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Short communication

# New haptens and antibodies for ractopamine

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ABSTRACT

In this work, three unreported immunizing haptens of ractopamine (RAC) were synthesized and used to produce highly sensitive and specific polyclonal antibody. The spacer arms of haptens for coupling to protein carrier were located on different position of RAC with different length. High affinity polyclonal antibodies were obtained and characterized in terms of titer and sensitivity by using enzyme-linked immunosorbent assay (ELISA). The best antibody employed in a heterologous competitive ELISA exhibited an $IC_{50}$ value as low as 0.12 ng $mL^{-1}$ and could not recognize other 10 β-agonists including clenbuterol and salbutamol. The heterologous competitive ELISA was preliminary applied to swine urine and the results showed the new antibody was sufficiently sensitive and specific, and potentially used for the detection of RAC at trace level in real samples.

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## 1. Introduction

Ractopamine (RAC) is a phenylethanolamine β-adrenoceptor agonist and was first approved by the FDA in 1999 for pigs, and then cattle and turkeys to boost growth and leanness, however, more than 150 countries has currently banned RAC in animals production including the European Union (EU) and China due to its potential health risks for humans (De Brabander et al., 2007; Lafontaine, Yu, Espourteille, & Shi, 2012). The maximum residue limits (MRLs) of RAC have been set at 30 μg $kg^{-1}$ for beef and 50 μg $kg^{-1}$ for pork in the United States of America (FDA, 2000). The EU commission proposed 10 μg $kg^{-1}$ as the minimum required performance limits (MRPLs), while, the action level of RAC in China is not exceed 2 μg $kg^{-1}$ in all edible tissue (European Commission, 2002; Ministry of Agriculture of China, 2002).

Antibody-based methods, i.e., immunoassay, are relatively cheap, rapid and sensitive screening methods to detect veterinary drug residues and form a very cost-effective alternative to instrumental methods in large-scale monitoring programs. There are some reports about immunoassays for the detection of RAC residue, mainly including enzyme linked immunosorbent assay (ELISA) and lateral flow immunoassay (Li, Chen, & Zhu, 2007; Liu et al., 2012; Ren et al., 2014; Shelver & Smith, 2000; Wang, Zhang, & Shen, 2006; Zhang et al., 2009). Antibody with high affinity and featured specificity is a key reagent to assay development, while, appreciate hapten design is the critical step in the process of antibody production. The structure of hapten partly determines the affinity and specificity of antibody, then performance of immunoassay. Up to now, few attempt has been made to prepare hapten of RAC in the literatures (Haasnoot et al., 1994; Shelver, Smith, & Berry, 2000). The aim of the study was to synthesize new RAC haptens in order to produce polyclonal antibody with high sensitivity and specificity. The obtained antibodies were characterized by ELISA in homologous and heterologous coating format to select the best combinations of antibody and coating antigen for developing immunoassay in the further study.

## 2. Materials and methods

### 2.1. Hapten synthesis and conjugate preparation

Three haptens (Hapten 1, Hapten 2 and Hapten 3) were synthesized as schematized in Fig. 1. Hapten 1 was prepared previously by our group (Wang, Liang, Wen, Zhang, & Shen, 2014).

*Hapten 2:* A mixture of RAC (300 mg) and malonic ester (200 mg) in ethanol (10 mL) containing 5% formaldehyde and 50 μL of acetic acid was stirred at room temperature for 24 h, then the mixture was evaporated by rotary evaporation. The residue was re-dissolved in water containing LiOH for hydrolysis. After that, the water phase was adjust to pH < 2 with concentrated HCl, then extracted with ethyl acetate (3 × 50 mL). The combined extract were washed by 30 mL of 0.9% NaCl, dried over $MgSO_4$, and concentrated in vacuo to give a solid. The crude solid was

