An immunochromatographic strip sensor for Marbofloxacin residues

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Article

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Abstract

Marbofloxacin (MBF) was once widely used as a veterinary drug to control diseases in animals. MBF residues in animal food endanger human health. In the present study, an immunochromatographic strip assay (ICSA) utilizing a competitive principle was developed to rapidly detect MBF in beef samples. The values of 50% inhibitory concentration ($IC_{50}$) and the limit of detection (LOD) of the ICSAs were 2.48 ng/mL and 0.54 ng/mL, respectively. The recovery rates of MBF in spiked beef samples were from 83.1–91.0%. The coefficients of variation (CV) for intra-assay and inter-assay were below 10%. In addition, when the same authentic beef samples were detected in a side by side comparison between the ICSAs and HPLC-MS, there was no statistically significant difference. Therefore, the proposed ICSAs can be a useful tool for both qualitative and quantitative monitoring of MBF residues in beef samples.

Introduction

Marbofloxacin (MBF) is a synthetic third-generation fluoroquinolone (FQ) antibiotic. Since the middle of the 1990s, MBF has been used exclusively in veterinary practice to treat respiratory, digestive, urinary, and skin infections in pet animals such as dogs and cats in Europe and the United States. MBF blocks the growth of Gram-negative pathogens, some Gram-positive pathogens and Mycoplasma by inhibiting DNA transcriptase. MBF is also proposed for the treatment of disease in respiratory tract, soft tissues, digestive tract and breast tissue of food-producing animals (cattle, swines) since 1997. While MBF was allowed for use in companion animals, it was prohibited by many countries for the use in all edible animals in order to reduce FQ-resistant campylobacter. MBF residues in animal tissues and milk can even cause development of resistant strains of bacteria, allergic hypersensitivity reactions, etc. in the human body. In order to prevent MBF residues from entering the food chain and reducing antibiotic resistance, many countries has also set maximum residue limits (MRLs) for MBF. The European Union (EU) has specified MRLs for MBF: 150 µg/kg in muscle, liver and kidney, 50 µg/kg in fat, 75 µg/kg in milk (Commission Regulation of the EU No 37/2010). In China, MBF is not permitted for use in animals. However, because of its wide antimicrobial spectrum, it has been illegally used by producers of animals to treat animal diseases. The Chinese Ministry of Agriculture and Rural Affairs (Bulletin NO.89) approved three veterinary drug products of MBF injection (CEVA SANTE ANIMALE S.A.) to be registered in China, and has set MRLs in bovine adipose tissue to be 50 µg/kg for MBF. Therefore, it is important to be able to monitor MBF residues in animal-derived foods.

Existing analytical methods to determine MBF in biological samples, include high performance liquid chromatography (HPLC), HPLC- tandem mass spectrometry (HPLC-MS/MS), reverse phase-HPLC (RP-HPLC) and HPLC with ultra-violet detection or fluorescence detection. Although these methods have relatively high sensitivity and good selectivity for detecting MBF, the drawbacks of chromatography are that it is time-consuming, labor intensive, and limited to laboratory use due to its dependency on complex sample pretreatment, huge and expensive instruments, and professional technicians. Furthermore, these drawbacks limit the amount of screening that can be done feasibly. In
contrast, immunoassays could overcome some of these shortcomings. While enzyme-linked immunosorbent assays (ELISAs) do eliminate the need for sample pretreatment and can tolerate certain matrix interferences by means of washing between steps, ELISAs require a lab setting, technical training, and multiple steps. Immunochromatographic strip assays (ICSAs) on the other hand, offer a more simple and convenient solution that has been proven to be effective in detecting drugs $^{12,13}$, pesticides $^{14,15}$, toxins $^{16-19}$, allergens $^{20,21}$, bacteria $^{22}$, and viruses $^{23}$. ICSAs are all-inclusive, can provide results in a timely manner, and can easily yield both qualitative and quantitative results.

Monoclonal antibodies (mAbs) of FQ mainly focused on SAR (Sarafloxacin), Enrofloxacin (ENR), Ciprofloxacin (CIP), Norfloxacin (NOR), Ofloxacin (OFL) and Difloxacin (DIF) in the ELISAs $^{24}$ and ICSAs $^{25,26}$ based on those mAbs had been developed for the detection of FQ in animal feeds, livestock carcasses and milk samples (Figure 1a). Though MBF is widely used in veterinary clinics, the need for the simple detection of MBF residues in food has rarely been addressed yet $^{4,27}$.

