Analytical Methods

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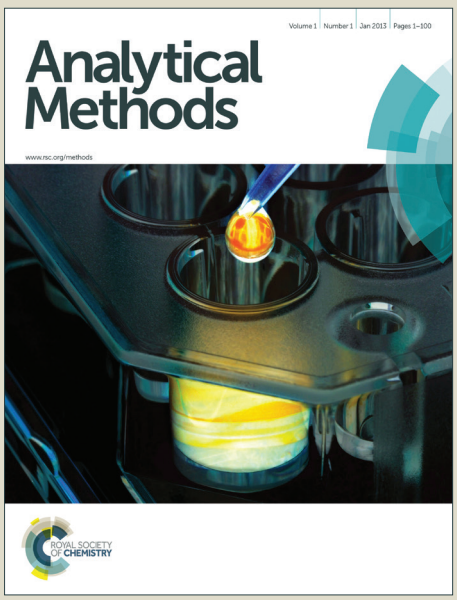

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Structure-specific hapten design for the screening of highly sensitive and specific monoclonal antibody to salbutamol

Liqiang Liu[a], Huang Kuang[a], Chifang Peng[a], Libing Wang*[b], Chuanlai Xu*[a]

[a] State Key Lab of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, JiangSu, 214122, PRC. E-mail: xcl@jiangnnan.edu.cn. Fax: +86 510-85329076, Tel: +86 510-85329076
[b] Technological Centre of Hunan Entry-Exit Inspection and Quarantine Bureau, Changsha, 410001, China

Abstract

Salbutamol (SAL) is a short-acting β2-adrenergic receptor agonist (β-agonist) forbidden in livestock production. To develop a more sensitive and specific detection method for SAL, a new hapten was synthesized, keeping the structure of benzene ring side of SAL while changing the tert-butyl group structure that SAL shares with other β-agonists. Ten mice were immunized with a SAL hapten-keyhole limpet hemocyanin (KLH) conjugate, and cells were selected within a urine sample at the cell fusion stage. The best monoclonal antibody (Mab) had an IC_{50} value of 0.31 ng/mL in PBST buffer and an IC_{50} value of 0.19 ng/mL in a swine urine sample diluted five times (with a lower B_0 value), and had no cross-reactivity with clenbuterol and other β-agonists. These results showed that the hapten design was successful and that the Mab can potentially be used in highly sensitive immunoassays for specifically monitoring the illegal use of SAL in real samples.

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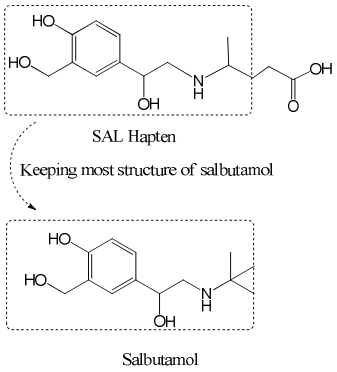

A new hapten was designed and synthesized for the production of highly sensitive and specific monoclonal antibody to salbutamol.

Introduction

Salbutamol (SAL) is a short-acting beta-adrenoceptor agonist (β-agonist) used for the treatment of asthma due to its bronchodilation and anti-inflammatory effects.[1, 2] However, like other β-agonists, salbutamol has a growth-promoting effect and has been applied in livestock breeding for illegal purposes. The use of SAL as a growth promoter is forbidden in China, the United States, and European Union member countries; however, SAL can be easily and legally bought as a crude drug for treating asthma and has the potential to replace clenbuterol as the mostly used growth promoter. Thus the demand from regulatory agencies for a highly sensitive, selective, and rapid detection of SAL residues has increased.

