Monoclonal antibody-based homogeneous immunoassay for three banned agonists and molecular modeling insight

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ABSTRACT
A homogeneous fluorescence polarization immunoassay (FPIA) was developed for the detection of an agonist salbutamol (SAL). Based on salbutamol-succinate-fluoresceinithiocarbamyl hexylenediamine (SAL-SUC-HDF), a fluorescein tracer with 6-carbon bridge was found to be more sensitive, the optimal FPIA showed the working range from 31.7 to 264.1 ng/mL with an IC50 value of 91.4 ng/mL and a limit of detection 16.9 ng/mL. The cross-reactivity to brombuterol and clenbuterol was unexpectedly found to reach 447.3% and 255.8%, respectively. Computer-assisted molecular modeling revealed that the steric structure and electron density surfaces’ effect appeared to be two important factors to the broad-specific recognition to other agonists.

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1. Introduction
Salbutamol (SAL) is a β-adrenergic receptor agonist that was highly useful to cure bronchial asthma, chronic obstructive pulmonary disease and other allergic diseases associated with the respiratory pathway (Goyal, Oyama, & Singh, 2007; Karuwan et al., 2009; Spyridaki et al., 2006). However, SAL may appear in urine and body organs after taking medicine for a few days, being retained in the liver for 10 days and in the retinal tissue for at least 4 months. After eating animal tissues containing higher SAL, people may have muscle pain, headache, heart palpitations, vomiting and other symptoms of poisoning, which have a greater harm for cardiovascular patients. Large-scale poisoning incidents have taken place in Spain, the Netherlands, France and China by adding β-receptor agonists in meat. The European Union, the United States, China and many countries have...
legislated to prohibit using β-receptor agonists as feed additives in animal production (Kim et al., 2005). The World Anti-doping Agency has prohibited the oral use of SAL, and a concentration of more than 1000 ng/mL in urine is considered as an indication of doping (Pichon, Venisse, Krupka, Perault-Pochat, & Denjean, 2006).

Quantitative detection of SAL currently is limited to instrumental methods, high-performance liquid chromatography (Cooper & Shepherd, 1996; Halabi, Ferrayoli, Palacio, Dabbene, & Palacios, 2004; Maudens, Zhang, & Lambert, 2004; Mazhar & Chrystyn, 2009; Murtaza, Ahmad, & Akhtar, 2009), gas chromatography–mass spectrometry (Tanaka, Yoneda, Inoue, Sugiura, & Ueno, 2000; Wang & Shen, 2007; Zhang et al., 2006), capillary electrophoresis (Chen, Wang, Duan, Chen, & Chen, 2005; Chu, Geng, Zhou, & Ye, 2007; Felix et al., 2006), etc. Most of these methods share a number of important drawbacks. They usually require costly apparatus and professional personnel, their sample throughput is limited and they are not suitable for on-site analysis.

Immunoassay technology had been increasingly used for screening food contaminants due to the sensitivity, selectivity, time efficiency, cost-effective and portability of the procedures. Such as enzyme-linked immunosorbent assay (ELISA) have been successfully used for both official and laboratory purposes. Over the past 20 years the importance and application of immunochemical methods, especially ELISA, have grown significantly. Generally, ELISA has many advantages over other techniques and allows direct analysis of a large number of samples. The detection limits of ELISA can be comparable to or even lower than that obtained with instrumental methods. However, ELISA is time-consuming and cannot be applied in a field test. These drawbacks of heterogeneous immunoassay can be overcome by fluorescence polarization immunoassay (FPIA), which is based on interactions between fluorescently labeled antigen (tracer) and specific antibody allowing the determination of the analyte within a short period of time (Smith & Eremin, 2008).

A number of FPIAs for pesticides (Eremin et al., 2002; Xu et al., 2011), detergent metabolite (Yakovleva et al., 2004), veterinary drugs (Chen et al., 2016; Zhang, Wang, Nesterenko, Eremin, & Shen, 2007), biological toxins (Lippolis, Pascale, & Visconti, 2006; Shim et al., 2004) and therapeutic drugs (McCann, White, & Keevil, 2002; Solnica, 2004) have been developed in the past few years. However, FPIA of SAL has not yet been reported.

