint, either the secondary amine or the amide would likely produce a suitable hapten. We believed that in the zilpaterol-conjugated protein, the three-carbon distance between the carboxyl group conjugated to the protein and the zilpaterol molecule would be adequate for efficient coupling and the appropriate exposure of the zilpaterol moiety to promote antibody specificity. The synthesis of the hapten, zilpaterol-butyric acid proved to be routine.

We attempted to identify the actual site of 4-bromobutyrate conjugation to zilpaterol via the use of mass spectrometry. The proposed fragmentation of zilpaterol-butyric acid is shown in **Figure 2**. Although the fragmentation did not conclusively locate the nitrogen to which the 4-butyric acid is attached, the data clearly indicated that only one butyrate "tether" was attached to zilpaterol and the molecular weights of the fragmentation species are compatible with those of the proposed structure. On the basis of Erlanger's method (*14*) the binding ratios were 3.2 mol of zilpaterol per mol of BSA and 274 mol of zilpaterol per

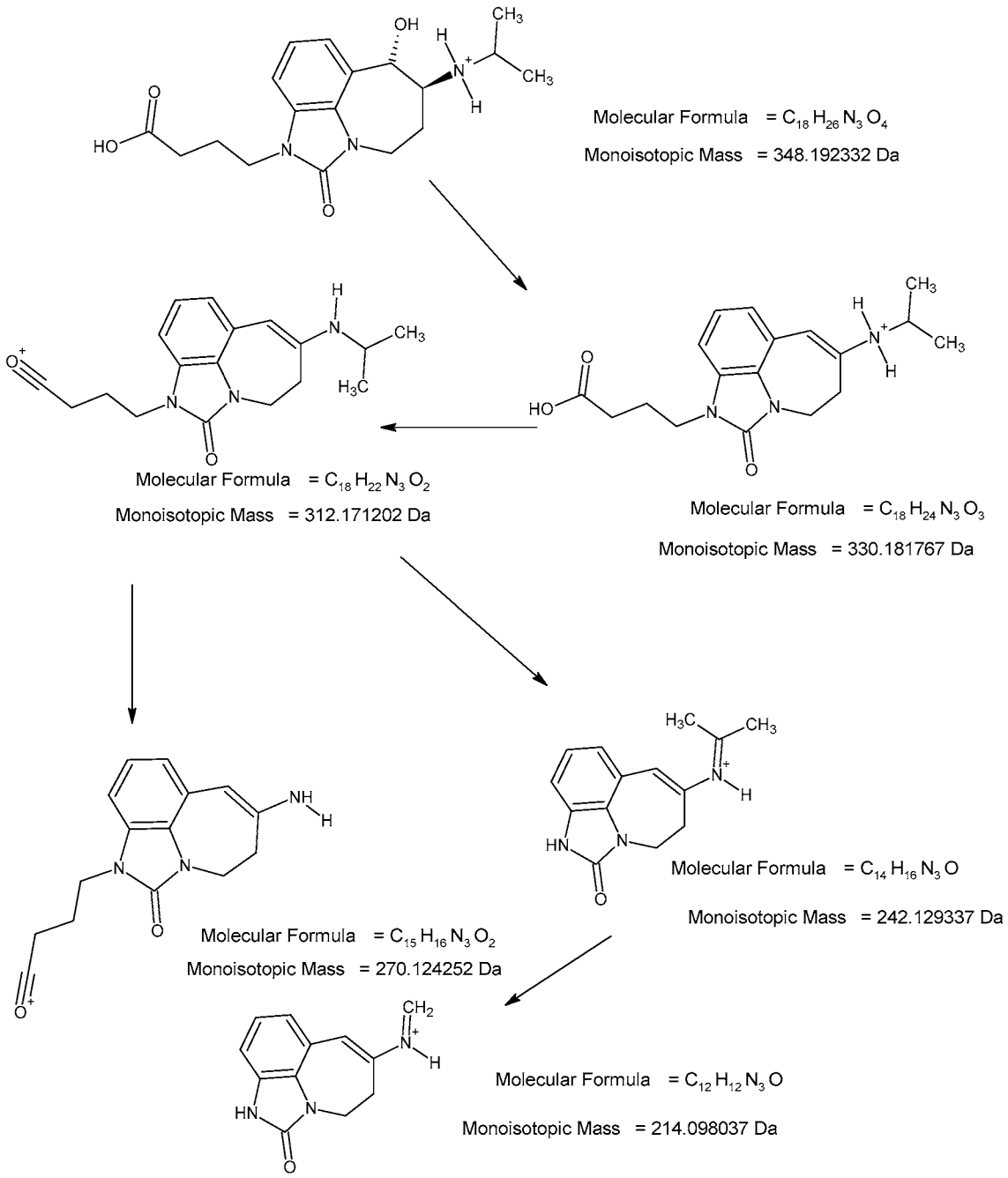


Figure 2. Molecular and fragment ions from Q-TOF mass spectrometry of the zilpaterol–butyrate conjugate.

mol of KLH. The result of SDS-PAGE confirmed a successful conjugation of zilpaterol hapten to BSA. The protein conjugate showed higher apparent molecular weight in comparison to the BSA alone (data not shown).