For over 40 years, immunoassays have been developed to become the primary method in rapid detection technology due to their specificity, simplicity, speed, cost-effectiveness and high-throughput. Among immunoassays, the ELISA and lateral flow assay (LFA) have been most successful for detecting contaminants or monitoring specific molecules in food and water. In 1985, Beaulieu first synthesized a salbutamol succinate derivative as a hapten, produced polyclonal antibody, and applied the antibody in a radioimmunoassay with a sensitivity in the low picogram range.[3] After 5 years, Adam produced monoclonal antibody with a 75% cross-reactivity for clenbuterol using the same hapten, then developed a radioimmunoassay[4] and alkaline phosphatase enzyme immunoassay[5] for screening SAL in horse urine at a detection limit of 28.8 fmol/tube (equivalent to 0.068ng/ml) and 26 fmol/well (equivalent to 0.12ng/ml), respectively. Dozens of researchers subsequently focused on the rapid detection of SAL using immunoassays, including preparing antibodies to SAL[6-11] and developing ELISA methods,[6-9, 12, 13] test strip assays,[10, 14-16] and immunosensor assays.[17-24] Among this research, only Chen developed a new hapten using the Mannich reaction, and obtained specific polyclonal antibody with an IC_{50} value of 0.736 ng/mL.[11] Other researchers have used antibodies generated by the same hapten developed using Beaulieu's method.[3] Wang's silver-palladium alloy nanoparticle-based electrochemical biosensor achieved the best LOD of 1.44 pg/mL in swine samples.[22] However, in all previous immunoassay approaches, antibody performance was a key factor and fundamentally determined the final sensitivity of the method. Thus, the design and synthesis of a suitable hapten is the first step in the successful development of a highly sensitive and specific immunoassay. Although Chen[11] has

obtained specific polyclonal antibody, the IC_{50} was only assessed in a standard solution and there is no data on the performance in a matrix.

In this study, we designed and synthesized a new hapten for SAL, generated a highly sensitive and specific monoclonal antibody, and evaluated the properties of the antibody in swine urine using the ELISA. The results of this study may allow the development of a more highly sensitive and specific immunoassay for regulating the illegal use of SAL in livestock production

Experimental

Chemicals and Reagents

Salbutamol was purchased from Energy Chemical (Shanghai, China) , and dibenzylamine and lithium aluminum hydride were purchased from Aladdin Industrial Corporation (Shanghai, China), 2-bromo-1-(4-hydroxy-3-(hydroxymethyl)phenyl)ethanone (BHHMPE) was purchased from Nanjing Chemlin Biomedical Technology Co., Ltd (Nanjing, China). Ethyl levulinate, sodium triacetoxyborohydride ($NaBH(OAc)_3$), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), Freund's complete and incomplete adjuvants, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween 20, TMB (3,3',5,5'-tetramethylbenzidine), and a polyethylene glycol solution (PEG, Hybri-Max, 50% (w/v)) were purchased from Sigma Chemical Co. (Shanghai, China).

HAT supplements, HT supplements, 1640 cell culture reagents, and fetal calf serum were purchased from Life Technologies Corporation (Shanghai, China). The Sp2/0-Ag14 murine myeloma cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A HRP-goat anti-mouse IgG conjugate was purchased from Jackson ImmunoResearch Laboratories (PA, USA). Ultrapure water was produced in our laboratory using a Milli-Q Integral system (Millipore, MA, USA). All other solvents and chemicals were analytical reagents and used without further purification.

Preparation of SAL hapten

The synthesis of the SAL hapten was initiated through a modification of the synthesis method for salmeterol[25] (Figure 1). First, BHHMPE (500 mg, 2.04 mmol) was dissolved in 20 mL of

