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Dual-label quantum dot-based immunoassay for simultaneous determination of Carbadox and Olaquindox metabolites in animal tissues

Tao Le [a,*], Liqian Zhu [b], Huan Yu [c]

[a] College of Life Science, Chongqing Normal University, Chongqing 401331, PR China
[b] College of Veterinary, Yangzhou University, Yangzhou 225009, PR China
[c] Wuhan Institute of Animal Health Supervision, Wuhan 430012, PR China

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ABSTRACT

A novel and reliable dual-label direct competitive fluorescence-linked immunosorbent assay (dc-FLISA) based on quantum dots (QDs) was developed for the simultaneous determination of the major metabolites of Carbadox and Olaquindox residues in animal tissues, using anti-QCA monoclonal antibodies and anti-MQCA polyclonal antibodies labeled with QD520 and QD635, respectively. The limits of detection for QCA and MQCA were 0.05 and 0.07 ng/ml, respectively. The method was used to analyze fortified samples and analyte recoveries ranged from 81.5% to 98.2% (QCA) and 84.2% to 95.7% (MQCA). Good correlations between the dc-FLISA method and HPLC were demonstrated for the determination of QCA and MQCA residues in swine tissue samples, confirming the reliability of the proposed method.

1. Introduction

Carbadox, methyl-3-(2-quinoxalinylmethylene)-carbazate-N^1, N^4-dioxide, is an antibacterial agent that has been used as a medicinal feed additive for the prevention and control of swine dysentery and bacterial enteritis in young swine (Joint Expert Committee on Food Additives (JECFA), 1991). Metabolic studies show that Carbadox rapidly converts into monooxy and desoxy metabolites to quinoxaline-2-carboxylic acid (QCA) in animal tissues (Sin, Chung, Lai, Siu, & Tang, 2004). QCA has a relatively long half-life in tissues and has, therefore, been designated as a marker residue for Carbadox in livestock animals (Wu et al., 2007). Olaquindox, *N*-(2-hydroxyethyl)-3-methyl-2-quinoxaline carboxamide-1,4-di-*N*-oxide, is normally used to promote growth, improve feed efficiency, and control both swine dysentery and bacterial enteritis during pig production (Liu et al., 2010). Metabolism of the drug, again via mono and desoxy compounds, produces methyl-3-quinoxaline-2-carboxylic acid (MQCA). MQCA, a compound structurally similar to QCA, is the last major remaining detectable metabolite of Olaquindox in animal tissue, and has, therefore, been designated as a marker for the drug (Wu et al., 2007). However, the EU withdrew the product licenses for both drugs in 1998, because of serious concerns regarding possible carcinogenic, mutagenic and photoallergenic effects of the drugs (JECFA, 1991, 2003). In the USA, Carbadox is approved for use in feed at 55 μg/kg for swine up to 35 kg bodyweight with a 10-week withdrawal period (Sin et al., 2004). In China, under recently implemented regulations, the maximum residue limit (MRL) for Olaquindox in porcine liver is set at 50 μg/kg and in porcine muscle at 4 μg/kg (Wu et al., 2007).

Traditionally, chromatography methods, including liquid chromatography–mass spectrometry (Boison, Lee, & Gedir, 2009; Horie & Murayama, 2004; Hutchinson, Young, & Kennedy, 2005), high-performance liquid chromatography (HPLC) (Duan, Yi, Fang, Fan, & Wang, 2013; Huang, Wang, Tao, Chen, & Yuan, 2008; MacIntosh, Lauriault, & Neville, 1985; Rose, Bygrave, & Tarbin, 1995; Wu et al., 2007; Zhang et al., 2005) and gas chromatography–mass spectrometry (Lynch, Mosher, Schneider, Fouda, & Risk, 1991) have been used for the determination of QCA and MQCA in food products. Although the afore-mentioned methods provide simultaneous and accurate determination of QCA and MQCA, they require expensive equipment, time-consuming processing, and highly skilled technicians. Immunochemical methods have been developed and extensively used for rapid detection of Carbadox and Olaquindox metabolites because of their high sensitivity, specificity, rapidity, low cost and applicability to large numbers of samples (Le, Wei, Niu, & Liu, 2014; Le, Yu, & Niu, 2015a; Le et al., 2012; Peng et al., 2011; Song et al., 2011).

* Corresponding author.
E-mail address: hnxylt@163.com (T. Le).

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Quantum dots (QDs) have gained increasing interest over the past two decades due to the unique optical and electrical properties of these semiconductor nanocrystals. Their size-tunable emission, broad absorption, intense brightness, narrow emission spectra, and exceptional resistance to photobleaching have made QDs more attractive than traditional organic fluorophores for developing analytical and biomedical applications (Carrillo-Carrión, Simonet, & Valcárcel, 2011; Chan & Nie, 1998). Recently, several papers have reported on the use of QDs for the quantitative determination of small molecule chemicals (Chen et al., 2009; García-Fernández, Trapiella-Alfonso, Costa-Fernández, Pereiro, & Sanz-Medel, 2014; Peng et al., 2009; Wu et al., 2013; Zhang et al., 2014). Previous work in our laboratory, concerning the development of QD-based immunoassay for screening of Tylosin and Tilmicosin in edible animal tissues, has indicated that QDs are attractive fluorescent probes for detection of veterinary drug residues (Le, Zhu, & Yang, 2015b). However, there have been few reports on quantitative measurement of QCA and MQCA based on the use of QDs.

