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Antigens synthesis and antibodies preparation for furazolidone and its metabolite 3-amino-2-oxazolidinone

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Abstract

Two haptens of 3-[(5-amino-furan-2-ylmethylene)amino]oxazolidin-5-one(FZ-NH_2) and 3-{[(4-carboxyphenyl)methylene]-amino}-2-oxazolidinone (CPAOZ) were synthesized. For FZ-NH_2, immunogens were prepared by glutaraldehyde and diazo salt methods. For CPAOZ, immunogens were connected by the methods of the active ester and mixed acid anhydride. Compared with the combination, indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) was developed with coating antigen of FZ-NH_2-OVA via the glutaraldehyde method and immunogen of CPAOZ-KLH via active ester method. For furazolidone and its metabolite AOZ (NPAOZ as derivative), the sensitivities (IC_{50}) were 2.0 μg/L and 2.5 μg/L, limits of detection (IC_{15}) were 0.09 μg/L and 0.25 μg/L, respectively. A sensitive method was developed for the simultaneous determination of furazolidone in feed and its metabolite AOZ in tissue.

Keywords: Furzolidone; AOZ; Antigen; Antibody; ELISA

Furazolidone [*N*-(5-nitro-2-furfurylidene-3-amino)-2-oxazolidinone] belongs to the nitrofuran antibiotic drugs, which have been employed for the prevention and treatment of gastrointestinal infections. Because the parent drug and its metabolite 3-amino-2-oxazolidinone (AOZ) showed carcinogenic and mutagenic characteristics [1], since furazolidone was prohibited for food animals in the European Union in 1995, it has also been banned in the China, United States, and other countries. However, furazolidone was still used due to its low cost and high benefit till now.

The detection for furazolidone residue was mainly based on the AOZ derivative 3-[(2-nitrophenyl)methylene-amino]-2-oxazolidinone (NPAOZ) [2–4]. Various instrumental methods were used to detect the residues of furazolidone and AOZ by HPLC, LC–MS, LC–MS/MS. But these instrumental analytical procedures were not fit for the well-accepted convenient, so ELISA is accepted and performed in the worldwide due to the low cost, high sensitive and rapid screening.

To obtain a specific antibody for furazolidone and its metabolite AOZ, the design of the corresponding immunogen is important. Furazolidone molecule has no active group and is hard to connect with carrier proteins, directly. Therefore, we utilized its NO_2 group to obtain active NH_2 group by Zn/HCl systems as Fig. 1. Then the immunogens of

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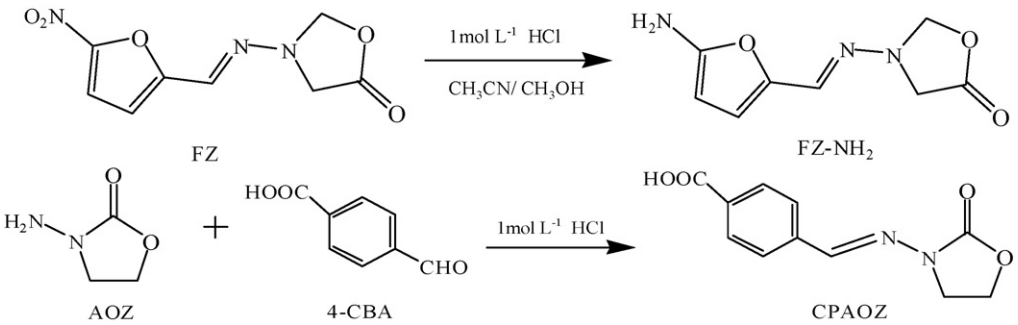

Fig. 1. Synthetic route for haptens.

FZ-NH_2-1 (I, II) and FZ-NH_2-2 (III, IV) were respectively connected by the diazotization and glutaraldehyde method [5,6], but the immune result for the furazolidone or AOZ are unsatisfactory, it is concerned that the furazolidone of low chemical stability might were damaged in rabbit body, and the released AOZ of light molecular is difficult to arouse the immune response. Subsequently, the hapten 3-[(4-carboxyphenyl)methylene]amino-2-oxazolidinone (CPAOZ) was synthesized as Fig. 1. A carboxylic acid moiety in the hapten was a convenient functional group for conjugation to carrier protein, and this similar structural element could increase the chance of high cross-reaction with furazolidone drug. Then the antibodies were achieved from different immunogen haptens of CPAOZ-KLH-1 (V, VI) and CPAOZ-KLH-2 (VII, VIII) *via* the methods of the active ester and the mixed acid anhydride [7,8], respectively. The identification of conjugates was determined by UV spectrophotometer [9]. The UV absorbance spectra of FZ-NH_2-KLH (λ_{max}, 345 nm) and CPAOZ-KLH (λ_{max}, 290 nm) were different from the spectra of KLH (λ_{max}, 275 nm), FZ-NH_2 (λ_{max}, 365 nm) and CPAOZ (λ_{max}, 295 nm). It showed that haptens conjugate to the protein successfully.

The polyclonal antibodies were produced by immunizing eight rabbits [10], and the antiserums were purified by a Protein A-Sepharose 4B affinity column. Using the indirect ELISA [11], antibodies titer and sensitivity (IC_{50}) were showed in Table 1. Comparing the four conjugated methods, the antibodies raised from the CPAOZ-KLH-1 (V, VI) were better than others. It was due to the higher conjugate ratios by the active ester methods.

