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Design, synthesis and characterization of tracers and development of a fluorescence polarization immunoassay for the rapid detection of ractopamine in pork

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ABSTRACT

In this study, 10 fluorescein-labeled ractopamine (RAC) derivatives (tracers) were synthesized and characterized to develop a rapid fluorescence polarization immunoassay (FPIA) for the detection of RAC in pork, using previously produced RAC polyclonal antibodies. The effect of the tracer structure on the sensitivity of the FPIA was investigated. The specificity of the FPIA was evaluated with 70 β-agonists and β-blockers. The FPIA showed a limit of detection of 0.56 μg kg^{-1} for RAC in pork, with recoveries ranging from 74.8% to 86.6% in spiked samples. The total analysis time, including sample pretreatment, was less than 1 h. The FPIA was used to screen 150 commercial pork samples for RAC residues and the results were consistent with those of an enzyme-linked immunosorbent assay (ELISA) and ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Our results demonstrate that the FPIA developed here is a rapid, accurate, and sensitive screening method for RAC residues in pork.

1. Introduction

The use of growth-promoting β-agonists, such as clenbuterol and salbutamol, in food-producing animals has been prohibited in almost all countries around the world because they have unacceptable side effects. However, the use of ractopamine (RAC) as a feed additive in animal husbandry is still highly controversial. RAC is a typical phenylethanolamine member of the β-adrenergic agonists, which was first approved as a feed additive for swine by the U.S. Food and Drug Administration (FDA) in 1999. The maximum residue limits for RAC have been set at 10 and 50 μg kg^{-1} for pork by the United States and the World Trade Organization, respectively (FDA, 2000). However, because of its potential effects on human health, including muscle fremitus, palpitations, tachycardia, and dizziness, more than 160 countries have currently banned RAC in animal production, including the European Union (EU) and China (Niño, Granja, Wanschel, & Salerno, 2017). The EU Commission proposed 10 μg kg^{-1} as the minimally required performance limit, whereas the active level of RAC in China cannot exceed 2 μg kg^{-1} in any edible tissue (European Commission, 2002; Ministry of Agriculture of China, 2002). Therefore, developing accurate, sensitive, simple, and rapid analytical methods for the detection of RAC in food samples of animal origin is both important and necessary.

Many instrument-based methods have been developed and applied to the detection of RAC, including gas chromatography–tandem mass spectrometry (GS–MS/MS) (He, Su, Zeng, Liu, & Huang, 2007), high-performance liquid chromatography (HPLC) (Vichapong, Burakham, & Srijaranai, 2016), and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Gonzalez-Antuna, Rodriguez-Gonzalez, Centineo, & Garcia Alonso, 2014). These methods usually require technically sophisticated, costly equipment, and time-consuming sample pretreatment. Some antibody-based methods, or immunoassays, have been developed to screen for RAC, which do not require costly equipment. The most frequently used immunoassay formats are the enzyme-linked immunosorbent assay (ELISA) (Gu, Liu, Song, Kuang, and Xu, 2016), lateral flow immunoassay (LFIA) (Hu et al., 2017; Ren et al., 2014), and chemiluminescence immunoassay (CLIA) (Han, Gao, Wang, Wang, & Fu, 2013; Wang, Su, Ouyang, Wang, & Fu, 2016). ELISA and CLIA are solid-phase assays and require a series of reagents, multiple washing

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steps, and long immunoreaction times, whereas LFIA is a real-time analytical method rather than a high-throughput screening method.

Simplifying the assay and shortening the analysis time are top priorities in the development of high-throughput screening methods. The fluorescence polarization immunoassay (FPIA) is a homogeneous assay, with no separation or washing steps, which can be completed within 10 min. FPIA has been widely used in medical diagnosis and in monitoring therapeutic drugs in serum since the 1980s (Sánchez-Villalobos, et al., 2011; Wu et al., 2012). Recently, FPIA has been applied to the high-throughput screening of chemical contaminants, such as veterinary drugs (Pennacchio, et al., 2016; Xu et al., 2013; Zhang, Wang, Nesterenko, Eremin, & Shen, 2007), mycotoxins (Valenzano et al., 2013), pesticides, and other environmental contaminants (Xu et al., 2011), in foods, feeds, and environmental samples. Compared with other immunoassays, FPIA is especially suitable for screening purposes and remains one of the most promising techniques for expanded use in food safety.

However, we have found very few published reports of monitoring RAC in food samples with FPIA. This may be attributable to the difficulties in finding a suitable tracer and in preparing a highly sensitive and specific antibody, which are both crucial for the development of FPIA. Recently, Zvereva et al. developed an FPIA for RAC in turkey meat (Zvereva et al., 2016). In their study, the FPIA was developed using only one pair of tracer and antibody without any selection, resulting in lower the sensitivity. Moreover, the specificity of the FPIA was evaluated by only 3 β-agonists (salbutamol, clenbuterol, and mabuterol), which was obviously not enough to guarantee highly specificity of the FPIA since there are at least 11 β-agonists was required to be monitored (Ministry of Agriculture of China, 2010). In this study, we aim to establish a highly sensitive and specific FPIA by synthesizing 10 tracers and paring with two previous prepared polyclonal antibodies (pAbs) against RAC (Wang et al., 2015). After careful selection and comparison, a highly sensitive and specific FPIA was developed, which was proved by a low limit of detection and cross-reactivity with 70 β-agonists and β-blockers. The practicality of the FPIA was compared with that of ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) and an ELISA kit.