acetone, then dibenzylamine (429 µl, 2.24 mmol) and potassium carbonate (563.8 mg, 4.08 mmol) were added, and the reaction refluxed for 12 h at 60°C. The reaction solution was then evaporated and dissolved in 20 mL tetrahydrofuran. At -20°C, 232 mg of lithium aluminum hydride was slowly added into the reaction solution. After 12 h, 3 mL water was slowly added to quench excess lithium aluminum hydride, then the reaction solution was evaporated and dissolved in absolute methanol. After being reduced by hydrogen under Pd/C, a reductive amination reaction similar to a previously published ractopamine hapten synthesis was introduced to produce the SAL hapten.[26] Briefly, while stirring at room temperature, 0.6 mL of triethylamine, 0.3 mL of ethyl levulinate, and 430 mg of sodium triacetoxyborohydride were added to the reductive product. After 12 h, 6 mL of 1 mol/L NaOH was added, and stirring was continued for 4 h. The reaction solution was evaporated using a rotary evaporator, and the solid residue was dissolved in 10 mL water. The pH of the solution was adjusted to approximately 3.0 using 6 mol/L HCl, and a cloudy white precipitate appeared. The solid (SAL hapten) was filtered and washed using diethyl ether, then dried under vacuum at 37°C for 2 h. Mass spectral analysis of the SAL hapten indicated masses of *m/z* 283, compatible with the structure proposed in Figure 1.

Conjugation of SAL hapten with Carrier Proteins

The conjugation procedure for the SAL hapten was modified from the synthesis method of melamine by Sun et al[27]. Briefly, the SAL hapten (18 mg, 0.064 mmol) was dissolved in 1 mL of DMF, then 1 mL of 0.1 M pH 5.5 2-[N-morpholino]ethane sulfonic acid (MES) was added to provide an acidic reaction environment. After the SAL hapten dissolved, 60 mg of EDC and 30 mg of NHS were added and stirred for 4 h. 25 mg of ovalbumin (OVA) and keyhole limpet hemocyanin (KLH), chosen as carrier proteins, were dissolved in 0.1 M pH 9.6 carbonate-bicarbonate buffer at a concentration of 10 mg/mL. The SAL hapten solution was then divided into two aliquots and slowly dropped into OVA or KLH solution. The reaction was continued for 12 h at room temperature and then dialyzed with PBS (0.01 M pH 7.4). The protein concentrations of the conjugates were measured using the Bradford method. After characterizing the UV-Vis spectra, the conjugates were aliquoted and stored at -20°C until use.

Immunization and generation of monoclonal antibody

Animal welfare and experimental procedures were strictly carried out according to recommendations in the Guide for the Care and Use of Laboratory Animals (23a) of the National Institutes of Health. All protocols were approved through the Institutional Animal Care and Use Committee (IACUC) of Jiangnan University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Ten female BALB/c mice, 6 weeks old, were initially immunized through an intradermal injection at four sites on the back with 50 μg of a SAL hapten-KLH mixed with complete Freund's adjuvant. Six booster immunizations, consisting of 25 μg antigen emulsified with incomplete Freund's adjuvant, were then administered at 3-week intervals. Seven days after the last booster immunization, tail blood was collected and tested for antibody titer and SAL affinity by indirect competitive ELISA (icELISA) using SAL hapten-OVA as the coating antigen. The mice producing antibody with the best affinity to SAL received an intraperitoneal injection of 20 μg of antigen before cell fusion. SP2/0 myeloma cells were fused with the splenocytes from the best mice at a ratio of 1:5 in the presence of 50% PEG 1500. After culture in RPMI 1640 with 20% FCS (v/v) and HAT in 96-well culture plates (200 μL per well) for 1 week, the hybridoma cells were selected by icELISA. SAL hapten-OVA (0.1 μg/mL) was used as a coating antigen, negative swine urine was used as blank sample, and swine urine spiked with 0.5 ng/mL SAL was used as a positive sample. The holes showing a highly positive and good inhibition effect to SAL were subcloned three times by limiting dilution assay. Hybridoma cell selection by icELISA was conducted every week after each subcloning. After the last selection, the hybridomas with the best inhibition effect were chosen for ascites generation using the method of Jackson et al.[28]. The collected ascites were purified using the caprylic acid–ammonium method.[29] The purified antibodies were aliquoted and frozen at -20°C until used.

