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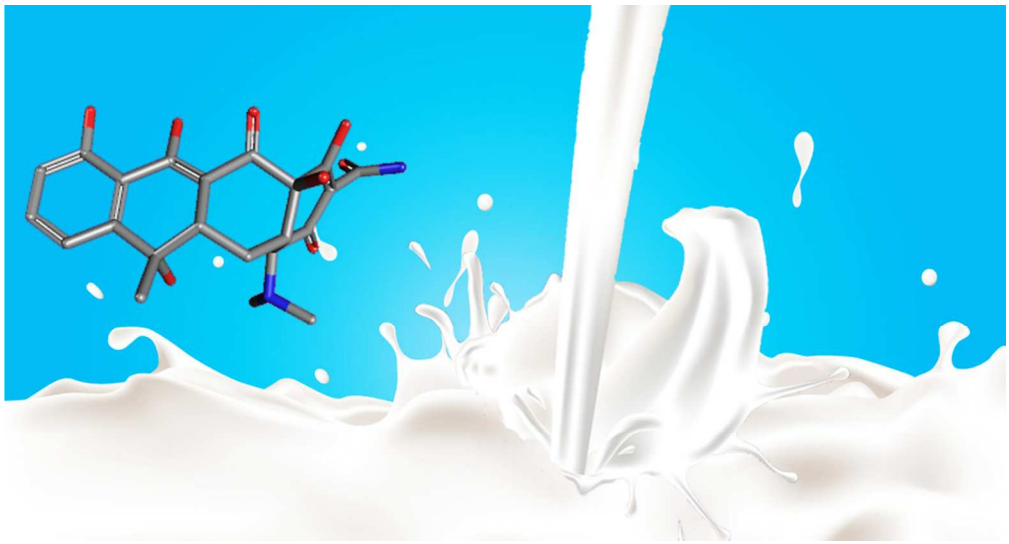

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ARTICLE

New haptens synthesis, antibody production and comparative molecular field analysis for tetracyclines

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In this work, two novel haptens of tetracycline (TC) were synthesized and used to produce a polyclonal antibody for TC. A selective heterologous enzyme-linked immunosorbent assay (ELISA) using the novel antibody was optimized to exhibit an IC_{50} value as low as 0.7 ng mL^{-1} in buffer. The assay was sufficiently sensitive for the analysis of the maximum residue limit (MRL) of TC in milk (100 μg L^{-1}). The analysis of TC in fortified milk samples indicated average recoveries of 84.9-111.8% with inter-assay CV values of 9.34-14.5% after 100-fold buffer dilution without any clean-up step or organic-solvent usage. The cross-reactivities of 17 other TC analogs were tested using the optimized ELISA, and a comparative molecular field analysis (CoMFA) was performed at the 3D level in a quantitative manner to explain the observed cross-reactivities. The results indicated that the CoMFA was a useful tool for understanding the recognition profiles of antibodies in competitive immunoassays for TCs.

Introduction

Tetracyclines (TCs) are broad-spectrum antibiotics that are extensively used to treat bacterial infections in both humans and animals throughout the world (Fig.1). TCs are used in pure form or mixed with other analogs, such as oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC).[1] According to reports, TCs represent the largest antibiotics sales for animal use in the United States, and the Union of Concerned Scientists has estimated that nearly 5 million pounds of TCs are given to healthy swine each year.[2] In China, the annual TC usage was approximately 9413 tons in 1999, and the annual OTC production was approximately 10,000 tons (65% of the world total) in 2003.[3] Over 60 percent of all antibiotics used for animals in Japan are TCs.[4] As a consequence, residues of TCs are often found in foodstuffs of animal origin, resulting in toxicological, economical and ethical concerns.