The competitive ELISA when performed in 1% BSA/PBST had an IC_{50} of 3.94 ± 0.48 ng/mL ($n = 25$), which is sensitive enough for a screening assay. The calibration curve (**Figure 3**) indicated that the working range (20−80% B/B_0) for the assay was 0.4 to 33 ng/mL. The limit of detection, based on 80% B/B_0, was 0.4 ng/mL.

Antibody Specificity. The hapten was originally designed to generate a zilpaterol specific antibody. To test this design we evaluated a number of chemicals with structural elements resembling portions of zilpaterol's structure. We divided β-agonists into separate classes: those with an *N*-alkyl substituent (**Figure 4**) and those with *N*-arylalkyl substituents (**Figure 5**). No cross reactivity of the antibody to any of the tested β-agonists was observed, indicating excellent antibody-analyte specificity. No β-agonists produced any binding inhibition for concentrations up to 1 μg/mL. 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 in some instances. Although a phenethanolamine β-agonist, zilpaterol is structurally distinct relative to other phenethanolamine β-agonists, having a unique benzimidazole nucleus. This uniqueness 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; neither compound was bound by the antibody, again for concentrations up to 1 μg/mL, and no binding inhibition was observed. The specificity

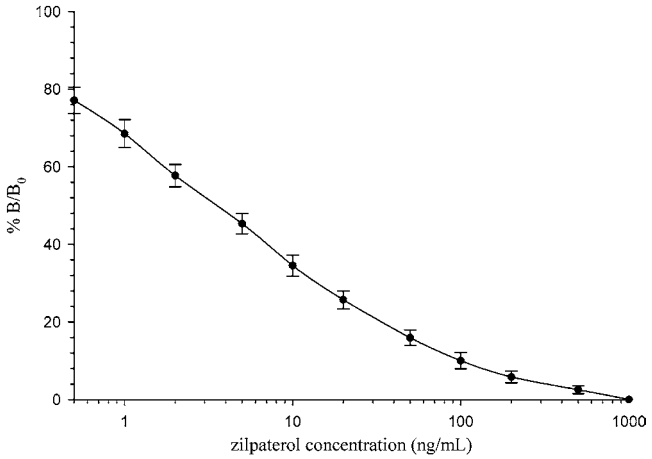
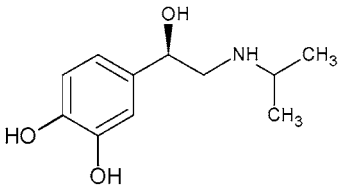
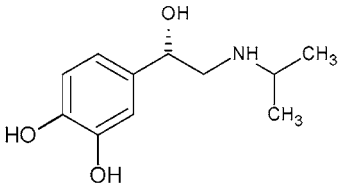


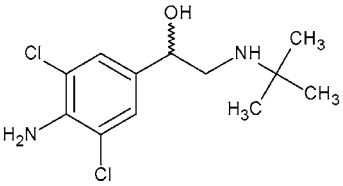
Figure 3. Zilpaterol competition curve run in 1% BSA+PBST; data represent means and standard deviations of 25 determinations.