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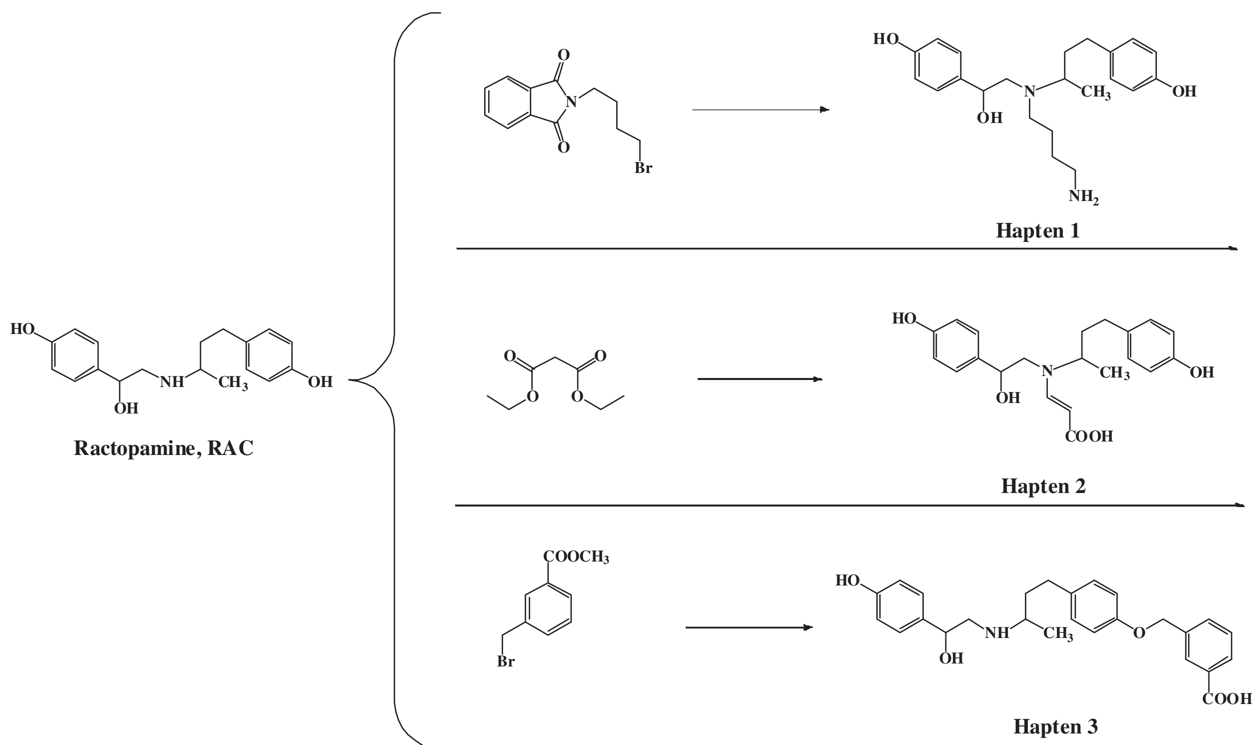


**Fig. 1.** The structure of ractopamine and haptens.

purified by silica gel chromatography with methanol–dichloromethane (v/v, 2:8) to give Hapten 2 (Rf = 0.5, 156 mg, 52%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 10.80 (br, 1H) 7.20 (d, 2H, $J$ = 7.5 Hz), 7.17 (d, 2H, $J$ = 7.6 Hz), 6.72 (dd, 2H, $J$ = 7.5, 1.6 Hz), 6.66 (dd, 2H, $J$ = 7.6, 1.6 Hz), 6.40 (d, 1H, $J$ = 7.6 Hz), 5.42 (d, 1H, $J$ = 7.6 Hz), 5.32 (s, 1H), 5.22 (s, 1H), 4.86 (m, 1H), 4.53 (s, 1H), 3.40 (m, 2H), 2.98 (m, 1H), 2.55 (d, 2H, $J$ = 7.0 Hz), 1.90 (d, 2H, $J$ = 7.0 Hz), 1.32 (d, 3H, $J$ = 6.9 Hz). MS $m/z$ (ESI): 372.2 $[M+H]^+$.