2. Materials and methods

2.1. Reagents and equipment

RAC, nadolol, salbutamol, bambuterol, sotalol, propranolol, carazolol, timolol, atenolol, bisoprolol, mabuterol, terbutaline, fenoterol, clenproperol, clenpenterol, salmeterol, and metaproterenol were purchased from Dr. Ehrenstorfer GmbH (Ausburg, Germany). Tulobuterol, cimaterol, clorprenaline, cimbuterol, clenhexerol, ritodrine, brombuterol, mapenterol, formoterol, clenisopenterol, bromchlorbuterol, and clencyclohexerol were purchased from Witega (Berlin-Adlersh, Germany). Pirbuterol acetate, oxoxprenolol, and levobunolol were purchased from U.S. Pharmacopeia (Rockville, MD, USA). Zilpaterol, phenylethanolamine a, α-hydroxymethyl, α-hydroxymetaprolol, *o*-desmethyl metoprolol, letaprolol, acebutolol, 7-hydroxy propranolol, nebivolol, labetalol, clenbuterol, prenalterol, reproterol, procaterol, clenisopenterol, bromchlorbuterol, carbuterol, colterol, isoxsuprine, clencyclohexerol, reproterol, isoxsuprine, procaterol, dobutamine, and bamethan hemisulfate were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Carteolol, esmolol, bevantolol, bufuralol, labetalol, celiprolol, alprenolol, bitolterol, cycloclenbuterol hydrochloride, arotinolol, and bopindolol were purchased from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, P. R. China). Isoproterenol sulfate was from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan).

N,*N*-Dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), fluorescein isothiocyanate, ethylenediamine, butanediamine, and hexamethylenediamine were from Aladdin (Shanghai, P. R. China). 5-Aminofluorescein (5-AF) and 4-(aminomethyl) fluorescein (4-AMF) were from Life Technologies., Ltd (Eugene, OR, USA). Normal solvents and salts were of analytical reagent grade and were supplied by Beijing Reagent Corporation (Beijing, P. R. China). Two immunizing haptens of RAC Hapten3 and Hapten4 (Fig. S1, Supplementary material) were conjugated to fluorescein, and two pAbs (pAb Hapten1#1 and pAb Hapten1#2) were previously prepared (Wang et al., 2015). Three fluorescein derivatives, fluoresceinthiocarbamyl ethylenediamine (EDF), fluoresceinthiocarbamyl butanediamine (BDF), and fluoresceinthiocarbamyl hexamethylenediamine (HDF), were synthesized as described in our previous report (Mi et al., 2014). The ELISA kit for RAC was purchased from WDWK Biotechnology Co., Ltd (Beijing, P. R. China). Nonbinding-surface black microplates were obtained from Corning Life Sciences (New York, NY, USA). Thin-layer chromatography (TLC) silica gel 60 aluminum sheets (F254) were purchased from Merck (Darmstadt, Germany).

Borate buffer (0.05 M, pH 8.0) was used as the working buffer for all FPIA experiments. A stock standard solution of RAC (5 μg kg^{-1}) was prepared by dissolving 5 mg of the RAC standard in 1 mL of methanol, and stored at −20 °C until use. A SpectraMax M5 microplate reader (Downingtown, PA, USA) was used to measure the fluorescence polarization (FP). The water used in this study was obtained from a Milli-Q synthesis system (Bedford, MA, USA). A Thermo Scientific Microplate Reader MK3 was used to measure the optical density values. The UPLC/MS-MS analyses were conducted on a Waters 2695 Separations Module and a Waters 2475 Multi-Wavelength Fluorescence Detector equipped with a reverse-phase Symmetry C18 250 mm × 4.6 mm column (Milford, MA, USA).

2.2. Preparation of fluorescein-labeled RAC derivatives

All tracers were synthesized with the active ester method by the condensation of the amino group of fluorescein with the carboxyl of the hapten, as follows: 5 mg of Hapten3 dissolved in 500 μL of *N*,*N*-dimethylformamide was mixed with 2.3 mg of *N*-hydroxysuccinimide and 4.1 mg of EDC, and the mixture was stirred overnight at room temperature to activate the carboxyl of Hapten3. After centrifugation at 5000*g* for 5 min, the precipitate was removed and 3.3 mg of EDF was added to the supernatant and stirred for 2 h at room temperature. An aliquot (20 μL) of the mixture was purified with TLC using methanol/trichloromethane (1:4, v/v) as the developing solvent. The main bands on TLC (Table S1, Supplementary material), with a retardation factor (R_f) 0.35, were collected and redissolved in methanol. The tracer Hapten3–EDF obtained was first characterized with a specific pAb and FPIA and then identified with HPLC–MS/MS. Other fluorescein-labeled tracers were synthesized, purified, and characterized with FPIA. In total, 10 tracers of RAC were generated (Table 1, Fig. 1, and Table S1).

2.3. Development of FPIA

2.3.1. Selection of antibody–tracer pairs

Three analytical parameters, the detection window (δmP), the titer of the pAb, and the half-maximal inhibitory concentration (IC_{50}) of the FPIA, were used as the main criteria for the selection of the optimal antibody–tracer pairs. $\delta mP = mP_{max} - mP_{min}$, where mP_{max} is the observed FP value of the tracer bound to a saturating amount of antibody, and mP_{min} is the observed FP value of the free tracer. Firstly, the binding abilities of the 10 tracers and two pAbs were examined. A tracer with a suitable δmP (> 100 mP) was required for the following experiment. The tracer solutions were diluted with borate buffer to working concentrations with FP values 10–15 times that of the borate buffer background (Li et al., 2015). The δmPs of all the tracers were calculated as follows: 70 μL of each tracer and 70 μL of 100-fold-diluted antibody were added to 70 μL of borate buffer solution. After incubation for 1 min in the dark, the FP value of the mixture was measured at