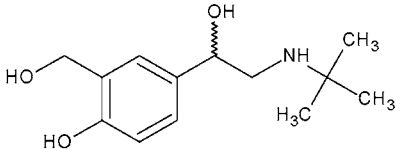
(R-Isoproterenol)



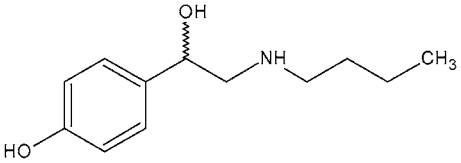
(S-Isoproterenol)



(Clenbuterol)



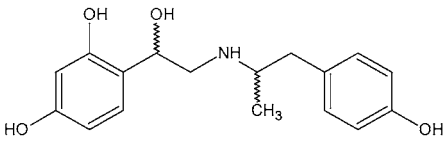
(Salbutamol)



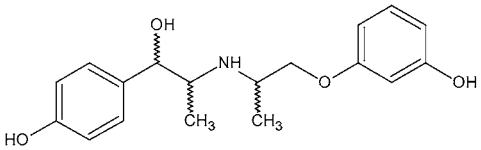
(Bamethane)

Figure 4. Structures of phenethanolamine β-agonists having alkylaminoethanolamine moieties.

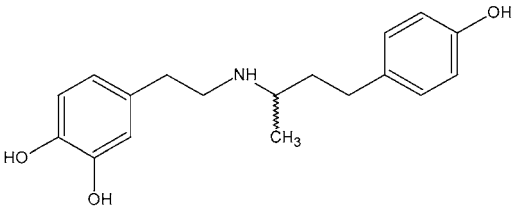
of this antibody is quite unique when compared to antibodies developed for the analysis of other β-agonists; nearly all other antibodies cross react to some extent with other β-agonists. The



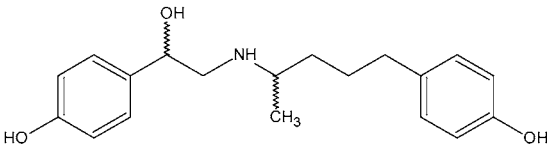
(Fenoterol)



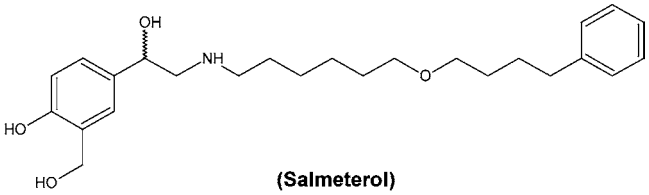
(Isosuprine)



(Dobutamine)



(Ractopamine)



(Salmeterol)

Figure 5. Structure of phenethanolamine β-agonists having arylalkylaminoethanol moieties.

major metabolite of zilpaterol, purported to be de-isopropyl zilpaterol (*17*), could cross react significantly with the antibody; to date, the lack of availability of the metabolite has precluded cross reactivity testing.

Media Effects. Increasing salt concentration up to 1 M in the assay media had no effect ($P > 0.05$) on the measured IC_{50} (**Figure 6**). In fact, the only statistically significant ($P < 0.05$) difference in the IC_{50} values relative to the control (0.15 M sodium chloride) concentration occurred when the assay salt concentration was zero, indicating that the assay and the antibody was robust relative to salt concentration. The change in IC_{50} values from a salt concentration of 0.05 to 1.0 M was slightly greater than 10%. If the salt concentration were kept relatively constant near 0.15 M during the assay procedure, small fluctuations in actual salt values would have little effect on the assay performance. The B_0 decreased with increasing salt concentrations, although the change was small (20%).