The objective of this study was to develop an ICSA for the rapid detection of MBF in beef samples. MBF was coupled with bovine serum albumin (BSA) and ovalbumin (OVA) via a mixed EDC/NHS method. The mAbs against MBF were produced and then conjugated with colloidal gold nanoparticles (CGNs) for the probe. The ICSA utilizes a competitive principle and was validated in terms of specificity, sensitivity, accuracy, and precision in spiked recovery experiments, and verified by HPLC-MS for used with detecting authentic edible animal tissue samples.

**Materials And Methods**

**Chemicals and materials.** MBF, N-Hydroxy succinimide (NHS), N, N-dimethylformamide (DMF), Na$_2$B$_4$O$_7$, 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC), Freund's adjuvant, and a mouse monoclonal antibody isotyping kit were purchased from Sigma (St Louis, MO, USA). ENR, CIP, NOR, OFL, DIF, and Danofloxacin (DAN) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). HRP conjugated goat-anti-mouse IgG was purchased from Sino-American Biotechnology Co., Ltd (Luoyang, China). BSA and OVA were all obtained from Yuanye Biotechnology Co., Ltd (Shanghai, China).

**Preparation of immunogen, coating antigen and anti-MBF mAbs.** The EDC/NHS method for the preparation of MBF-BSA and MBF-OVA conjugates was modified from a previous report $^{28,29}$. Briefly, 18.12 mg BMF were dissolved in 3 mL DMF, and mixed with 5.75 mg NHS and 9.59 mg EDC at room temperature (RT) for 12 h. The mixture was centrifuged at 3950 rpm for 5 min to collect the supernatant. In an ice bath, the active final products was slowly added drop-by-drop into 2 mL phosphate buffer saline (PBS, 0.01M) containing 33.22 mg of BSA or 22.25 mg OVA and the mixture was the stirred for 12 h at 4°C. The final mixture was dialyzed against PBS nine times for three days and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected and stored at -20°C.

BALB/c mice (7 weeks of age) were subcutaneously immunized with 70 µg immunogen MBF-BSA at intervals of 3 weeks for five times. Freund's complete adjuvant and Freund's incomplete adjuvant with
isometric immunogen were used for the first and 2–5 times immunization, respectively. The titer and specificity of antiserum in mice were detected by indirect and indirect competitive ELISAs (ic-ELISAs) using microplate readers (Multiskan FC, Thermo Fisher Scientific Instrument Co., Ltd., USA) as previously described \(^\text{30,31}\). The mouse giving the best half-maximal inhibitory concentration (IC\(_{50}\)) in the ic-ELISA was selected to be vaccinated intraperitoneally with 100 \(\mu\)g of immunogen before cell fusion. The detailed cell fusion between splenocytes and myeloma cell procedure was as described in a previous study \(^\text{32,33}\). Briefly, hybridomas secreting anti-MBF mAbs were selected by ic-ELISA, subcloned with limiting dilution, and cultured to prepare ascites fluids in paraffin-primed mice. Non-competitive ELISA were performed for characterization of affinity and isotype of the mAbs using a mouse mAbs isotyping kit \(^\text{34}\).

**Conjugation of anti-MBF mAbs with colloidal gold and Preparation of ICSAs.** Anti-MBF mAbs 7A12 were labeled with spherical colloidal gold nanospheres (CGNs). The CGNs were produced via deacidizing of HAuCl\(_4\) with sodium citrate reduction \(^\text{35}\). To conjugate the anti-MBF mAbs 7A12 with CGNs, the antibody concentration and pH value were adjusted to the best condition with 10% NaCl and 0.2 M K\(_2\)CO\(_3\), respectively. To produce CGNs-mAbs, 1.5 ml mAbs 7A12 solution (2 \(\mu\)g/mL) were incubated with 7.5 ml colloidal gold solution (pH 9.0) for 25 min at RT. The mixture was incubated at RT for another 12 min with 1.5 mL of 10% BSA. The labeled mAbs were then washed twice; and centrifuged at 15000 \(\times\) g for 30 min at 4°C. The sediment was finally resuspended in sodium borate solution including 1% BSA (W/V) and stored at 4°C before use. To capture hapten MBF or MBF-BSA, CGNs-mAbs were sprayed on the conjugate pad. The ICSAs were assembled according to a previously described method \(^\text{36}\).