Determination of antibody specificity

The checkerboard method was used to optimize the concentration of the coating antigen, SAL hapten-OVA (0.05 ~ 1 μg/mL), and SAL antibody (0.05 ~ 1 μg/mL) for icELISA before specificity determination. The icELISA procedure followed the method of Hoffman et al[30] with some modifications carried out to determine the 50% inhibition concentration (IC_{50}) for SAL and other analogs. Briefly, 96-well flat-bottom ELISA plates were coated with 100 μL/well antigen in 50 mM pH 9.6 carbonate-bicarbonate buffer and incubated at 4°C for 12 h. Then the plates were

washed 3 times with PBST (10 mM pH 7.4 PBS containing 0.05% Tween 20) and blocked with 2% gelatin in 50 mM pH 9.6 carbonate-bicarbonate buffer for 2 h at 37°C. Then the plates were washed 3 times to remove blocking buffer. Fifty microliters of competitors at different concentrations in PBST and 50 μL primary antibody were sequentially added to plate wells and incubated for 30 min at 37°C. After 3 times washing, 100 μL of HRP-goat anti-mouse IgG conjugate (diluted 1:3000) was added to each well and incubated for 30 min at 37°C. After the final wash, the plates were developed with 100 μL/well of TMB substrate solution (0.1 mL of 10 mg/mL TMB-glycol mixed with 10 mL of citrate-phosphate buffer and 2 μL of 30% hydrogen peroxide) for 15 min at 37°C. 2 M sulfuric acid was added at 50 μL/well to stop the color development. The optical density (OD) of the plates was read at 450 nm and the data were analyzed by a four-parameter logistic equation.

Matrix effect on antibody in swine urine

Matrix effects were evaluated following the method of Shelver et al.[31] with some modifications. Swine urine samples without beta-adrenergic agonist were collected from an organic swine farm (Shandong, China). The urine sample and diluted urine sample with PBST at 1:2, 1:5, and 1:10 were spiked with SAL at a series of concentrations. The icELISA procedure in the urine matrices was the same as described above, and the standard curve was plotted through fitting to a four-parameter logistic equation. The B_0 value and IC_{50} were compared with the values generated from the PBST standard curve.

Results and Discussion

Hapten Design and Antigen Conjugation

The conventional SAL hapten is directly derived from the benzene ring side of SAL, keeping the tert-butyl side structure of SAL. This type of hapten usually has a high cross-reactivity to other β- agonists with a tert-butyl group.[8, 13, 32] Therefore, a new hapten was designed (Figure 1) by reserving the benzene ring side structure of SAL to keep its specificity compared to other β-agonists. In the present study, 4-(2-amino-1-hydroxyethyl)-2-(hydroxymethyl)phenol, an intermediate (intermediate D, Figure 1) in the routine synthesis of salmeterol[25] was chosen for the introduction of a carboxyl group by a reductive amination reaction with ethyl levulinate. The

hapten finally obtained contains almost all the structure of SAL, except for its tert-butyl group, which therefore theoretically eliminates cross-reactivity to other β-agonists with a tert-butyl group. The introduced carboxyl group of the hapten can form amide bonds with proteins using EDC and NHS. KLH, a highly effective immunogenic high-molecular-weight protein, was chosen as the immunizing carrier, and OVA, which is heterologous to KLH, was chosen as the coating antigen carrier. The UV spectrum absorbance of the hapten, protein, and conjugates were recorded from 200 to 500 nm (Figure 2). The maximum characteristic absorbance of hapten is around 278 nm, which is the same as the protein; therefore, successful conjugation cannot be determined from a shift in maximum characteristic absorbance. However, there was an obvious shift of the peak base to the left beside the maximum characteristic absorbance of the SAL hapten-KLH compared to the KLH. In addition, there was a shift of the peak base to the left beside the maximum characteristic absorbance of the SAL hapten-OVA compared to the OVA. Because the concentration of the conjugate was similar to the corresponding protein, the wavelength shift can only be contributed by the hapten, indicating the SAL hapten was definitely conjugated to carrier proteins.