Variation of assay pH caused significant fluctuations in the IC_{50} values. A plot of IC_{50} vs pH (**Figure 6**) shows a U-shaped curve with the optimum assay pH occurring between pH 7.4 and 8.1. IC_{50} values at pH 7.4 and 8.1 were not different ($P > 0.05$), but IC_{50} values measured at other pHs did differ ($P < 0.05$) from that obtained at 7.4. Fitting the data with a power series indicated that the minimum IC_{50} (maximum sensitivity)

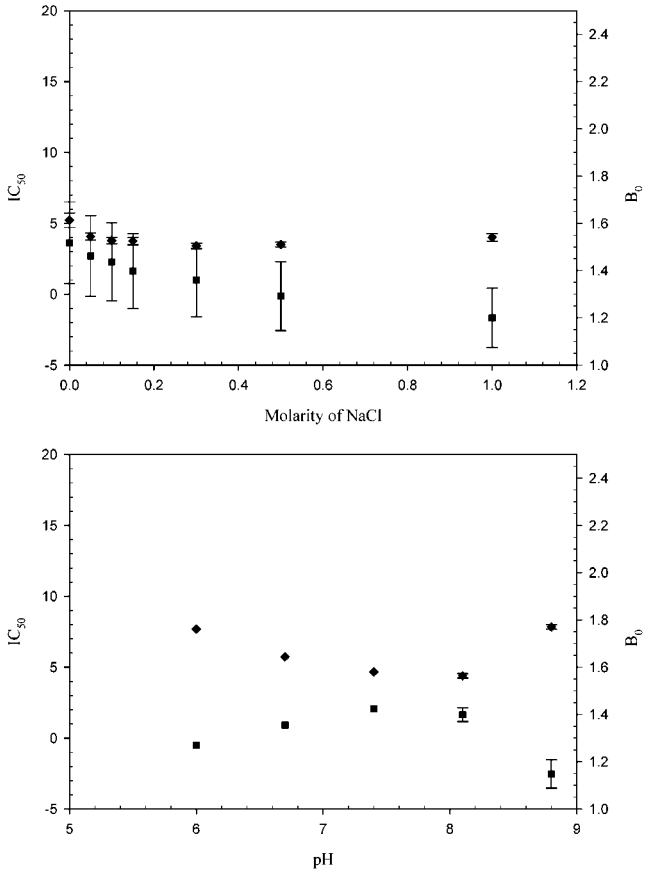


Figure 6. The effects of pH and salt concentrations on IC_{50} and B_0 values ($n = 3$). Data are expressed as IC_{50} (diamonds; ng/mL) or B_0 (squares; absorbance units at 450 nm).

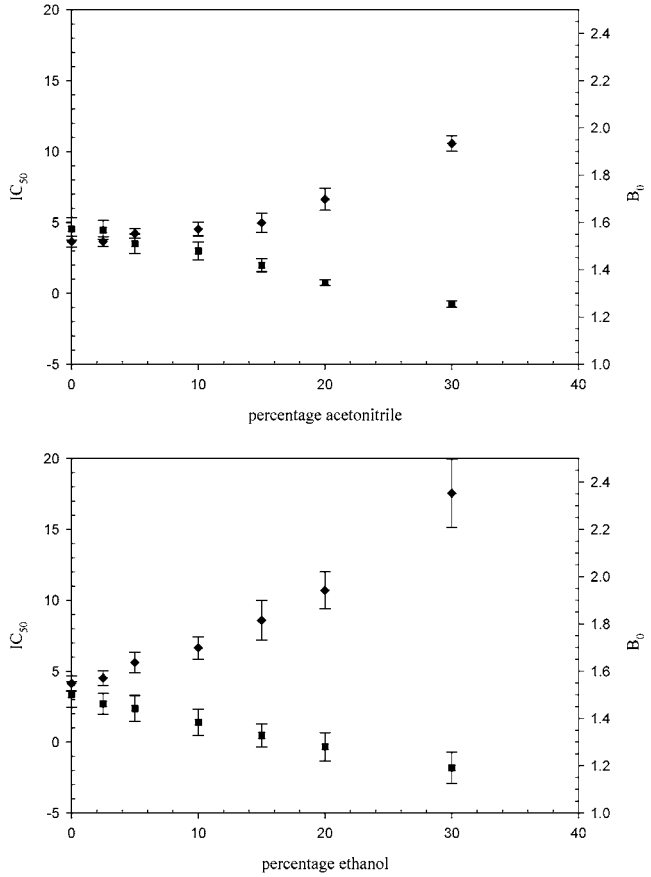


Figure 7. The effects of percentage solvent on IC_{50} (diamonds; ng/mL) and B_0 (squares; absorbance units at 450 nm) for acetonitrile and ethanol for the zilpaterol immunoassay ($n = 3$).

was obtained at pH 7.2. The IC_{50} values were nearly doubled at pH 6.0 and 8.8, indicating that pH has a critical role in assay performance.