*Hapten 3:* An amount of 300 mg RAC was dissolved in 5 mL of acetone containing 200 mg of $K_2CO_3$ and then 230 mg of 3-bromobenzonic methyl ester was added and heated to 60 °C for 18 h. The mixture was extracted by 20 mL of ethyl acetate twice and the combined extract were removed under reduced pressure. The residues were re-dissolved in water containing LiOH for hydrolysis at 70 °C for 2 h. After that, the water was washed by 30 mL of ether and then adjust to pH < 2 with concentrated HCl and extracted with ethyl acetate (3 × 50 mL). The combined extract was washed by 30 mL of 0.9% NaCl, dried over $MgSO_4$, and concentrated in vacuo to give a solid. The crude solid was purified by silica gel chromatography with methanol–dichloromethane (v/v, 1:9) to give Hapten 3 (Rf = 0.4, 114 mg, 38%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 10.23 (br, 1H), 7.96 (d, 1H, $J$ = 7.5 Hz), 7.88 (s, 1H), 7.71 (d, 1H, $J$ = 7.5 Hz), 7.45 (t, 1H, $J$ = 7.5, 7.5 Hz), 7.20 (d, 2H, $J$ = 7.5 Hz), 7.17 (d, 2H, $J$ = 7.5 Hz), 6.82 (dd, 2H, $J$ = 7.5, 1.5 Hz), 6.71 (dd, 2H, $J$ = 7.5, 1.5 Hz), 5.43 (s, 1H), 5.15 (s, 2H), 4.59 (s, 1H), 4.56 (m, 1H), 4.52 (s, 1H), 2.90 (m, 2H), 2.76 (m, 1H), 2.55 (d, 2H, $J$ = 7.0 Hz), 1.70 (d, 2H, $J$ = 7.0 Hz), 1.23 (d, 3H, $J$ = 6.8 Hz). MS $m/z$ (ESI): 436.2 $[M+H]^+$.

### 2.2. Antibody production and characterization

The polyclonal antibodies derived from 9 rabbits immunized with three immunogens were produced. The immunization, antisera screening and characterization protocol as described in Supplementary material.

### 2.3. ELISA development

The competitive ELISA were performed as described previous reports and provided in Supplementary material (Wang et al., 2013). A four parameter logistic equation was used to fit competitive ELISA data.

### 2.4. Matrix effect and recovery

Negative swine urine samples were supplied by National Reference Laboratory of Veterinary Drug Residue (Beijing, China). The urine samples were centrifuged at 3000g for 5 min at 4 °C before use. Matrix effect were accessed by preparing RAC standard curves in swine urine at several dilution factors (0, 2, 5 and 10 times) and used to investigate the parallelism with the RAC standard curve prepared in buffer.

Swine urine samples were fortified with 1 μg $mL^{-1}$ solution of RAC to give levels of 0.1, 0.5 and 1 μg $L^{-1}$. Spiked samples were diluted 10 times in assay buffer and 50 μL of this diluted samples was submitted to ELISA detection.

## 3. Results and discussion

### 3.1. Hapten and conjugate preparation

There were two methods frequently employed for preparing RAC conjugates in the literature (Haasnoot et al., 1994; Shelver et al., 2000). Shelver et al. firstly described the synthesis of RAC hapten derivatives by using glutaric anhydride (GA), a five atoms of linear alkane with a carboxyl group was introduced to RAC. Haasnoot et al. reported the preparation of RAC conjugates by using butane-1,4-dioldiglycidylether (BDDE) as crosslink reagent. Derivation of RAC by BDDE was a rudimentary strategy because several sites in RAC molecule could be reactive with BDDE and no purified hapten could be obtained. Recently, Liu et al.

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