Visual flow-through column biomimetic immunoassay using molecularly imprinted polymer as artificial antibody for rapid detection of clenbuterol in water sample

Yiwei Tang\textsuperscript{a,b}, Yuchen Zhang\textsuperscript{a}, Hong Zhang\textsuperscript{a}, Xiuying Liu\textsuperscript{a}, Xue Gao\textsuperscript{a}, Changxin Lv\textsuperscript{a}, Tao Ma\textsuperscript{a}, Xiaonan Lu\textsuperscript{b} and Jianrong Li\textsuperscript{a}

\textsuperscript{a}College of Food Science & Project Engineering, Bohai University, Jinzhou, People’s Republic of China; \textsuperscript{b}Food, Nutrition, and Health Program, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, Canada

ABSTRACT

A novel visual flow-through column biomimetic immunoassay using horseradish peroxidase-labeled clenbuterol (HRP-CLB) as a tracer and molecularly imprinted polymers (MIPs) with high specificity as the artificial antibody has been developed for rapid detection of CLB. Different intensities of blue color products on test layer of the column could be observed after reaction. This optimized assay provided a preliminary qualitative result in the concentration range of 0 to 1000 \( \mu \text{g/L} \) by judging color density of the test layer without any equipment, and the detection could be finished within 15 min. This assay was evaluated using CLB spiked water samples and results were comparable to high performance liquid chromatography method. The visual detection limit of 5 \( \mu \text{g/L} \) in water sample was obtained. This developed flow-through column biomimetic immunoassay was demonstrated as a suitable tool for rapid, sensitive, low-cost, and qualitative determination of CLB residues on site.

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Introduction

Clenbuterol (CLB) as a number of \( \beta \)-adrenergic agonist was not only widely used as a bronchodilator and a tocolytic agent in clinical management, but also illegally used as a growth promoter in animal production (Xu et al., 2014). However, CLB residue in the meat products would cause serious adverse effects on human health including muscle tremors, headache, and heart palpitations (Du et al., 2014; Yang, Wu, Chen, & Lin, 2014). Therefore, China, Mexico, and European Union prohibited it as a growth promoter in meat-producing animals (Morales-Trejo, León, Escobar-Medina, & Gutiérrez-Tolentino, 2013). Accordingly, developing methods for the monitoring of CLB residue in foods is of great importance.

Currently, many analytical methods including enzyme-linked immunosorbent assay (ELISA) (Prezelj, Obreza, & Pecar, 2003), lateral test strip (Khaemba et al., 2016), high...
performance liquid chromatography (HPLC) (Morales-Trejo et al., 2013), gas chromatography coupled with mass spectrometry (GC-MS) (Ramos et al., 2003), and liquid chromatography coupled with mass spectrometry (LC-MS) (Courant et al., 2009; Domínguez-Romero et al., 2013) for screening CLB residue have been reported. However, these methods are relatively time consuming, require expensive equipment and well-trained personnel, and can only be used in laboratories (Meng et al., 2014). Therefore, a rapid and economical method for the detection of CLB should be developed.

Molecularly imprinted polymers (MIPs) as a type of bionic antibodies have attracted considerable interest due to their favorable selectivity and specificity for target analytes, good physiochemical stability, long usage life, simple preparation procedure, and low cost (Ren & Chen, 2015). Currently, MIPs have been used in solid-phase microextraction (Zhao, Guan, Tang, Ma, & Zhang, 2015), sensor (Chen, Huang, Zeng, Tang, & Li, 2015; Ding et al., 2015), membrane (Kobayashi, Takeda, Ohashi, Makoto, & Sugiyama, 2009), and artificial antibody (Tang, Fang, Wang, Sun, & Qian, 2013). Evidently, MIPs will be a powerful molecular receptor to improve the selectivity of the recognition material.

In the present study, taking advantage of the MIPs (an artificial antibody) produced in our laboratory by covalent imprinting approach, we prepared a novel flow-through column for rapid determination of CLB using horseradish peroxidase-labeled CLB (HRP-CLB) as a tracer. This assay offers many advantages such as no requirement of instruments, easy and rapid result evaluation, low cost of detection, and simple operation for on-site testing.