The change in IC_{50} with pH suggests that ionic interactions between zilpaterol and the zilpaterol antibody are critical for binding. The decrease in IC_{50} from a high pH to 7.4 could indicate protonation of a group involved in binding possibly zilpaterol's amine group and the increase below 7.4 could indicate protonation of a negative group involved in the binding of the protonated zilpaterol. This pH dependence is not definitive for this behavior, but is certainly compatible with our explanation. B_0 also changes with variations in pH in a parabolic fashion with an optimum at approximately 7.4. Clearly, the data indicate that assay performance is optimized when the assay is run in a moderate concentration buffer (0.15 M) at pH 7.4, providing a sensitive IC_{50} and an acceptable B_0 value.

Acetone, methanol, ethanol, acetonitrile, and DMSO are commonly used for the extraction of organic analytes from tissues and excreta (*18*). Because of their utility in such extractions, we tested the effects of these solvents on the performance of the zilpaterol immunoassay. Dunnett's test was used as a statistical means to identify the lowest concentration of solvent that would alter the assay IC_{50} significantly relative to solvent-free controls. The greatest solvent percentage that caused no statistical alteration in IC_{50} represented the maximum solvent concentration the assay would tolerate. The ELISA performance was unaltered by 10% (v/v) acetone, 10% (v/v) methanol, 10% (v/v) ethanol, 15% (v/v) acetonitrile, and 15% (v/v) DMSO. For all solvents the IC_{50} increased as the solvent percentage increased in a curvilinear fashion as exemplified for acetonitrile and ethanol (**Figure 7**). B_0 decreased in a linear fashion as the percentage of any given solvent increased. The decrease in B_0 was small, relative to the changes in IC_{50}. For maximum sensitivity, analytical procedures should minimize solvent present in the ELISA. Alternately, if higher solvent concentrations are necessary, consistent results could be obtained by maintaining constant solvent concentrations in both the standards and the samples. Such a strategy would result in a slight loss of sensitivity, but precision could be maintained. Because of the curvilinear response, assay sensitivity could rapidly decrease if solvent percentage were increased relative to the maximum percentage of solvent shown above.

Matrix Effects. 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 urine on the IC_{50} as a measure of the matrix effect. 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.

Bovine urine decreased the assay sensitivity as measured by the IC_{50}, even at relatively low concentrations (**Figure 8**). For example, the IC_{50} increased ($P < 0.05$) from 3 ng/mL to 6.5 ng/mL when the assay included 20% bovine urine. When 10% bovine urine was included in the assay, the IC_{50} values were not statistically different ($P > 0.05$) although the values were 39% higher; consequently, the addition of 10% bovine urine was used for zilpaterol immunoassay.

Interestingly, the assay was much more tolerant of porcine urine than bovine urine (**Figure 8**). Although the IC_{50} with 20%