**Test procedure and principle of ICSAs.** The ICSA is composed of a sample pad, a conjugate pad, the nitrocellulose (NC) membrane, an absorbent pad, and a backing card. The operating principle of the ICSAs used free MBF in the sample solution to compete with the MBF-BSA for binding to CGNs-mAbs at the test line (T line). The sample solution is added to the sample pad and flows toward the absorbent pad via capillary effect. If MBF is present in the sample, it will bind to the CGNs-mAbs, lessening the amount of CGNs-mAbs available to bind with MBF-BSA at the T line; the CGNs-mAbs will then be captured by a second antibody (goat-anti-mouse IgG) at the control line (C line). Thus, the T line will show a light red or achromatic color that is inversely proportional to the amount of MBF present in the sample, while the C line shows as red. Within the linear range, the concentration of MBF in the sample solution is inversely correlated with the intensity of red at the T line. If there is no MBF in the tested sample, the CGNs-mAbs will be bound to the MBF-BSA at the T line and the second antibody at the C line, respectively. Note that a visible red of the C line should always appear to indicate a valid ICSA. Otherwise, the test strip is considered invalid and a new test strip should be used (Fig. 2) \(^\text{37}\).

**Sample pretreatment for the ICSAs.** Fresh beef was purchased from a local market. It was tested by HPLC-MS to be negative for MBF. The beef sample was then minced and homogenized. The tissue sample (2 \(\pm\) 0.01 g) was transferred into 50-mL centrifuge tubes, was dissolved in PBS (8 mL) at ratio of 1:4 to prepare 0.25 g/mL negative beef solution. MBF was spiked with negative beef solution to give a
final concentration of 6, 10, 20, 80, 160, 320 ng/mL. The sample was then extracted by adding 5 mL of 5% trichloroacetic acid and acetonitrile solution containing 1% acetic acid (8:2, v/v) and vibrating for 10 min. Following centrifugation at 10000 × g for 10 min at 4°C, 1.5 mL of the supernatant and 2 mL of n-hexane were added successively in a 10-mL centrifuge tube and vortexed for 1 min. After 5 min, 1 mL of the subnatant was filtered using a 0.22 µm of microporous membrane.

**Evaluation of the performance of ICSAs.** The sensitivity of the ICSAs was determined using standard solutions serially diluted including different concentration of MBF (0, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ng/mL). After about 150 µL of sample was dropped onto the sample pad, qualitative detection results were evaluated by the appearance of the coloration at T and C lines within 8 min. For quantitative detection, a TSR3000 membrane strip reader (Bio-Rad, USA) was used to read the relative optical density (ROD) of the T line. A standard curve was established by plotting the various concentrations of MBF and its corresponding B/B₀ value. B and B₀ represented the ROD values of the testing samples and the blank sample, respectively. Using GraphPad prism, the value of IC₅₀ was computed on the basis of the linear regression equation.

To evaluate specificity of the ICSAs, MBF and the structural analogues (OFL, ENR, NOR, CIP, DIF, and SAR) in the negative beef samples were tested, respectively. Cross-reactivity (CR) was assessed with the equation: CR (%) = (IC₅₀ of MBF)/IC₅₀ of the competitor) × 100.

The accuracy and precision of the ICSAs were evaluated by detecting beef samples containing 5.0, 30.0, and 100.0 ng/ml of MBF six times. The accuracy and precision were expressed as recovery and coefficient of variation (CV, %), respectively.

**Authenticity of ICSAs.** The negative tissues samples spiked with four different MBF concentrations (0, 8.4, 25.8, and 67.6 ng/mL), were detected in parallel by ICSAs and HPLC-MS, respectively. HPLC-MS was performed for instrumental analysis of MBF using a previous method with minor changes. Briefly, liquid chromatography conditions: Diamonsil C18 (2.0×150 mm×5 µm), the mobile phases were 1000 mL of double distilled water, 1 mL of formic acid and 500 µL of 1 M ammonium formate solution, flow rate of 0.25 mL/min, column temperature was 30 °C, and MBF was monitored at 295 nm. Mass spectrometry conditions: Electrospray ion source, positive ion scanning, auxiliary gas (N₂) at 50 psi and 350 °C, flow rate of auxiliary gas: 9.0 L/min, capillary outlet voltage:115 V, and collision energy was 12 eV. There was no peak at 295 nm for beef sample spiked without MBF (0 ng/mL). MS detection also get negative results.