Material and methods

**Chemical and reagents**

CLB, isoproterenol (ISOP), terbutaline (TER), salbutamol (SAL), and horseradish peroxidase were obtained from Sigma-Aldrich (Saint Louis, USA). The one-component, ready-to use TMB solutions were obtained from Innoreagents (Huzhou, China). Quartz sand (60–100 mesh) was received from Canspec Scientific Instruments Co., Ltd (Shanghai, China). Silica gel (60–100 mesh, 100–200 mesh, 200–300 mesh) was purchased from Branch of Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Methanol, NaCl, KCl, and Tween-20 were got from Tianjin Chemical Reagent Factory (Tianjin, China). The CLB MIPs were prepared in our laboratory by covalent imprinting method, and the adsorption property of the MIPs was studied and published (Tang et al., 2016). Herein, we used this CLB MIPs as biomimetic antibody to develop a visual flow-through column immunoassay.

**Solutions**

Phosphate buffer solution (PBS, 10 mmol/L, pH 7.5) contains 10 mmol/L sodium phosphate, 137 mmol/L NaCl, and 2.7 mmol/L KCl. Standard stock solutions of 1.0 mg/L CLB, ISOP, TER, and SAL in methanol were stored in a refrigerator and remained stable for at least 1 month. Washing buffer (PBS-T) was prepared using PBS with 0.05% (v/v) Tween-20.
**Apparatus**

HPLC was performed using an Agilent 1260 HPLC system with ultraviolet detector at 246 nm and a reversed-phase C$_{18}$ analytical column (4.6 × 250 mm, 5 µm, Agilent, USA). The mobile phase was 50 mmol/L NaH$_2$PO$_4$ and methanol (65:35, v/v), and the flow rate was maintained at 1.0 mL/min (column temperature, 25°C).

**Flow-through immunoassay column preparation**

The flow-through bio-immunoassay column (Figure 1) was prepared by adding a mixture of 0.2 g of quartz sand and 10 mg of the MIPs into a plastic column equipped with two frists at each end of the test layer. The MIPs should be mixed well with the quartz sand; this could provide homogeneous distribution of the colored products over the test layer.

**Preparation of HRP-labeled CLB tracer**

The HRP-labeled tracer (HRP-CLB) was synthesized according to the previous literature (Degand, Bernes-Duyckaerts, & Maghuin-Rogister, 1992).

**The flow-through column immunoassay procedure**

PBS solution (1 mL) and CLB standard solution or sample extracts were added into the control and allocated flow-through immunoassay columns, respectively, followed by preconditioning the columns with 1 mL PBS to activate the test layer. The solution was removed using the syringe after 3 min. Subsequently, the columns were washed using PBS-T solution (1 mL), and 1 mL of HRP-labeled tracer diluted with PBS (1:20000; v/v) percolated through the columns within 4 min. After washing using 4 mL of PBS-T.

![Figure 1. Schematic representation of the flow-through immunoassay column.](image-url)
solution, 400 μL of TMB solution flowed through the column within 4 min. Then, the color change of the test layer could be observed depending on the CLB concentration. A qualitative result was obtained by comparing the color intensity of the test layer and that of the negative control column (without standard solution or sample extracts). The whole assay procedure took approximately 15 min.

Sample preparation

Water samples without CLB were filtered by 0.22-μm membrane filters (Membrana, Wuppertal, Germany) and spiked at five selective concentration levels. The concentration was analyzed by flow-through column biomimetic immunoassay and HPLC.