The aim of the present research is to develop an FPIA for analysis of SAL. Two fluorescein-labeled SAL tracers containing linkers with different lengths were synthesized successfully and compared to gain the better sensitivity. Under the optimized conditions, the developed FPIA demonstrated high cross-reactivity (CR) to three agonists, and the observed CR was analyzed based on the molecular modeling for better understanding the broad-specificity of antibody.

### 2. Experimental section

#### 2.1 Reagents and instruments

Fluorescein isothiocyanate isomer I (FITC), N, N-dimethylformamide (DMF), N, N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, MO, USA). The analytical standards of salbutamol and cross-reactants (brombuterol (BRO), clenbuterol (CLE), isoproterenol (ISO), ractopamine (RAC),
noradrenalin (NOR), epinephrine (EPI) and chloramphenicol (CHL)) were obtained from Yunhui Corporation (Guangzhou, China). Ethylenediamine (EDA), hexylenediamine (HDA) and tributylamine were purchased from Jingke Corporation (Guangzhou, China). The fluoresceinthiocarbamyl ethylenediamine (EDF) and fluoresceinthiocarbamyl hexylenediamine (HDF) were prepared by our laboratory (Wang et al., 2011). The salbutamol-succinate (SAL-SUC) was prepared according to literature (Peng et al., 2010). Anti-salbutamol (anti-SAL) monoclonal antibody (MAb) was generated previously by our laboratory (Peng et al., 2010). Other organic solvents and reagents were analytical grade unless specified otherwise. Fluorescence polarization values were conducted on a Multilabel Counter (Wallac 1420 Victor3, PerkinElmer, USA).

Borate buffer (BB) (50 mmol, pH 8.5) with 0.01% sodium azide was used as working buffer for all FPIA experiments. The salbutamol stock solutions (20 mg/mL) of and other related cross-reactants were prepared by dissolving 20 mg of each in 1 mL of methanol and were stored at −20°C before use. Standard solutions of analytes in the range 0.1–50,000 ng/mL were prepared by dilution of stock solution with borate buffer, standard solutions were stored in methanol at 4°C before use.

2.2 Preparation of fluorescein-labeled salbutamol tracers

Fourteen milligrams (40 μmol) of SAL-SUC, 16.0 mg (80 μmol) of DCC and 10 mg (80 μmol) of NHS were mixed and dissolved in 0.4 mL DMF, and kept overnight at room temperature before centrifugation to remove the precipitate of dicyclohexylurea. Then, the supernatant was added to 5 mg (10 μmol) EDF (or 10 μmol HDF). After 5 min, a small portion of the reaction mixture was purified by Thin Layer chromatography (TLC) using dichloromethane to methanol ratio = 4:1 (V/V) (or trichloromethane: methanol = 10:1 (V/V) for synthesis of SAL-SUC-HDF). The TLC plates were dried, and major yellow bands of varying $R_f$ values were collected and eluted with 0.5 mL methanol. The concentration of tracer was determined spectrophotometrically at 492 nm, and it was assumed that the absorbance in borate buffer (0.2 mol/L, pH 8.0) is the same for fluorescein ($\varepsilon = 8.78 \times 10^4$ L/(mol cm)).

2.3 FPIA procedure

Several physicochemical factors influencing the FPIA were studied. Modification of $\delta$mP and IC$_{50}$ parameters of the standard curves were evaluated under different conditions. Buffer pH and ionic concentration were optimized using tracers with higher sensitivity. For buffer pH, competitive curves were performed with buffers of different pH values in constant ionic concentration of 200 mmol. A stock solution of 200 mmol boracic acid (pH = 5.4) and 200 mmol borate sodium (pH = 9.5) were prepared. Buffer of different pH was mixed to the two stock solutions to reach desired pH. For buffer ionic concentration, standard curves were studied with buffers of different ionic concentration values in constant pH 8.0. A stock solution of 200 mmol BB was diluted by double distilled water to 10, 25, 50, 100, 200 and 400 mmol.