Antibody Generation and Cell Screening

Low dose immunization produced more sensitive antibody to salmeterol in work previously reported by Fodey.[33] However, Fodey's work focused on the immunization of rabbits, so the current study made a modification to his immunization program. Each mouse was immunized with 50 μg of SAL hapten-KLH conjugate, then a booster immunization dose of 25 μg was administered to avoid immunological tolerance. Conjugates were chosen as the coating antigen. In Fodey's work, the best affinity was obtained after the 6th booster. Therefore, in the current study, mice sera was collected and only evaluated after the 6th booster. One of the mice sera showed the best reactivity against SAL (IC_{50} = 3.2 ng/mL) at an 8000 fold dilution. As the best IC_{50} was obtained using 0.1 μg/mL SAL hapten-OVA, the ELISA plates for hybridoma cell selection were also coated at this concentration.

To screen for antibody that can resist the matrix effect of urine, urine samples were used in place of PBS buffer. After three rounds of selection by limited dilutions, one of the cell clones (3G6) with a highly positive and best recognition of SAL were obtained. The inhibition ratio of

the cell supernatant to 0.5 ng/mL SAL were all greater than 50%, which indicated a stable and sensitive antibody production against SAL. Thus, 3G6 was chosen for further evaluation.

Antibody Specificity and Matrix Effects

The selectivity of the antibody produced by hybridoma 3G6 is shown in Table 1. The antibody had a similar selectivity for salbutamol and SAL hapten. However, the antibody did not recognize other β-agonists with a tert-butyl group, demonstrating that the benzene ring side of SAL was the main epitope, as planned through the initial hapten design. This result demonstrated that the 3G6 antibody was highly specific to SAL. This means that designing a SAL derivative with a carboxyl group in place of the tert-butyl group can mimic the antigenicity and specificity of SAL very well.

In China, beta-agonists are commonly added to swine feed illegally; therefore, swine urine was chosen and spiked with SAL to simulate the real-world situation. A standard curve in PBST and in swine urine at 0, 2, 5, and 10 times dilution was produced (Figure 3). The B_0 values for PBST and urine dilutions at 0, 1:2, 1:5, 1:10 were 1.578, 0.914, 1.142, 1.431, and 1.457, respectively. The IC_{50} values were 0.31, 0.21, 0.25, 0.19, and 0.22 ng/mL, respectively. The corresponding B_0/IC_{50} values were 5.09, 4.35, 4.57, 7.53, and 6.62, respectively. These results showed that swine urine has strong matrix effects on antibody recognition. At 0 and 1:2 urine dilution, the IC_{50} values were better than that in PBST; however, the B_0 values were too low, which suggested little practical value. The B_0 and IC_{50} value only became acceptable when the urine was diluted more than 5 times. The IC_{50} was best at a 1:5 urine dilution. Although the B_0 was lower than that in PBST, these parameters were acceptable for real-world sample detection. Thus a five times dilution of swine urine was sufficient using the current ELISA method. This property may be due to the early selection of antibodies during cell fusion within a real urine sample. The ELISA IC_{50} value to SAL in the urine sample was much better than those in other published reports[5-7, 13]; therefore, the 3G6 antibody can be used to develop practical immunoassays for the highly sensitive and specific detection of SAL in urine samples.

Conclusions

This study describes the design and synthesis of a new hapten for the highly sensitive and specific

production of monoclonal antibody to salbutamol. To develop a more sensitive and specific detection method for SAL, a new hapten was synthesized, keeping the benzene ring of SAL while changing the tert-butyl group structure that SAL shares with other β-agonists. The antibody produced was highly sensitive and specific to salbutamol. In addition, the antibody demonstrated a good performance in a swine urine sample due to the selection of antibody within a urine sample at the cell fusion stage. The IC_{50} of the antibody was 0.19 ng/mL in a swine urine sample diluted five times, demonstrating its potential application in real samples.