Results and discussion

Principle of the flow-through column immunoassay

The flow-through column assay is based upon the selectivity of the MIPs to CLB (Figure 2). When standard solution or sample extracts passed through the column, CLB and (or) interferences were bounded to the cavities of MIPs. The cavities in MIPs could specifically adsorb CLB because of both spatial structure and functional groups. These cavities were obtained in the process of MIPs synthesis and are only complementary to the imprinted molecule. After removing interference by washing with PBS-T solution, HRP-labeled tracer was added into the columns, which could bind to the cavities of the MIPs not occupied by analytes. After the columns were washed with PBS-T solution, the TMB solution was added. Then, the blue colored test layers could be obtained. The higher the concentration of the analytes (i.e. CLB) in the standard or sample solution is, the weaker the blue color could be observed. Positive result was obtained when the blue color was weaker than that of the control column or no color was developed. No color development was related to high concentration of analyte in sample or standard solution, resulting in occupying all of the cavities of MIPs and subsequently no cavities

\[ \text{SUB} \quad \text{a: MIPs against to CLB; b: standard solution or sample extracts is passed through the column; c: washing step with PBS-T to remove unbounding materials; d: HRP-CLB is added to the column and binds to the MIPs; e: washing step with PBS-T to remove unbounding materials; f: TMB substrate solution is added to obtain color.} \]

Figure 2. The principle of the assay.
binding to the HRP-CLB tracer. The negative result was evaluated when the color densities of test layer of control and test column were the same. If no blue color was presented in the test layer of the control column, the test was invalid.

**Optimization of the flow-through column immunoassay**

To choose a suitable inert matrix that cannot bind with HRP-CLB tracer, 0.4 g of quartz sand (60–100 mesh) and silica gel (60–100 mesh, 100–200 mesh, 200–300 mesh) were employed in this study. The results shown in Figure 3 indicated that quartz sand has little effect on the color of the test layer. Therefore, a certain amount of quartz sand was used in the subsequent assay.

In order to easily distinguish color differences of the test layers, a suitable thickness of the test layer is also required. In the present study, 0.2 g of quartz sand was determined. In addition, the amounts of MIPs and HRP-CLB tracer were optimized according to the following criteria: (a) appropriate blue shade of the control test layer; (b) high sensibility; (c) low cost. Ten milligrams of MIPs mixed with 0.2 g of quartz sand and 1 mL HRP-CLB tracer diluted with PBS (1:20000, v/v) were determined. Subsequently, washing times were also optimized to avoid blue color development at the test layer of the columns filled with quartz sand only or mixed by 0.2 g quartz sand and 10 mg of NIPs. A total of 4 mL of PBS-T washing solution was chosen after HRP-CLB tracer solution flowed through the column. In order to save testing time, 4 min for color development was chosen to meet the test requirement.

Under the optimal conditions, the flow-through column immunoassay was carried out using various concentrations of CLB standard solutions. The results are shown in Figure 4, indicating that 10 μg/L of CLB standard solution resulted in a slight but distinguishable color change.

**Figure 3.** The color of the test layer based on quartz sand and silica gel.
difference compared to the control flow-through column, and 1000 μg/L of CLB standard solution inhibited the color development completely.

**Specificity of the developed assay**

The specificity of the flow-through column immunoassay based upon MIPs was determined by cross-reactivity using TER, ISOP, and SAL as the structural analog of CLB. The results are shown in Table 1, indicating that blue color was developed even at the concentration from 100 to 1000 μg/L of TER, ISOP, and SAL standard solution, and all of these results demonstrated that the MIPs was only specific to CLB. The preparation of MIPs with high selectivity to the analytes was necessary to achieve sensitive cross-reactivity detection.

**The stability of the flow-through immunoassay column**

The stability of the flow-through immunoassay column was determined by running the flow-through immunoassay at zero concentration of CLB using the same batch of the columns, which were stored for selective days (2, 15, 30, and 60 days) and at different

![Figure 4. Flow-through columns immunoassay for different concentration of CLB in standard solution.](image)

Table 1. Cross-reactivity of CLB with TER, ISOP, and SAL.

| Analogs | 100.0 μg/L | 1000.0 μg/L |
|---------|------------|-------------|
| CLB     | −          | +           |
| TER     | − − − − − − | − − − − − − |
| ISOP    | − − − − − − | − − − − − − |
| SAL     | − − − − − − | − − − − − − |

Notes: (+), no blue color development of the test layer of the test column within 4 min; (−): blue color development of the test layer of the test column within 4 min. The more “−” is, the darker the blue color is.
temperatures (4, 20, and 37°C), respectively. The density of the test layers of all the columns was not significantly different by visual judgment, indicating that MIPs and quartz sand were insensitive to the environmental conditions and this immunoassay column is suitable to rapid testing on-site.

