Development of a sensitive chemiluminescent immunoassay for the determination of 3-methyl-quinoxaline-2-carboxylic acid and quinoxaline-2-carboxylic acid in edible animal tissues using immunomagnetic beads capturing

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Reasons for Urgent Publication

My colleagues and I think that our work is significant because we established a fast and sensitive method for simultaneous determination of MQCA and QCA. The method is proven to provide quantitative, sensitive, and stable monitoring of MQCA and QCA residue in edible animal tissues.

Therefore, this method provides a new reference for the development of MQCA and QCA detection in edible animal tissues.
Abstract

We have reported a sensitive chemiluminescent competitive indirect enzyme-linked immunosorbent assay (CL-ciELISA) based on immunomagnetic beads separation, purification and enrichment for simultaneous determination of 3-methyl-quinoxaline-2-carboxylic acid (MQCA) and quinoxaline-2-carboxylic acid (QCA) in edible animal tissues. Forty field samples were analyzed with the developed CL-ciELISA, and the results correlated well with those obtained in liquid chromatography-tandem mass spectrometry (LC-MS/MS), confirming the utility of CL-ciELISA for quantitation of MQCA and QCA in fish, shrimp, pork and chicken with a good accuracy.

Keywords: immunomagnetic beads, chemiluminescent competitive indirect enzyme-linked immunosorbent assay (CL-ciELISA), 3-methyl-quinoxaline-2-carboxylic acid (MQCA), quinoxaline-2-carboxylic acid (QCA)
Olaquindox (OLA) and carbadox (CBX) are the member of quinoxaline antibiotics, which are used for hastening growth and anti-infectious activities on animals, poultry and fish.¹ In animal tissues, OLA and CBX can be rapidly metabolized and converted into 3-methyl-quinoxaline-2-carboxylic acid (MQCA) and quinoxaline-2-carboxylic acid (QCA), respectively.²⁻⁴ But, MQCA and QCA have carcinogenic, mutagenic and teratogenetic effects on people, threatening the safety of food.⁵ More and more countries banned or limited the use of CBX and OLA in animal cultivation because of the negative effects on health.

Much effort has been devoted to establish sensitive, fast and reliable analytical strategies applicable to determine the OLA, CBX and their metabolites (MQCA and QCA), such as high performance liquid chromatography,¹,⁶,⁷ high performance liquid chromatography-tandem mass spectrometry (HPLC–MS).⁸,⁹ Instrumental detection is the widely accepted technique for veterinary drug residue analysis in complex food matrixes thanks to its sensitivity and accuracy. However, the expensive equipment, time-consuming sample preparation and professional knowledge for operator restrict its extensive use in supervise. Immunoassay is considered to be the rapid technique for veterinary drug residue analysis for high throughput test. Some strategies were proposed, such as enzyme-linked immunoassay (ELISA),¹⁰ time-resolved fluoroimmunoassay (TR-FIA)¹¹ and gold immunochromatographic assay (GICA).¹² Among these methods, ELISA suffers from complicated steps, long test time and low sensitivity; TR-FIA is a rapidly developing technology with high sensitivity and little background interference, but it is susceptible to fluorescence attenuation and other factors, and the development of this method is limited by special equipment; GICA is simple and fast, which is very suitable for field detection, but has low sensitivity. Moreover, this method is mainly used for qualitative
detection, while the quantitative range of quantitative detection is narrow. These characteristics limit the application of this method in quantitative detection with high sensitivity. In this study, we firstly established a sensitive chemiluminescent competitive indirect enzyme-linked immunosorbent assay (CL-ciELISA) for simultaneous determination of MQCA and QCA in edible animal tissues using immunomagnetic beads capturing (Fig. 1), the use of immunomagnetic beads decreased sample pretreatment time, increased assay sensitivity and addressed the interferences from the complex samples.

The design and synthesis of hapten is the most crucial step in the generation of antibody with highly specificity and affinity, so a novel hapten MQCA-NH$_2$ was synthesized by conjugating a methyl and amidogen to MQCA, MQCA-NH$_2$ was conjugated to BSA and OVA by the active amides method for synthesis of immunogen and coating antigen, respectively (Fig. 2). The synthesized hapten was identified by LC-MS/MS and the result showed hapten was synthesized successfully (Fig. S1). Then the antibody with high sensitivity was generated from BALB/c mice. The IC$_{50}$ of the mAb (0.2 μg L$^{-1}$) preceded the most sensitive antibody for MQCA in previous report (1.46 μg L$^{-1}$). Meanwhile, the structure of MQCA is consisted of quinoxaline, carboxy, amidogen and methyl, resulting in high cross-reactivity (CR, 40%) with QCA (QCA has the structure of quinoxaline, carboxy and methyl). And the related compounds (MEQ, QCT, CYA and CBX) had hardly cross-reactivity with MQCA (<1%) (Table S1). The curve fitting of CL-ciELISA and traditional ELISA method were designed and are reported in Fig. 3. The IC$_{50}$ of traditional ELISA and CL-ciELISA were 0.2 and 0.02 μg L$^{-1}$, respectively. Sensitivity of the CL-ciELISA for MQCA was about 10 times greater compared to the traditional ELISA method developed under the same optimum condition; about 73 times more sensitive than the
most sensitive ELISA method (IC_{50}=1.46 \mu g L^{-1})^{10} in previous report; about 155 times more sensitive compared to the ELISA for the similar hapten synthetic method (IC_{50}=3.1 \mu g L^{-1})^{11} about 167 times more sensitive than the latest quantitative GICA (IC_{50}=3.35 \mu g L^{-1})^{12} which confirmed that the developed CL-ciELISA has great sensitivity compared to the other immunoassay, affording the possibility of establishing a sensitive method for determination of MQCA and QCA in animal edible tissue.