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In addition, the excessive use of TCs has been associated with the dramatically increasing prevalence of multidrug-resistant pathogens.[5] The Europe, United States and China have set maximum residue levels (MRLs) for TCs that range from 100 μg L^{-1} in milk to 1200 μg kg^{-1} in the kidneys.[6-8]

At present, the most frequently used methods of analyzing TCs are microbiological methods and instrumental methods. [1, 9-11] Microbiological methods are generally complicated and time-consuming and lack specificity, whereas instrumental methods require expensive equipment, large volumes of organic solvents and time-consuming sample clean-up procedures. Antibody-based analytical methods offer a simple, rapid and cost-effective alternative if suitable antibodies are available.[12] To date, there have been only a few reports concerning the preparation of antibodies for TCs. [13-20]

In this work, we describe the synthesis of two novel haptens and the generation of a specific polyclonal antibody for TC that was found to exhibit significantly improved sensitivity. A highly specific and sensitive enzyme-linked immunosorbent assay (ELISA) based on the novel antibody was developed and optimized and was then applied to milk samples. Moreover, the specificity of the optimized ELISA was evaluated using 18 TC analogs and analyzed via comparative molecular field analysis (CoMFA).

Results and discussion

Haptens and conjugates

The objective of this study was to generate a highly sensitive and specific polyclonal antibody for TC. The preparation of the immunogen is a critical step in the process of antibody production, particularly for antibodies for small molecules. Few attempts have been made to prepare immunogens for TCs in the literature,[17] moreover, most studies have employed rudimentary strategies based on the direct coupling of TC to the carrier protein using homobifunctional aldehydes, carbodiimide (CDI) or diazonium conjugation via Mannich condensation.[20-21] Recently, several authors have synthesized TC derivatives as haptens with active carboxyl or amino groups, such as TC, CTC, OTC and methacycline.[14, 15, 17, 20, 22] In these previous reports, the conjugation sites in the TCs or haptens

were generally situated in the D ring of the TCs (Fig. 1). Among TCs, the D ring and C ring can vary, whereas the A ring is highly conserved. Thus, to generate a specific antibody for TC, the unique moiety of the TC molecule should be exposed to the immune system of the animal; therefore, the conjugation site for TC immunogens should be located in the A ring. Pastor-Navarro *et al.* and Javier Adrian *et al.* have synthesized haptens of TCs characterized by a carboxyl or an amino group at position 2 or 4 in the A ring, respectively.[15, 17] However, adequate antibodies could not be obtained using these haptens. This may be because the spacer arms in TC-3, OTC-2 and CTC-2 in the former work were too short, whereas the dimethylamino group at position 4 of hapten TC2 in the latter work played an important role in immune response.[15, 17]

In this work, we synthesized two innovative haptens of TC (TC-AA and TC-AB) with the aim of maximizing the exposure of the discriminative D ring and C ring of TC (Fig. 1). Haptens TC-AA and TC-AB were prepared via the formation of carboxamido derivatives from the corresponding TC using 4-aminobutyric acid and aminoacetic acid. The experimental conditions were selected to favor Mannich condensation, wherein the primary carboxamido functions on the A ring undergoes *N*-alkylation.[17] The haptens maintained their tetracyclic nucleus with an aliphatic spacer arm at position 2 of the A ring. The two novel haptens were covalently coupled through their carboxylic groups to the lysine amino-acid residues of BSA and OVA using an activated ester method to form an immunogen and a coating antigen. In addition, the conjugates TC-F-BSA and TC-F-OVA were also prepared via the Mannich reaction. The hapten densities of the three BSA conjugates were estimated using MALDI-TOF MS (Table 1). Each immunogen was used to raise four antisera in the study.