Acknowledgments

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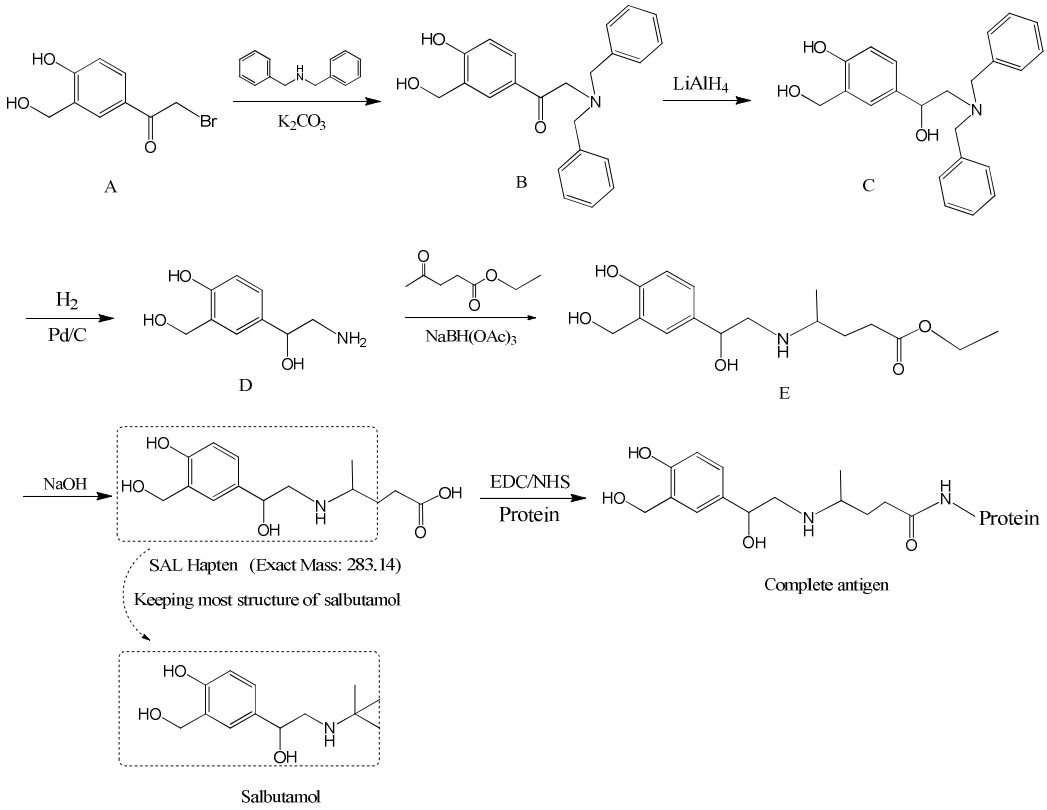
Captions

Figure 1. Synthesis of specific hapten for salbutamol and coupling of hapten to protein.
Figure 2. UV-Vis spectrum of SAL-hapten, OVA, KLH, and their conjugates.
Figure 3. Standard curve in PBST and in swine urine with non-, 2, 5 and 10 dilute times.
Table 1. IC_{50} and percentage cross-reactivities towards salbutamol and other analogs.



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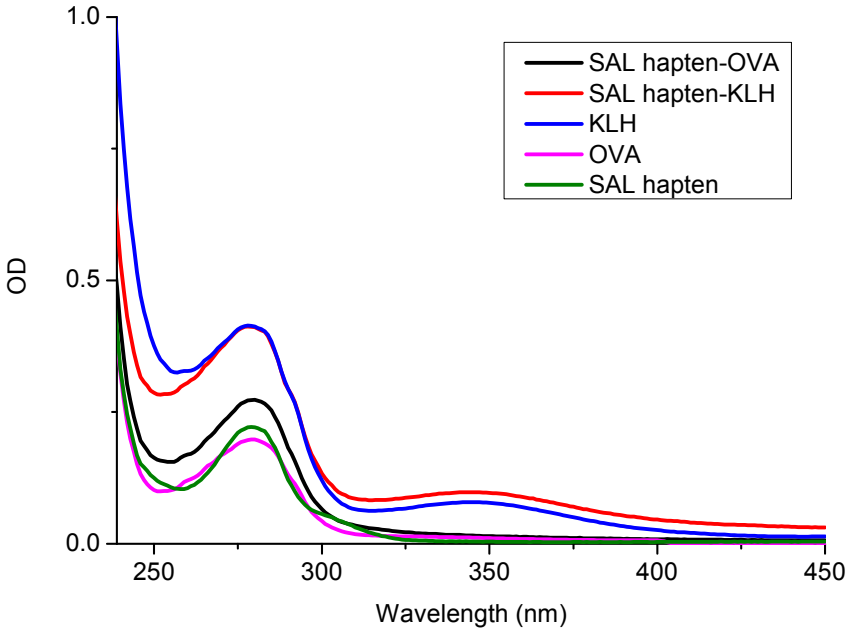