**Sample analysis and accuracy of the assay**

Water samples were spiked with CLB at the concentrations of 1, 5, 10, 100, and 500 μg/L. Each spiked sample was evaluated with six replicates and analyzed by the column assay and HPLC, respectively. The results are shown in Table 2, indicating that negative results were observed when the concentration of CLB in the water was 5 μg/L while positive results were obtained when at or over 5 μg/L. Thus, a visual detection limit was determined to be 5 μg/L. The results were confirmed by HPLC analysis, and the accuracy of this developed assay was validated.

**Conclusion**

A visual flow-through column biomimetic immunoassay based upon MIPs as the artificial antibody was developed for rapid determination of CLB for the first time. The preparation of flow-through column was simple, and the procedure of the developed assay was rapid (∼15 min) and without aid of any instrument. This method was applied to determine CLB in the spiked water samples, and good correlation between data obtained using this assay and HPLC was observed. This currently developed assay is a rapid, sensitive, and reliable method for determination of CLB.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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| Sample | Spiked level (μg/L) | Column assay | HPLC Mean ± SD (μg/L) |
|--------|---------------------|--------------|-----------------------|
| Water  | 1.0                 | − − − − − −   | 1.04 ± 8.65           |
|        | 5.0                 | + + + + + +   | 5.12 ± 5.98           |
|        | 10.0                | + + + + + +   | 9.03 ± 6.21           |
|        | 100.0               | + + + + + +   | 101.36 ± 5.87         |
|        | 500.0               | + + + + + +   | 499.28 ± 4.32         |

Notes: (−), negative result, CLB is absent or the CLB concentration is lower than the limit of detection; (+), positive result, the presence of CLB.
Notes on contributors

Yiwei Tang got his PhD in Food science in 2011 from Tianjin University of Science & Technology, Tianjin, China and then became a faculty in College of Food Science & Project Engineering of Bohai University. His research interests are molecularly imprinted polymers design and application.

Yuchen Zhang got his bachelor in Shenyang Normal University (2015), Shenyang, China. His research interests are molecularly imprinted polymers design and application.

Hong Zhang got her bachelor in Bohai University (2016), Jinzhou, China. Her research interests are antibodies development and immunoassay and applications.

Xiuying Liu was born in 1987, received her PhD in Food Science in June 2014. Since July 2014, she has worked at College of Food Science & Project Engineering of Bohai University. Her research field is development of aptasensors and sensors on the basis of molecularly imprinted polymer for the detection of different compounds in foodstuffs.

Xue Gao was born in 1986, received her PhD in Analytical Chemistry in June 2013. Since July 2013, she has worked at College of Food Science & Project Engineering of Bohai University. At present her main research interests focus on the development of nanostructured sensors for ultrasensitive detection of food contaminants.

Changxin Lv was born in 1965; he is now a Professor in College of Food Science & Project Engineering of Bohai University. His research interests focus on detection of food contaminants, and food microbiology.

Tao Ma was born in 1962; he is now a Professor in College of Food Science & Project Engineering of Bohai University. His research interests focus on detection of food safety.

Xiaonan Lu was born in Tianjin, received his BSc (Food Science) from Ocean University of China in 2007 and PhD (Food Science) from Washington State University before he joined in UBC in 2013. He focuses on developing innovative and rapid sensing, instrumentation systems and detection methods for ensuring food safety and preventing food bioterrorism.

Jianrong Li was born in 1964; he is now a Professor in College of Food Science & Project Engineering of Bohai University. His research interests focus on optical chem/biosensors, tailored (bio) molecular recognition interfaces, molecularly imprinted materials, and aquatic products storage and processing.

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