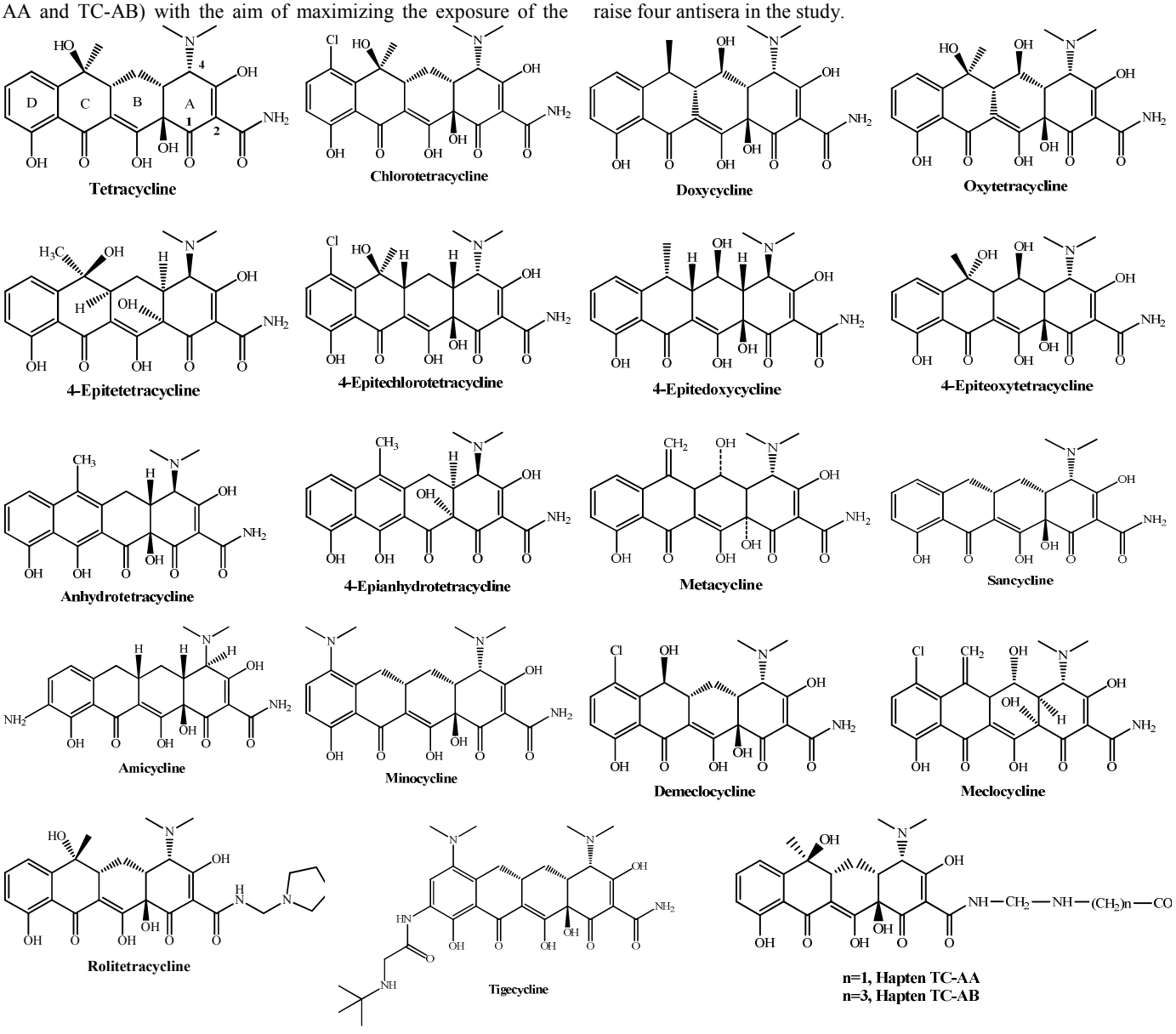

Fig. 1 Chemical structures of the most commonly used tetracyclines and haptens.

Screening and selection of antisera

The antisera of 12 rabbits immunized with three immunogens were screened against their homologous coating antigens using indirect noncompetitive and competitive ELISA, in which the same hapten was used as both the coating antigen and the immunogen. The titers and IC_{50} values of all antisera obtained after the sixth booster injection are summarized in Table 1. All antisera exhibited relatively low titers to their homologous assays (all below 1/4000) in comparison with other antisera for small molecules.[23-24] The highest titer of an antiserum was obtained from the rabbit immunized with TC-AB-BSA, whereas the lowest titer was from TC-F-BSA. It should be noted that the titers of all antisera from the rabbits immunized with TC-F-BSA were below 1/1000. The affinities of the majority of the antisera to free TC, expressed as IC_{50} values, were also relatively low. The best antiserum, TC-AB-BSA-2, yielded the lowest IC_{50} value of 40.7 ng mL^{-1}, and all other antisera exhibited IC_{50} values ranging from 106.4 to 1576.7 ng mL^{-1}. In the group of rabbits immunized with TC-F-BSA, only antiserum-3 exhibited affinity to TC, and the other antisera yielded not only low titers but also no affinity. Although the immunogen TC-AA-BSA resulted in three antisera with observed affinities, the lowest IC_{50} value of any of these antisera was 154.9 ng mL^{-1}. These results indicate that all three TC immunogens induced weak immune responses and that the immunogen TC-AB-BSA, which contained a longer spacer arm, was the best one among them. We conclude that the low chemical stability of TC may contribute to the low immunocompetence of the immunogens. Because the antiserum-2 from TC-AB-BSA exhibited the highest titer and affinity, it was selected for further assay development and assay optimization.