FPIA calibration curves were obtained by adding 100 μL of the tracer solution in BB and 20 μL standard solution or sample to the microplate well, then 100 μL of the optimal dilution of antiserum were added to the mixed well. The reaction mixture was
mixed and fluorescence polarization (FP) was measured and plotted against the concentration of analyte. The four parameter logistic equations in OriginPro7.0 software package as defined below were used to fit the immunoassay data.

\[ y = \frac{(A - D)}{[1 + (X/C)^B]} + D, \]

where \( A \) represented a high asymptote, \( B \) is the slope factor, \( C \) is the concentration corresponding to 50% specific binding, \( D \) is the response at low asymptote and \( X \) is the calibration concentration. The limit of detection (LOD) represents the standard concentration at 10% of tracer binding (IC\(_{10}\)) (Kong, Liu, Song, Kuang, & Xu, 2016), and the working range was defined as the standard concentration at 20–80% of tracer binding (IC\(_{20}\) – IC\(_{80}\)) (Zhu, Song, Liu, Kuang, & Xu, 2016).

2.4 Specificity

Eight kinds of cross-reactants were selected for the specificity of the FPIA under optimized FPIA conditions. CR was calculated according to the following equation, where IC\(_{50}\) is the concentration at which 50% of the anti-SAL is bound to the analyte:

\[ CR\% = \left( \frac{IC_{50}(\text{salbutamol})}{IC_{50}(\text{structurally related compounds})} \right) \times 100\%. \]

2.5 Molecular modeling

Minimum energy conformations of all structures were calculated using the Becke3LYP level of density functional theory (DFT-B3LYP) with Gaussian 16 packages (Revision B.05, Gaussian, Inc., Pittsburgh, PA; Lee, Yang, & Parr, 1988). The basis set used was 6-31G**. The minimum energy conformations and electrostatic potential molecular surfaces were obtained by SYBYL-X 2.1 program package (Tripos Inc., USA) running on an HP xw6600 workstation with an Intel Xeon E5430 2.66 GHz processor based on the optimized structures.

3. Results and discussion

3.1 Synthesis of the tracers

The structure of the tracer has a significant influence on the assay characteristics (Wang, Zhang, Nesterenko, Eremin, & Shen, 2007). To study the influence of the structure of the tracer on assay sensitivity, two tracers were synthesized for the SAL (Figure 1). The tracers were designed between the SAL and EDF (or HDF) with different bridge lengths (2- and 6-carbon bridge). It was found that SAL-SUC-EDF and SAL-SUC-HDF product at \( R_f = 0.3 \) showed sufficient binding capability with the antibody (Figure S1), suggesting that the compounds SAL-SUC-EDF and SAL-SUC-HDF at \( R_f = 0.3 \) were desired tracers with good binding response. Therefore, SAL-SUC-EDF and SAL-SUC-HDF were used for the further investigation.

3.2 Optimization of tracers and antibody concentration

The concentration of the tracer was an important parameter to be optimized for the development of a sensitive FPIA. In this study, tracers SAL-SUC-EDF and SAL-SUC-HDF were
diluted and mixed with a fixed antibody dilution titer to construct a series of calibration curves for evaluation by comparing the three parameters (\(\delta mP\), \(I_{C50}\) and \(\delta mP/I_{C50}\)), where \(\delta mP\) is the difference between maximal and minimal FP signals. Higher \(\delta mP\), lower \(I_{C50}\) values and higher \(\delta mP/I_{C50}\) values indicate a more sensitive assay (Lei et al., 2011). As shown in Table 1, the tracer SAL-SUC-HDF at 0.5 nmol/L exhibited the highest \(\delta mP\) (113) and maximal \(\delta mP/I_{C50}\) (0.4216). Therefore, 0.5 nmol/L was selected as the optimal tracer concentration of SAL-SUC-HDF. Similarly, the tracer SAL-SUC-EDF at 1 nmol/L exhibited the higher \(\delta mP\) (43), lowest \(I_{C50}\) (75 ng/mL) and bigger \(\delta mP/I_{C50}\) (0.5733). The \(I_{C50}\) value at the concentration 1.0 nmol/L was the lower than the \(I_{C50}\) value at the concentration 0.1 nmol/L. Thus, the concentration of SAL-SUC-EDF of 1.0 nmol/L was regarded as the best concentration in the overall evaluation.