The specificity of the antibody was evaluated by the cross-reactivity with nitrofuran drugs (furazolidone, nitrofurantoin, furaltadone, nitrofurazone) and their metabolites [3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) and semicarbazide (SEM)] in Table 2. The furazolidone (125%) showed a high cross-reactivity, because the CPAOZ structure is similar to furazolidone. It is well known that antibodies show a preferential recognition to the moiety of the hapten that is farthest to the carrier protein.

Table 1
Antibody titer and sensitivity of each immunization.

	I	II	III	IV	V	VI	VII	VIII
Titer	2000	2400	3000	2500	6000	5000	3000	3500
IC_{50}(μg/L)	600	700	400	450	2.5	3.2	16	18

Antibodies of I–VIII were obtained respectively via the diazotization, glutaraldehyde, active ester and a mixed acid anhydride reaction.

Table 2
Cross-reactivity of antibody with various drugs and marker residues.

Competitor	IC_{50} (μg/L)	Cross-reactivity (%)
NPAOZ	2.5	100
Furazolidone	2.0	125
AOZ	6500	0.038
AMOZ,	5500	0.045
AHD, SEM	100000	0.003
Nitrofurantoin	5000	0.050
Furaltadone, nitrofurazone	4000	0.063

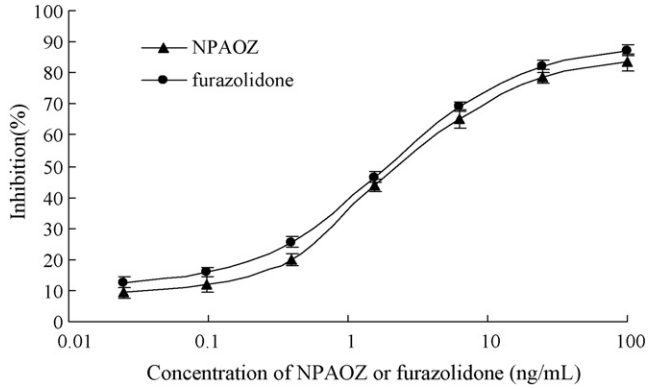

Fig. 2. Competitive indirect ELISA standard curve of NPAOZ and furazolidone.

But the cross-reactivity of AOZ was only 0.038%, so the C=N group played an important role in antibody recognition, despite it is closer to the carrier protein.

In indirect ELISA, the different coating antigens were studied. The OVA conjugates of FZ-NH_2 were achieved by the glutaraldehyde method, and the CPAOZ-OVA were obtained *via* the active ester and the mixed acid anhydride methods. Compared the three coating antigens, the effect of the heterologous conjugates were better than the homologous conjugates. The standard curves were shown in Fig. 2, IC_{50} (the concentration of 50% inhibition in inhibition curve) values for NPAOZ and furazolidone were 2.5 and 2.0 μg/L, IC_{15} (the concentration of 15% inhibition in inhibition curve) were 0.25 and 0.09 μg/L, respectively.

In conclusion, this paper provided a highly sensitive ELISA method for rapidly analyzing furazolidone and AOZ residues. It established the foundation to determine furazolidone in animal feed and its metabolite AOZ in animal tissues in the future.

1. Experimental

Furazolidone (200 mg) in 10 mL solutions of methanol/acetonitrile (30:70, v/v) were heated to 50 °C, then 30 mL 1 mol/L HCl and zinc powder (200 mg) were added slowly and heated to 80 °C for 40 min. The dark red solution was cooled down to 4 °C. After filtration, the solution was extracted with ethyl acetate three times. The combined organic solutions were dried over Na_2SO_4, and solvent was removed by reduced pressure. The residues were chromatographed on silica [ethyl acetate/petroleum ether/acetic acid, 5:2:0.05, v/v/v] to give FZ-NH_2 in 46% yields. The target compound was identified by TLC $R_f = 0.67$ and HPLC–MS/MS [ESI-MS: $2M^+$+H: 319.4 ($C_8H_9N_3O_3$, MW = 195.16)].

4-Carboxybenzaldehyde (42.3 mg) in 200 μL DMF were slowly dropped into the solution of AOZ (20.4 mg) in 1 mL of 0.1 mol/L HCl, the mixture solution was stirred overnight at 60 °C. The mixture was extracted with ethyl acetate. The organic layer were dried over Na_2SO_4 and concentrated by reduced pressure. The residues were purifies on silica [ethyl acetate/petroleum ether/acetic acid, 5:1:0.05, v/v/v] to give 4-CPAOZ in 62% yields. The obtained CPAOZ was characterized by TLC $R_f = 0.45$, HPLC–MS/MS [ESI-MS: M^-: 233.76 ($C_{11}H_{10}N_2O_4$, MW = 234.21)], ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.94–3.99 (t, 2H, J = 8.0 Hz), 4.49–4.54 (t, 2H, J = 8.0 Hz), 7.82 (s, 1H), 7.84 (s, 1H), 7.89 (s, 1H), 7.99 (s, 1H), 8.02 (s, 1H), 13.08 (br, 1H).

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