Optimization of the ELISA

Heterologous coating antigens usually provide weaker recognition of the antibody to the coating antigen compared to homogenous coating antigens, thus allowing the competitor to compete at lower concentrations with the heterologous coating antigen and higher sensitivities to be obtained.[25-26] First, we evaluated the effect of heterologous coating antigens on the sensitivity of the ELISA for TC. It can be observed in Fig. 2 that the two heterologous coating antigens, TC-AA-OVA and TC-F-OVA, both significantly improved the ELISA sensitivity. TC-AA-OVA, which differed from TC-AB-BSA in its spacer arm, improved the sensitivity of the ELISA approximately 5.5-fold, providing an IC_{50} value of 7.42 ng mL^{-1}. TC-F-OVA, which differed in conjugation site from TC-AB-BSA, surprisingly resulted in an IC_{50} value as low as 1.95 ng mL^{-1}, which was an improvement of more than 20-fold compared to the homologous coating antigen TC-AB-BSA, which had an IC_{50} value of 40.7 ng mL^{-1}. Hence, the antiserum pair TC-AB-BSA-2 and TC-F-OVA was selected for the optimization of the physicochemical conditions.

A plot of the ratio of IC_{50}/A_{max} as a function of the physicochemical conditions is presented in Fig. S1A, including the pH value and the concentrations of Triton, salt and $Ca2^{+}$. It can be observed that the IC_{50}/A_{max} ratio dramatically changed as the pH value increased, and the best IC_{50}/A_{max} ratio occurred at a pH value of 7.4. Therefore, a pH of 7.4 was chosen for the next optimization step. The effect of Triton on the assay performance is illustrated in Fig. S1B. Concentrations of Triton ranging from 0 to 0.2% were tested. The OD_{max} and IC_{50} values changed gradually as the Triton concentration increased. In this study, 0.02% was chosen to achieve optimal values of IC_{50} and OD_{max}, and the IC_{50}/OD_{max} ratio was lowest at this Triton concentration. In addition, slight differences in the Triton concentration did not severely affect the OD_{max}, unlike the strong influence of the pH value.

Using the optimized Triton content and pH, the effect of the salt concentration (NaCl) on both the OD_{max} and the IC_{50} was studied (Fig. S1C). The results demonstrated that concentrations of NaCl between 0 and 0.5 M had obvious adverse effects on the OD_{max} and IC_{50} values. As the NaCl concentration increased, the OD_{max} and the IC_{50} values both dramatically decreased. The best IC_{50}/A_{max} ratio was achieved at a NaCl concentration of 0.15 M. We also evaluated the effect of Ca^{2+} in the assay because TC can form chelate complexes with various metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, etc.) that are frequently found in food samples. The results indicated that the

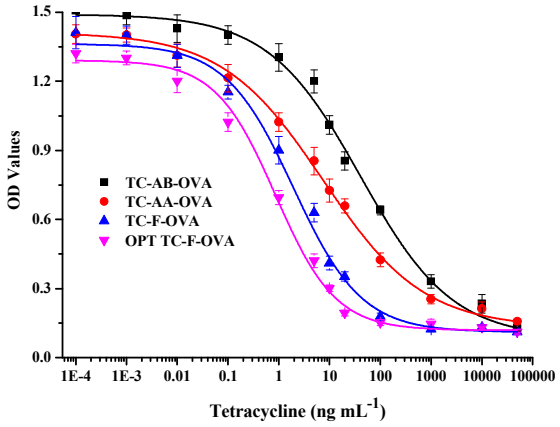

Fig. 2 Standard curves for the ELISA based on the antiserum TC-AB-BSA-2 using heterologous and homologous coating haptens for the determination of TC in buffer and milk samples. The standard curves were prepared using three well replicates.