Figure 1. Structures of salbutamol-succinate-fluoresceinthiocarbamyl hexylenediamine (SAL-SUC-HDF) and salbutamol-succinate-fluoresceinthiocarbamyl ethylenediamine (SAL-SUC-EDF).

| Table 1. Optimum concentrations of the two tracers. |
|-----------------------------------------------|
| Concentration (nmol) | \(I_{C50}\) (ng/mL) | \(\delta mP\) | \(\delta mP/I_{C50}\) |
|----------------------|-------------------|-------------|-------------------|
| SAL-SUC-HDF          |                   |             |                   |
| 0.1                  | 252               | 84          | 0.3333            |
| 0.5                  | 268               | 113         | 0.4216            |
| 1.0                  | 237               | 96          | 0.4051            |
| 2.0                  | 234               | 81          | 0.3462            |
| SAL-SUC-EDF          |                   |             |                   |
| 0.1                  | 94                | 59          | 0.6277            |
| 0.5                  | 222               | 52          | 0.2342            |
| 1.0                  | 75                | 43          | 0.5733            |
| 2.0                  | 101               | 42          | 0.4158            |
After the optimal tracer concentrations were determined, antibody dilution curves were established using serially diluted antiserum with two different tracers (Figure 2). Generally, it can be seen that tracer binding has a great difference among the two tracers. The antibody concentration was selected to be at an unsaturated level, which results in about 70% binding to ensure a strong enough signal and good sensitivity (Smith & Eremin, 2008). And the result showed that the titers of antiserum were 1/1700 and 1/1200 for SAL-SUL-HDF and SAL-SUL-EDF, respectively.

Table 2 shows the antiserum titers, IC$_{50}$, LOD, δmP, δmP/IC$_{50}$ and working range of calibration curves obtained with the two tracers. SAL calibration curves were obtained under optimal antiserum dilution and tracer concentration. Obviously, the influence of the tracer structure on assay sensitivity is observed in Table 2. SAL-SUC-HDF gave better assay sensitivity than SAL-SUC-EDF. The IC$_{50}$ and LOD obtained with SAL-SUC-EDF and SAL-SUC-HDF indicated that a spacer of six methylene groups instead of two gave the more sensitive assay. Compared to SAL-SUC-EDF, SAL-SUC-HDF gave a strong signal as well as a more sensitive assay. Therefore, FPIA calibration curves were recorded using SAL-SUC-HDF which showed higher sensitivity.

### 3.3. Effects of physicochemical factors on FPIA

**Effect of buffer pH.** Immunoassay performance is often influenced by chemical parameters, such as pH and ionic concentration. The effects of these parameters were assessed by comparing IC$_{50}$, δmP and δmP/IC$_{50}$. δmP/IC$_{50}$ reflects the MAb recognition of the tracer in the absence of analyte, and IC$_{50}$ reflects the MAb affinity for the analyte itself. In order to examine the influence of pH on the assay, the pH value range of the buffer was changed (from 6.0 to 9.0), as shown in Table S1. The IC$_{50}$ increased gradually but δmP/IC$_{50}$ was reduced gradually between pH 6.6 and 9.0. It could be found that the conditions (pH value from 6.0 to 6.6) have a great effect on the assay, while it was lesser in neutral and alkaline conditions (pH value from 7.0 to 9.0), especially when pH ranging from 7.0 to 8.0. δmP value was the highest, IC$_{50}$ and δmP/IC$_{50}$ value were also under a superior condition with sensitivity being obtained as high and signal responses strongly at pH 7.5;
therefore, pH 7.5 was selected as the optimal pH. The marked effect of pH on FPIA parameters could be related to analyte ionizing in various pH conditions.

**Effect of ionic concentration.** Because the ionic strength of the assay system can affect antibody binding, the buffer ionic concentration was adjusted within 10–400 mmol (Table S2). For the FPIA method, higher salt concentration greatly reduced $\delta mP/IC_{50}$, whereas the IC$_{50}$ rose dramatically. The solution was not very stable with variation of pH when ion concentration was 10 mmol in the experiment process; therefore, 25 mmol was regarded as the optimal ion concentration to guarantee stability of FPIA. As the salt ion concentration increased, the primary effect was that the hydrophobic bond formation and stability was reduced, and the interaction of antibody–analyte would decline.

**Calibration curve.** Under the optimal conditions, the FPIA for SAL was obtained using tracer SAL-SUC-HDF (Figure 3). The LOD and IC$_{50}$ for SAL were 16.9 and 91.4 ng/mL, respectively, and the working range was 31.7–264.1 ng/mL.