**Results And Discussion**

**Preparation of complete antigen.** The molecular weight of hapten MBF is 362.35 Da. It can bind to homologous antibodies, has no immunogenicity, and does not stimulate the immune response of humans and animals. Complete antigen MBF-BSA is necessary to produce specific antibodies against MBF. Haptens with carboxyl groups are normally coupled with carrier proteins using one of two methods:
the EDC/NHS method or the mixed anhydride (MA) method. The conjugating conditions in the EDC/NHS method need to be completely anhydrous, but compared with the EDC/NHS method, this method produce more outgrowth. Therefore, we synthesized the immunogen MBF-BSA and coating antigen MBF-OVA using the EDC/NHS method (Fig. 1b). The coupling rates of MBF with BSA and OVA were calculated by ultraviolet (UV) scanning at 19.2:1 and 14.6:1, respectively. The immunized mouse with its antibody titer of \(1.512 \times 10^4\) and an IC\(_{50}\) value of 20.51 ng/ mL was chosen for subsequent cell fusion.

**Characterization of mAbs against MBF.** The 3F4, 3F10, 7A12, and 8A4 cell lines were obtained after screening. mAbs 7A12, which showed a highest antibody titer of \(1.024 \times 10^6\) and the greatest IC\(_{50}\) value of 2.29 ng/mL, was selected to be labeled by CGNs with a diameter of 25 nm. The affinity constant (Ka) and the subtype of 7A12 were determined by ELISA to be \(1.2 \times 10^{10}\) L/mol and immunoglobulin G 1, respectively.

**Sensitivity of the ICSAs.** To demonstrate the sensitivity of ICSAs for MBF, we detected reference solutions of MBF at concentrations of 0, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ng/mL by the ICSAs. The ROD value for each ICSAs was read using scanning the T line with a TSR3000 membrane strip reader (Fig. 3, Table 1). A quantitative calibration curve was constructed by plotting the value of B/B\(_0\) against the concentrations of MBF in beef samples (Fig. 4). ROD values have a negative correlation with the MBF concentration samples. The regression equation was \(y = -0.4576 + 0.6805 (R^2 = 0.9901)\). According to the linear equation, the IC\(_{50}\) value was 2.48 ng/mL, and limit of detection (LOD) was 0.54 ng/mL. The ranges is defined as extending from the IC\(_{20}\) to the IC\(_{80}\). The detection ranges of the ICSA were 0.54–11.22 ng/mL for MBF. The qualitative LOD of the ICSAs was identified to be 4.0 ng/mL based on unaided visual assessment (Fig. 5).

<table>
<thead>
<tr>
<th>MBF concentration (ng/mL)</th>
<th>G/D×area-ROD (pixel)</th>
<th>G/Peak-ROD (pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>280.9586</td>
<td>0.1492</td>
</tr>
<tr>
<td>1.0</td>
<td>190.1199</td>
<td>0.1185</td>
</tr>
<tr>
<td>2.0</td>
<td>155.6601</td>
<td>0.0966</td>
</tr>
<tr>
<td>4.0</td>
<td>113.8419</td>
<td>0.0715</td>
</tr>
<tr>
<td>8.0</td>
<td>77.8907</td>
<td>0.0474</td>
</tr>
<tr>
<td>16.0</td>
<td>23.1068</td>
<td>0.0229</td>
</tr>
<tr>
<td>32.0</td>
<td>5.9140</td>
<td>0.0062</td>
</tr>
</tbody>
</table>

**Specificity of the ICSAs.** The specificity of the ICSAs was evaluated in comparison other structural analogues of MBF including OFL, ENR, NOR, CIP, DIF, and SAR. When these competitors were added at a final concentration of 1000 ng/ml, the color of the T lines remained the negative. The CR of the ICSAs
based on mAbs 7A12 to OFL, ENR, NOR, and CIP were 61.23%, 31.96%, 22.81%, and 23.31%, respectively; however, the CR for DIF and SAR was less than 0.1% (Table 2). One explanation for this is that the FQs like OFL, ENR, NOR, CIP, DIF, SAR, and MBF have the same or similar basic chemical structure, (for example a fluorine atom in the C-6 position of the quinoline ring and piperazinyl at C-7), but DIF and SAR have an additional unique fluorobenzene structure. The MRLs established by the EU for FQs in muscle, kidney, and egg et al. were greater than 30 ng/mL. Therefore, the ICSAs of MBF are passable tools to test OFL, ENR, NOR, and CIP residues in animal food.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (ng/mL)</th>
<th>CR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBF</td>
<td>2.48</td>
<td>100</td>
</tr>
<tr>
<td>OFL</td>
<td>4.05</td>
<td>61.23</td>
</tr>
<tr>
<td>ENR</td>
<td>7.76</td>
<td>31.96</td>
</tr>
<tr>
<td>NOR</td>
<td>10.87</td>
<td>22.81</td>
</tr>
<tr>
<td>CIP</td>
<td>10.64</td>
<td>23.31</td>
</tr>
<tr>
<td>DIF</td>
<td>&gt; 300</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>SAR</td>
<td>&gt; 300</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