Table 1 Characterization of the antisera and their homologous coating haptens used for the ELISA[a]

immunogens	δ-hapten [b]	rabbit no. 1			rabbit no. 2			rabbit no. 3			rabbit no. 4		
		Ab[c]	Ag[c]	IC_{50}[d]	Ab	Ag	IC_{50}	Ab	Ag	IC_{50}	Ab	Ag	IC_{50}
TC-AB-BSA	7.85	2	1	106.4±8.9	4	2	40.7±5.1	0.5	0.5	458±19.7	0.8	0.5	338±15.7
TC-AA-BSA	6.24	3	0.8	1576.7±35.1	0.8	1	375.3±10.5	1	1	ni	1	2	154.9±13.2
TC-F-BSA	9.10	1	0.5	ni[e]	1	1	ni	1	0.5	182.4±7.	0.5	0.5	ni

[a] Assay parameters represent the average of three independent assays. [b] Analyses were performed using MALDI-TOF MS. [c] Dilution factors of antisera and coating antigen are provided as values×10^3. [d] Sensitivity using tetracycline as the competitor. [e] Indicates no inhibition

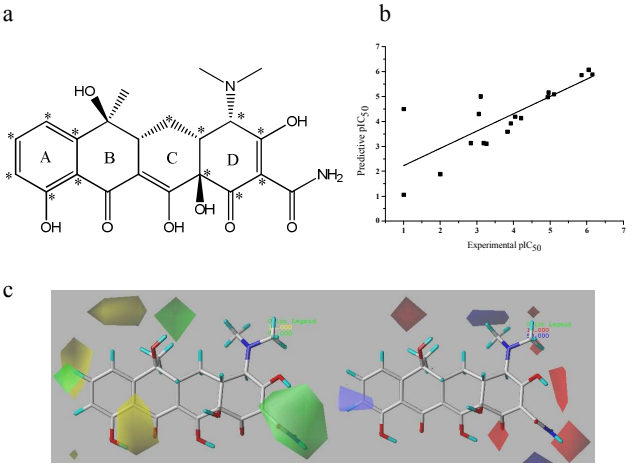

Fig. 3 (a) The molecule used as the template for molecule alignment. The atoms indicated by an asterisk are the core for alignment. (b) The contour plots of the CoMFA steric and electrostatic fields. Green contours indicate regions where bulky groups increased antibody affinity, and yellow contours indicate regions where bulky groups decreased antibody affinity. Blue contours indicate regions where positively charged groups increased antibody affinity, and red contours indicate regions where negatively charged groups increased antibody affinity. (c) Plot of experimental versus predictive affinity values derived from the CoMFA.

performance of the ELISA deteriorated if even 0.5 mM Ca^{2+} was present in the buffer (Fig. S1D), implying that the presence of metal ions should be avoided as much as possible.

The parameters of the ELISA before and after optimization are provided in Table 2. The IC_{50} value and LOD of the optimized ELISA were 0.7 and 0.047 ng mL^{-1}, respectively. The IC_{50} value was improved nearly 3-fold after the optimization procedure was performed.

When the results obtained in this work are compared with those achieved by other authors, it can be observed that the ELISA we developed for TC offered significant advantages in terms of sensitivity.[13, 17, 20] The IC_{50} values of the ELISAs developed by Pastor-Navarro *et al.*, Zhang *et al.* and Burkin *et al.* were 6.52, 3920 and 2.1 ng mL^{-1}.

Sample preparation

To evaluate the effect of the milk matrix on the performance of the optimized ELISA, a standard curve generated in PBS was compared with curves obtained in raw milk diluted at several dilution factors. The results revealed that the milk samples could not be directly analyzed (Fig. S2). A dilution factor of 100 was necessary toeliminate the matrix effect of the milk, which placed the LOD at 2.89 μg L^{-1}, still far over the MRL of TC in milk (Table 2). To assess the accuracy and precision of the ELISA method, a spike-and-recovery analysis was conducted. The milk samples were spiked with known amounts of TC at 50, 100 and 200 μg L^{-1} and then assayed using the proposed ELISA. Before being submitted to the ELISA, the raw milk was diluted 50-fold and 100-fold with buffer. The results of the accuracy and precision tests are listed in Table 3. When the samples were diluted 100-fold, the recoveries ranged from 84.9 to 111.8%, with an inter-assay coefficient of variation (CV) in the range of 7.62-12.5% and an intra-assay CV of 9.34-14.5%. Meanwhile, recoveries of 88.2-133.4% were obtained in the case of the 50-fold-diluted milk, with intra-assay and inter-assay CVs of 7.4-13.2% and 8.4-15.2%, respectively (Table S1). According to the standard set by the European Commission, the MRLs for TCs in milk are between 100 and 1000 μg kg^{-1}, the recoveries should be in the range of 80 to 110%, and the intra- and inter-assay CVs should be no more than 15%. Thus, the accuracy and precision of the ELISA were acceptable when the raw milk was diluted 100-fold prior to analysis.