The objective of this research was to explore the possible application of QDs for the identification and quantitation of both the Carbadox metabolite QCA and the Olaquindox metabolite MQCA in animal tissue. To this end, two antibodies (anti-MQCA antibody and anti-QCA antibody) were conjugated with the corresponding QDs to establish a direct competitive fluorescent-linked immunosorbent assay (dc-FLISA) for the simultaneous detection of MQCA and QCA in one well of a microtiter plate. The Anti-QCA antibody and the anti-MQCA antibody when coupled to the QDs gave emission maxima at 520 nm and 635 nm, respectively. The reliability of results was confirmed by HPLC. To the best of our knowledge, this is the first report of using dual-label dc-FLISA for the simultaneous determination of the major metabolites of Carbadox and Olaquindox residues in edible animal tissues.

2. Materials and methods

2.1. Materials and reagents

Olaquindox, Carbadox, MQCA, QCA, *N*-butylquinoxaline-2-carboxamide (BQCA, derivatized QCA), ovalbumin (OVA), horseradish peroxidase-labeled goat anti-mouse IgG conjugate (HRP-GaMIgG) and horseradish peroxidase-labeled goat anti-rabbit IgG conjugate (HRP-GaRIgG) were purchased from Sigma (St. Louis, MO, USA). The polyclonal antibody (PcAb) against MQCA (Anti-MQCA PcAb), the monoclonal antibody (McAb) against derivatized QCA (Anti-QCA McAb), BQCA-OVA and MQCA-OVA conjugates were from our laboratory (Le et al., 2012, 2015a). QD520 and QD635 were purchased from Invitrogen Corp (Carlsbad, CA, USA). Tris–HCl buffer, carbonate buffer, Tween 20, phosphate-buffered saline (PBS) and other common solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., China. All chemicals and reagents were of analytical grade or better, and the percentage concentrations were by weight unless stated otherwise.

2.2. Labeling procedures

Anti-QCA McAb and Anti-MQCA PcAb were labeled with QD520 and QD635, respectively. Briefly, 3 ml of QD520, 0.09 ml of EDC (1 mg/ml) and 4 ml of methanol were stirred for 30 min at room temperature and 8 μl of β-mercaptoethanol was added. Ten milliliters of PcAb (5 mg/ml dissolved in PBS at pH 7.0) was added and the mixture was then stirred for 2 h at room temperature. After centrifugation at 16,300*g* for 3 min, the PcAb-QD520 conjugate was obtained by removal of the supernatants. The conjugate compound was stored in a refrigerator at 4 °C prior to use. McAb-QD635 was prepared similarly, except that QD520 was replaced with QD635.

2.3. Binding affinity of the conjugated McAb and PcAb

The biological activity of the McAb and PcAb before and after QDs coupling was examined by the standard ELISA technique. Briefly, 1 μg/ml of coating antigen (BQCA-OVA or MQCA-OVA) was diluted with coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6). After the plates were washed with PBST (PBS with 0.05 % Tween 20), binding sites were blocked by adding 200 μl of 0.1% gelatin in PBS and incubating for 1 h at 37 °C. After the washing step, 100 μl per well of serial dilutions of the McAb-QD520 conjugate (or PcAb-QD635) in PBST were dispensed into the wells and incubated for 1 h at 37 °C. After three washes with PBST, 100 μl per well of HRP-GaMIgG (1:20,000 in PBST) or HRP-GaRIgG (1:5000 in PBST) was added to each well and incubated at 37 °C for 45 min. After washing the plate with PBST, 100 μl of TMB substrate solution was added to each well and incubated at 37 °C for 15 min. The reaction was stopped by adding 50 μl per well of H_2SO_4, and the absorbance of each well was measured at 450 nm.

2.4. Quantum dot-based dc-FLISA

The wells of the opaque white microtiter plate wells were coated with coating conjugates (BQCA-OVA and MQCA-OVA) at a concentration of 1 μg/ml in 0.05 M $NaHCO_3$ (pH 9.6). The coating process was undertaken at 37 °C and after 2 h the plates were washed three times with PBST and then incubated (37 °C) with 200 μl per well of 0.1% gelatin in PBS for 2 h. After the blocking solution was removed, 50 μl of test sample extract, or a series of standards, were then pipetted into the coated microtiter well and then 25 μl each of McAb-QD520 and PcAb-QD635, at an optimal concentration (1:100) diluted in PBS, were added into each well. After incubation (37 °C) for 1 h, the plates were washed three times with PBST. Then the dried plates were imaged in a fluorescence microplate reader. Using an excitation wavelength of 335 nm and emission wavelengths of 520 nm (QCA) and 635 nm (MQCA), standard curves were plotted from which the IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) and the LODs (the limit of detection, defined as 10% inhibition) for QCA and MQCA were obtained.

2.5. Sample preparation and analysis of authentic samples

Commercial swine muscle and liver samples were purchased from a local supermarket in Chongqing. Samples without detectable QCA and MQCA, as determined by HPLC, were used as negative controls. To assess the reliability of the data, all collected samples were fortified with QCA and MQCA at different concentrations and analyzed by the dc-FLISA method. The swine muscle and liver samples were first minced and homogenized before fortification. The McAbs against derivatized QCA were sensitized when QCA was enlarged by reaction with *n*-butylamine or aniline. Thus, the derivatization of tissue homogenates was a necessary step for the determination of QCA residues in edible tissues of animals. Sample pre-treatments were suitably adapted from previous procedures (Le et al., 2015a). Recovery studies were performed by fortifying blank muscle and liver samples with QCA and MQCA, respectively, at different concentrations (2.5, 10 and 50 μg/kg) to evaluate the accuracy and precision of the methods. The assays were carried out with five replicates being performed on the same day to establish the intra-assay precision and also on five days to obtain the inter-assay precision, the results being based on evaluation of the standard curves for the respective dc-FLISA methods.