### 3.4 Specificity

To study the specificity of the obtained antibody, the CR against a range of compounds structurally related to SAL was tested. The results are shown in Table 3. Interestingly, the CR for brombuterol (BRO) and clenbuterol (CLE) were 447.3% and 255.8%, respectively. Other structurally related compounds showed a CR lower than 8%. Comparing the structures of SAL, brombuterol and clenbuterol revealed that if the hydrogen atom at position 6 for SAL was changed to a bromine atom (for brombuterol) or a chlorine atom (for

![Figure 3](image-url). FPIA calibration curve for salbutamol with tracer SAL-SUC-HDF.
### Table 3. CR of antiserum to salbutamol and related compound.

| Compound         | Molecular structure | IC$_{50}$ (ng/mL) | CR (%) |
|------------------|---------------------|-------------------|--------|
| Salbutamol       | ![Salbutamol](image) | 112.5             | 100    |
| Brombuterol      | ![Brombuterol](image) | 25.2              | 447.3  |
| Clenbuterol      | ![Clenbuterol](image) | 44.0              | 255.8  |
| Isoproterenol    | ![Isoproterenol](image) | 1466.0            | 7.7    |
| Ractopamine      | ![Ractopamine](image) | 5451.4            | 2.1    |
| Noradrenalin     | ![Noradrenalin](image) | $>10^5$           | <0.1   |
| Epinephrine      | ![Epinephrine](image) | $>10^5$           | <0.1   |
| Chloramphenicol  | ![Chloramphenicol](image) | $>10^5$           | <0.1   |

**Figure 4.** (a) Models of the minimum energy conformations of the compounds. (b) Models of the electrostatic potential molecular surfaces of the compounds.
clenbuterol), the CR dramatically increased from 100% for SAL to 447.3% for brombuter-olor and 255.8% for clenbuterol. This suggests that the atom at position 6 is an important structural factor that affects the affinity of the anti-SAL analyte. In addition, the difference among the CR of isoproterenol and that of epinephrine could be attributed to the group at position 4. To better explain the CR, molecular modeling method was used for further investigation.

3.5 Molecular modeling

Molecular modeling can provide useful information according to physicochemical properties of compounds and is often used as a tool to explain the CR of an antibody (Xu, Shen, et al., 2009). The antibody–antigen recognition was suggested based on steric criteria and interactions resulting from the electronic properties of the molecules (Xu, Shen, et al., 2009). Therefore, the minimum energy conformation and electrostatic potential isosurfaces were modeled, and the results are shown in Figure 4. The model of minimum energy conformation of SAL and the structurally related compounds (Figure 4(a)) indicated that the –C(CH₃)₃– was a bulky group and the characteristic group of SAL, BRO and CLE. Changing the –C(CH₃)₃– to –CH(CH₃)₂– resulted in a decreasing of CR to 7.7% for ISO. From the electrostatic potential isosurfaces (Figure 4(b)), we can see that the isosurfaces of SAL, BRO and CLE were similar and distinguished with other compounds. The results can well explain the high CR for SAL, BRO and CLE, and low CR for other compounds. The higher CR for BRO and CLE might be explained by the existing –NH₂ group and the halogen atoms. Both the –NH₂ group and the halogen atoms were electronegative and had strong electron-donating ability, which may improve the antibody recognition (Xu, Xie, et al., 2009). Compared with the chlorine atom, the bromine atom was more electronegative, which might be the reason why the CR for BRO was higher than that for CLE. In conclusion, the steric structure and electron density surfaces’ effect appeared to be two important factors with regard to the antibody binding to the haptens.

4. Conclusions

In this study, SAL-SUC-HDF and SAL-SUC-EDF were synthesized successfully, and the FPIA for SAL was established using the SAL-SUC-HDF tracer. The optimal FPIA for SAL showed the working range from 31.7 to 264.1 ng/mL with an IC₅₀ value of 91.4 ng/mL and an LOD of 16.9 ng/mL. CR for BRO and CLE were 447.3% and 255.8%, respectively. Based on the comparison of structure and biological activity data, the steric structure and electron density surfaces’ effect appeared to be two important factors to CR.

Disclosure statement

No potential conflict of interest was reported by the authors.

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