Cross-reactivities and CoMFA Study

The specificity of the optimized ELISA was assessed using 18 TC analogs (Fig. 1). The results for the IC_{50} values and cross-reactivities are presented in Table 3. It can be observed that the ELISA could detect at least 8 TCs with IC_{50} values of less than 100 ng mL^{-1} (0.7-91.9 ng mL^{-1}). Thus, the antisera employed in the ELISA could theoretically be used to develop a generic immunoassay for the qualitative detection of the most commonly used TCs. However, there were only two analogs, 4-epitetracycline and rolitetracycline, that provided significant cross-reactivities of 50% and 77.8%, indicating that the antisera was unable to establish a qualitative assay for TCs.

The cross-reactivities of the ELISA performed in this work were clearly different than those presented in previous reports.[13-15, 18, 19] Because we tested 18 TC analogs, explaining the recognition profile of the antisera by analyzing only the 2D structure of TCs was likely to provide only qualitative results. Thus, there was considerable interest in understanding the antibody-TC recognition at the 3D level and in a quantitative manner. Fortunately, CoMFA can provide electronic and conformational information regarding antibody

Table 2 Parameters for three different ELISA assays for the detection of tetracycline in buffer and milk samples

parameter	ELISA in buffer	optimized ELISA in milk	optimized ELISA in milk
OD_{max}	1.412±0.07	1.321±0.04	1.289±0.0014
OD_{min}	0.112±0.011	0.117±0.01	0.154±0.017
slope	0.59±0.07	0.62±0.13	0.48±0.09
detection range (IC_{20}-IC_{80}) (ng mL^{-1})	0.186±0.11-20.86±2.12	0.08±0.01-7.21±0.36	7.12±6.23-659.4±23.2
IC_{50} (ng mL^{-1})	1.97±0.11	0.7±0.04	90.8±0.11
dilution factor	-	-	100
LOD (ng mL^{-1})	0.02±0.13	0.047±0.032	2.89±0.14
R^2	0.994±0.006	0.996±0.004	0.996±0.009

Table 3 Experimental and predicted IC_{50} values and experimental cross-reactivities of the optimized ELISA for TCs

tetracyclines	TC-AB-BSA-2/TC-F-OVA			
	IC_{50} (ng mL^{-1})	pIC_{50} $M\times10^{-9}$ (exp.)	pIC_{50} $M\times10^{-9}$ (pre.)	CR
tetracycline	0.7	6.15	5.89	100%
chlortetracycline	7.9	5.10	5.10	8.9%
oxytetracycline	11.8	4.93	4.98	5.9%
doxycycline	63	4.20	4.14	1.1%
anhydrotetracycline	149	3.83	3.59	0.5%
4-epitetracycline	1.4	5.85	5.87	50%
4-hydroepitetracycline	555	3.26	3.11	0.1%
4-epichlortetracycline	666	3.18	3.14	0.1%
4-epioxytetracycliner	11.1	4.95	5.17	6.3%
4-epidoxycycline*	900	3.05	4.31	<0.1%
rolitetracycline	0.9	6.05	6.08	77.8%
metacycline*	800	3.10	5.01	<0.1%
sancycline	91.9	4.04	4.20	0.8%
minocycline*	$>1\times10^5$	1	4.51	<0.1%
amicycline	1473.5	2.83	3.14	<0.1%
tigecycline	$>1\times10^5$	1	1.06	<0.1%
demeclocycline	120	3.92	3.93	0.6%
meclocycline	$>1\times10^4$	2	1.89	>0.1%

[a] The values represent the mean of three separate experiments, each of which contained a minimum of three replicates. [b] $pIC_{50} = -\mathrm{Log}IC_{50}$
*Test set; the other TCs are the training set.