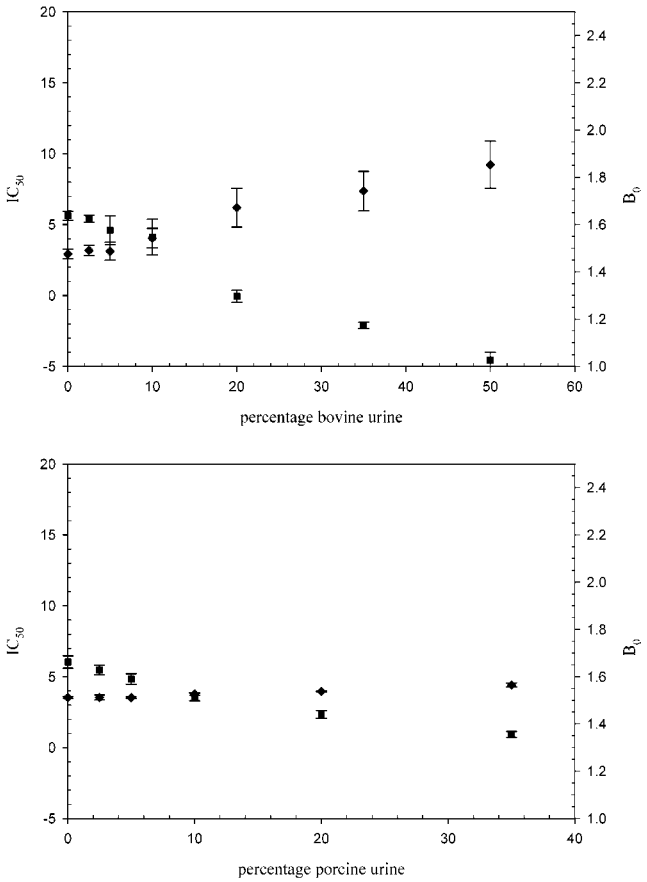


Figure 8. The effects of bovine or porcine urine on IC_{50} (diamonds; ng/mL) and B_0 (squares; absorbance units at 450 nm) for the zilpaterol immunoassay ($n = 3$).

Table 1. Inter- and Intraassay Variations Obtained for the Zilpaterol Immunoassay When Performed in Porcine and Bovine Urine at Various Zilpaterol Fortification Levels

	interassay variation				intraassay variation			
fortification level, ng/mL	*n*	measd, ng/mL	av recvd, %	CV, %	*n*	measd, ng/mL	av recvd, %	CV, %
				swine urine				
0.5	5	0.53 ± 0.05	106	9	12	0.47 ± 0.06	94	13
1	5	0.94 ± 0.07	94	7	12	1.04 ± 0.09	104	8
2	5	2.07 ± 0.12	104	6	12	2.02 ± 0.26	101	13
4	5	3.90 ± 0.13	97	3	12	3.81 ± 0.55	95	14
8	5	7.97 ± 0.09	100	11	12	8.31 ± 0.61	104	7
10	5	9.64 ± 0.18	96	2	12	10.44 ± 1.00	104	10
				cattle urine				
0.5	5	0.56 ± 0.07	111	12	12	0.53 ± 0.06	106	12
1	5	1.17 ± 0.09	116	8	12	1.02 ± 0.09	102	9
2	5	2.15 ± 0.28	107	13	12	2.04 ± 0.16	102	8
4	5	4.05 ± 0.11	101	3	12	4.42 ± 0.35	110	8
8	5	7.86 ± 0.54	98	7	12	8.36 ± 0.24	104	3
10	5	10.32 ± 1.04	103	10	12	10.48 ± 0.56	105	5

urine was statistically different from the control, the IC_{50} was only 12% higher. This statistically significant difference at a small change in the IC_{50} is a consequence of the small variance of the measurements in porcine urine. We elected to use 20% porcine urine in this analysis. By running the standard curve with blank urine of the same concentration as the samples, problems with accuracy and precision will be minimized.

Inter- and intraassay validation studies are shown in **Table 1**. Recoveries were generally within 15% of the targeted value with coefficients of variation of 15% or less. As one might expect, variation was greater at the lower fortification levels (0.5 and 1 ng/mL). Recoveries from cattle urine (diluted 1:10) were comparable to those obtained from porcine urine (diluted 1:5).

In conclusion, using a hapten generated by reacting zilpaterol with ethyl 4-bromobutyrate followed by alkaline hydrolysis an antigen was generated that produced a very specific antibody to zilpaterol. This antibody was used to develop an ELISA that was capable of determining zilpaterol with an IC_{50} of 3.94 ± 0.48 ng/mL, sufficient sensitivity for residue analysis. The assay was tolerant to low concentrations of organic solvents and demonstrated minimal matrix effects when used to analyze bovine and porcine urine providing the samples were diluted with 1% BSA/PBST.

ABBREVIATIONS USED

BSA, bovine serum albumin; CV, coefficient of variation; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; MES, 2-[*N*-(morpholino)ethanesulfonic acid]; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine.

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