A matrix elimination or masking needs to be considered when the developed CL-ci ELISA method was applied to the real sample, MQCA and QCA in tissue were separated and enriched by immunomagnetic beads (Fig. 1). After optimization of conditions (Fig. S2), a portion of (800 \mu L) the extractive supernatant of sample was taken out and 300 \mu L immunomagnetic beads (1 mg mL^{-1}) was added and incubated for 10 min to capture the MQCA or QCA. Interferences of samples were removed after washing, immunomagnetic beads conjugating with MQCA or QCA was heated for 5 min at 85 °C, the eluent was used to CL-ciELISA analysis. The superimposition of the calibration curves indicated that there was no significant matrix effect thanks to sample pretreatment by immunomagnetic beads (Fig. S3), the tissue sample can be analyzed using the standard inhibition curve instead of the sample matrix curve. The limit of detection (LOD) was based on the mean value of 20 blank samples plus three times the mean standard deviation. The LOD for MQCA in fish, shrimp, pork and chicken were 0.05, 0.043, 0.048 and 0.050 \mu g kg^{-1}, and the LOD for QCA in fish, shrimp, pork and chicken were 0.090, 0.10, 0.13 and 0.18 \mu g kg^{-1}. As shown in Table S2, the mean recovery ranged from 76.6% to 117.0% for four edible animal muscle tissues, with the intra-assay CVs in the range of 4.5% to 10.5% the inter-assay CVs in the range of 6.1-11.5%. The intra- and inter-assay CVs should be
no more than 15%. Thus, the accuracy and precision are acceptable, and the CL-ciELISA method has good repeatability. To evaluate the reliability of developed CL-ciELISA method, 40 field samples (10 samples per kind of tissue), were analyzed with the developed CL-ciELISA, and the results were compared with the results obtained in the LC-MS/MS. As shown in Table 1, the results of field sample measured by CL-ciELISA and LC-MS/MS were nearly equal.

Chemiluminescent detection with the separation and enrichment target in sample pretreatment by immunomagnetic beads has been demonstrated to be a useful analytical method for monitoring drug residues in foods because of its high sensitivity, low cost, environment-friendly (heating as the elution method), convenience of handling and high throughput in investigating. We have for the first time developed a sensitive CL-ciELISA based on immunomagnetic beads capturing for simultaneous quantitation of MQCA and QCA in fish, shrimp, pork and chicken with a good accuracy and reliability, which makes it a useful tool for screening purposes.

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Supporting Information

The supporting information contains detailed experimental procedures in this study and supplementary figures and tables. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Legends of Figures and Tables

Fig. 1 The scheme of CL-ciELISA with immunomagnetic beads for the determination of MQCA and QCA in animal tissues.

Fig. 2 Synthesis scheme for the novel hapten and hapten-protein conjugates.

Fig. 3 Normalized standard curve of CL-ciELISA for MQCA under optimized conditions compared to the standard curve obtained by traditional ELISA for MQCA.

Table 1 Determination of field animal meat samples collected from retail outlets in Chongqing by the CL-ciELISA and LC-MS/MS methods (n=3)
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| Sample | CL-ciELISA (μg kg\(^{-1}\)) | LC-MS/MS (μg kg\(^{-1}\)) |
|--------|-------------------------------|-----------------------------|
| MQCA+QCA | 0.93±0.08 | 1.05±0.06 | ND |
| MQCA   | 1.25±0.11 | 1.17±0.12 | ND |
| QCA    | 2.14±0.15 | 2.26±0.16 | ND |
| P2     | 1.92±0.11 | 2.13±0.15 | ND |
| P7     | 3.26±0.24 | 1.57±0.13 | 4.12±0.31 |
| P8     | 0.55±0.06 | ND | 1.24±0.14 |
| S3     | 2.31±0.18 | 1.5±0.11 | 1.89±0.21 |
| S9     | 0.78±0.09 | ND | 2.15±0.18 |

P1, P3, P4, P5, P6, P9, P10

C2, C3, C4, C6, C7, C8, C9, C10

F1, F2, F3, F4, F5, F7, F8, F9, F10

S1, S2, S4, S5, S6, S7, S8, S10

< LOD ND

ND: Not detected.
P1-P10: pork samples; C1-C10: Chicken muscle samples; F1-F10: fish samples; S1-S10: shrimp samples