recognition by generating steric and electrostatic contour maps of the region around the molecule with respect to changes in affinity.[27] This technique is a powerful tool for understanding the cross-reactivities of immunoassays when many homologues are detected, as in the case of TCs. Several papers have described the use of CoMFA to study the binding interactions between small molecules and antibodie.[28-29] Our group has previously reported the use of CoMFA to investigate the binding of sulfonamides and fluoroquinolones to antibodies.[30-31]

In this study, we used TC as a template to align the TC analogs, and the atoms indicated by an asterisk in Fig. 3a were used to align the backbone. The CoMFA model was then established, demonstrating an acceptable predictive ability with a cross-validation q^2 value of 0.510 and a non-cross-validated r^2 value of 0.989. The results demonstrated that a quantitative structure–activity relationship (QSAR) did exist between the TC analogs and the antiserum affinity. The contributions of the steric and electrostatic fields to the affinity were 57.6 and 42.4%, respectively, indicating that the steric factor played a more important role in the antibody affinity for the TCs than did the electrostatic factor. Fig. 3b features the steric and electrostatic contour plots from the CoMFA analysis. Higher affinities are correlated with more bulk near the green contours, less bulk near the yellow contours, more positive charges near the blue contours and more negative charges near the red contours. The antibody affinity was restricted by the presence of bulky groups in the region of the yellow contours, which were near the D ring of the TCs, whereas bulky groups near the green contours located near the A ring were not detrimental to the antibody affinity. Table 3 lists the experimental binding affinities and the predicted binding affinities from the CoMFA. Fig. 3c illustrates the correlation between the experimental affinities and the predicted affinities, which exhibits acceptable agreement (r^2=0.61), implying that the CoMFA analysis was a robust and useful tool for the investigation of the recognition mechanism of the antibody with respect to the haptens. This CoMFA study also demonstrated that the use of a conjugation site in TC at position 2 was a successful strategy for generating specific and sensitive antibodies for TC.

Experimental

Materials

All TC standards, bovine serum albumin (BSA), ovalbumin (OVA), 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), 4-aminobenzoic acid, aminoacetic acid, formaldehyde and Freund's complete and incomplete adjuvants were obtained from Dr. Ehrenstorfer (Augsburg, Germany) or from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was provided by Jackson ImmunoResearch (West Grove, PA, USA). White, opaque 96-well polystyrene microtiter plates were purchased from Costar Inc. (Milpitas, CA, USA). A SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used in this study.

Synthesis of the haptens

Hapten TC-AB. Selected amounts of TC (1.80 g, 4.1 mM) and 4-aminobutyric acid (0.45 g, 4.4 mM) were dissolved in 20 mL of ethyl alcohol that contained 400 μL of acetic acid. After 600 μL of 37% formaldehyde was added, the reaction was stirred at room temperature for 12 h. The mixture was recrystallized using ethyl acetate. A white crystal was obtained (0.67 g, 31%): ^{1}H NMR (500 MHz, DMSO-d6) δ1.34-1.78 (m, 7H), 1.88-1.96 (s, 2H), 2.34-2.45

(s, 2H), 2.48 (s, 6H), 3.49 (s, 2H), 3.76 (s,2H), 6.86 (d, 1H), 7.07 (d, 1H), 7.50 (d, 1H).

Hapten TC-AA. For the synthesis of TC-AA, the same procedure employed for hapten TC-AB was followed. In brief, 4.0 mM TC, 4.5 mM aminoacetic acid and 600 μL of 37% formaldehyde were mixed. A white crystal was obtained (0.51 g, 24%): ^{1}H NMR (500 MHz, DMSO-d_6) δ1.46 (s, 3H, CH_3), 1.47-1.78 (m, 2H), 2.48 (s, 6H), 2.73 (m, 2H), 3.49 (s, 2H), 6.99 (d, 1H), 7.27 (d, 1H), 7.52 (d, 1H).

Preparation of the protein-hapten conjugates

The haptens TC-AB and TC-AA were conjugated to BSA and OVA using as an immunogen and a coating antigen by using the activated ester method through their carboxylic acid moieties. In brief, the hapten (20 mg) was dissolved in 2 mL of dimethylformamide, and NHS (20 mg) and EDC (20 mg) were then added to the hapten solution, which was then stirred at room temperature overnight. BSA or OVA (30 mg) was dissolved in 5 mL of carbonate buffer and added drop-wise to the active NHS solution under continuous stirring, and the solution was then further stirred at room temperature for 4 h. The conjugates were dialyzed against PBS (pH 7.0) at 4 °C for 72 h.