**Accuracy and precision of the ICSAs.** Beef samples with MBF (5.0, 30.0, and 100.0 ng/mL) were determined. The ROD of the T line was scanned by the strip reader and MBF concentrations were evaluated by standard curve. The recoveries ranged from 83.1–90.0% (Table 3). Both assay CV of the ICSAs were less than 10.0%.

<table>
<thead>
<tr>
<th>Spiked MBF (ng/mL)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (ng/mL)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>5.0</td>
<td>4.15 ± 0.30</td>
<td>83.1 ± 6.0</td>
</tr>
<tr>
<td>30.0</td>
<td>26.15 ± 1.45</td>
<td>87.2 ± 4.8</td>
</tr>
<tr>
<td>100.0</td>
<td>90.35 ± 3.37</td>
<td>90.4 ± 3.4</td>
</tr>
</tbody>
</table>

**Comparison of ICSAs with HPLC-MS.** ICSAs and HPLC-MS were performed by testing beef samples. There was no significant difference between the results using the two methods (Table 4), which means that the ICSAs are reliable for the range of values tested.
Table 4  
Comparison between the ICSAs and HPLC-MS

<table>
<thead>
<tr>
<th>MBF (ng/mL) in beef samples</th>
<th>ICSAs (ng/mL)</th>
<th>HPLC (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4</td>
<td>7.06 ± 0.74(^a)</td>
<td>7.32 ± 0.51(^a)</td>
</tr>
<tr>
<td>25.8</td>
<td>22.20 ± 1.25(^a)</td>
<td>24.12 ± 0.68(^a)</td>
</tr>
<tr>
<td>67.6</td>
<td>59.60 ± 2.48(^a)</td>
<td>64.29 ± 1.28(^a)</td>
</tr>
</tbody>
</table>

\(^a\) the superscript represents no statistical significance between the results given by the ICSAs and HPLC (\(P > 0.05\))

**Conclusion**

In this study, we generated a high-affinity and specific mAbs 7A12 against MBF. ICSAs was established for detecting MBF residues in beef samples. The ICSAs showed the IC\(_{50}\) of 2.48 ng/mL and a LOD of 0.54 ng/mL. The recoveries of MBF in beef samples was determined to range from 83.1–90.0%. Certified by HPLC-MS, the ICSAs are a convenient on-screening tool for the detection of MBF residues in beef samples.

**Declarations**

**Ethical statement.** This study does not involve any human testing. The study protocol were approved by the animal Ethics committee of Zhoukou Normal University (ZKNU2021038) and were performed according to the Guide for the ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

**Acknowledgements**

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**Author Contributions**

X.Y. and X.H. conceived of the study, designed the study, and drafted the manuscript; Q.L., J.Y., Q.Z., M.Z. and M.G. carried out the experiments. Y.Y. and T.T. participated in data analysis and the design of the study; X.H. and S.K. designed the experiments and reviewed drafts of the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Data Availability**

All data generated or analysed during this study are included in this published article [and its supplementary information files].
References


Figures
Figure 1

a. Chemical structure of FQs for mAbs produced in previous literature. b. Synthesis of MBF-BSA using an activated ester method.
Figure 2

Structure and schematic diagram of the ICSAs.

![Diagram of ICSAs with labels: Sample pad, Conjugate pad, Sample without MBF, Sample with MBF, NC membrane, Absorbent pad, T line, C line, Backing card. Symbols for MBF, MBF-BSA, Anti-MBF mAbs, Colloidal gold-labelled mAbs, Goat-anti-mouse immunoglobulin G antibody.](image-url)
Figure 3

ROD curves of beef samples containing MBF at 0, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ng/mL were detected by ICSAs using a strip reader.

\[ y = -0.4576x + 0.6805 \]
\[ R^2 = 0.9901 \]

Figure 4

The quantitative calibration curve for MBF using ICSAs. The X-axis shown is the logarithmic concentrations of MBF. B/B_0 represents the percentage of ROD values determined by the different concentrations of MBF in beef samples divided by the zero-dose.
Figure 5

Standard MBF samples were tested using the ICSAs observed with the naked eye.