Figure 2. UV-Vis spectrum of SAL-hapten, OVA, KLH, and their conjugates.

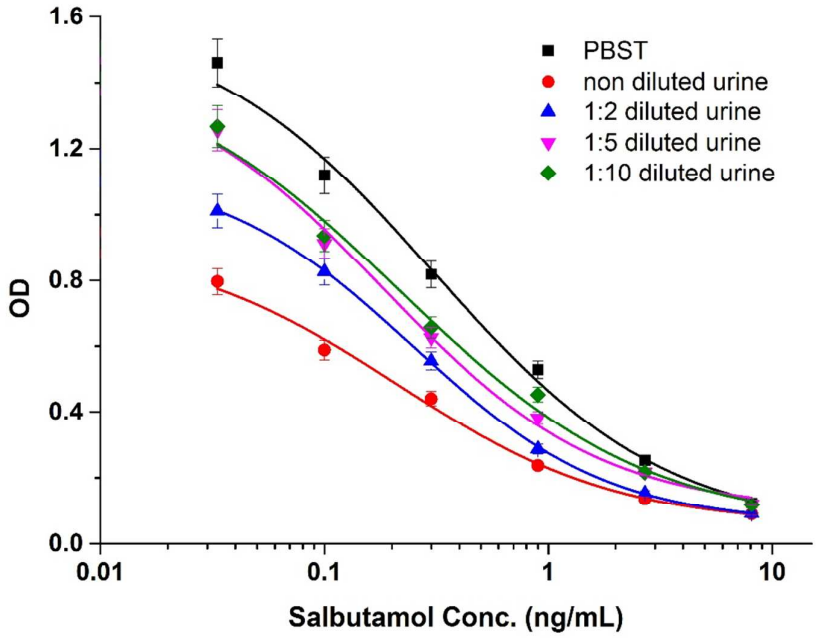
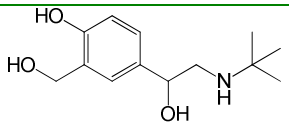
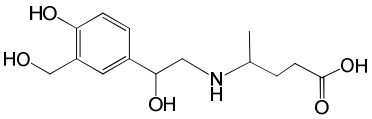
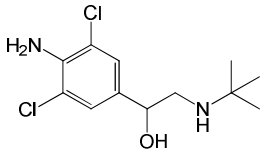
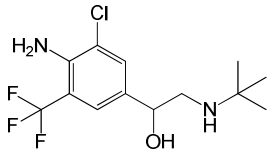
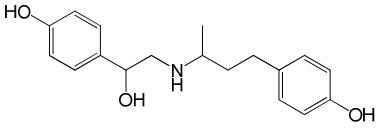


Figure 3. Standard curve in PBST and in swine urine with non-, 2, 5 and 10 dilute times.

Table 1. IC_{50} and percentage cross-reactivities towards salbutamol and other analogs.

Analogs	Structures	IC_{50} (ng/mL)	Cross-reactivity (%)
Salbutamol		0.31	100
SAL hapten		0.32	96.8
Clenbuterol		>500	< 0.1
Mabuterol		>500	< 0.1
Ractopamine		>500	< 0.1