In addition, TC was conjugated to BSA as an immunogen and to OVA as a coating antigen using a formaldehyde method in accordance with Burkin and Galvidis.[13] In brief, the TC (20 mg) was dissolved in water (1 mL) and added to a solution that contained the desired protein (30 mg) in 2 mL of water. To this solution, 3 M sodium acetate (1 mL, pH 5.5) and 37% (w/v) formaldehyde (0.4 mL) were added. The reaction mixture was stirred for 2 h at room temperature. The conjugates were dialyzed against PBS (pH 7.0) at 4 °C for 72 h.

The hapten densities of the BSA conjugates were calculated using MALDI-TOF MS by comparing the molecular weight of native BSA to that of the conjugates.

Antibody production

Twelve rabbits were used to produce antisera. The immunogen (0.5 mg) for each rabbit was dissolved in 1 mL of 0.9% NaCl and emulsified with 1 mL of Freund's complete adjuvant. This emulsion was injected intracutaneously in the first immunization. For the booster immunizations, immunogen (0.25 mg) emulsified with 0.5 mL of Freund's incomplete adjuvant was employed. The booster immunizations were repeated every 3 weeks. The rabbits were bled through an ear vein 7 days after every third booster injection. To obtain the antisera, the blood samples were stored to coagulate for 1 h at room temperature and then overnight at 37 °C, followed by centrifugation at 3500×g for 10 min. Each serum was collected, divided into aliquots and stored at -20 °C until use.

Competitive ELISA

The competitive ELISA approach can be described as follows: Microtiter plates were coated with coating antigen (100 μL per well) and then incubated at 4 °C overnight. After blocking, 50 μL of diluted TC standard solution and 50 μL of diluted antiserum were added to each well, and the plates were incubated for 30 min at 37 °C. After washing, 100 μL of diluted goat anti-rabbit IgG-HRP solution was added, and the plates were incubated for 30 min at 37 °C. The substrate solution was added after washing, and the reaction was terminated with 2 M H_2SO_4 after incubation at 37 °C for 10 min. The optical-density (OD) values were determined using a microplate reader in the dual-wavelength mode (450 nm for the test sample and 630 nm for the reference).

Competition curves were fitted using the following four-parameter logistic equation:

$$Y = (A–B)/[1+(X/C)^D]+B$$

where A is the response at high asymptotes of the curve, B is the response at low asymptotes of the curve, C is the concentration of the analyte that results in 50% inhibition, D is the slope at the inflection point of the sigmoid and X is the calibration concentration.

The titer in the antiserum screening procedure was defined based on the selected dilutions of antisera, providing an absorbance of approximately 1.0-1.5 when the competitor was absent.

ELISA optimization

The effects of the heterologous coating antigen, the pH values, the salt concentration, the Triton concentration and the Ca^{2+} concentration were evaluated sequentially to improve the sensitivity of the ELISA. The criteria used to evaluate the ELISA performance were the IC_{50} value, the OD_{max} and the ratio of IC_{50}/OD_{max}.

Sample analysis

TC-free milk samples were supplied by the Veterinary Drug Safety Inspection & Testing Center of the Ministry of Agriculture (Beijing, China). Before analysis, the milk samples were centrifuged at 10,000×g for 10 min to remove fat.

Matrix effect. The milk samples were serially diluted with the assay buffer and used to prepare standard curves. The parallelism of the sigmoidal curves was compared to that of the curve prepared using the assay buffer to evaluate the extent of the interference caused by the milk matrix.

Recovery study. Aliquots of milk (1 mL) were fortified with 1000 μg L^{-1} TC standard solution to yield levels of 50, 100 and 200 μg L^{-1} of TC. After 100-fold dilution of the milk, 50 μL of the supernatants was used in the ELISA assay.

Cross-reactivity and CoMFA

*Cross-reactivity***.** To determine the specificity of the optimized ELISA, a cross-reactivity study was conducted. The cross-reactivity of the polyclonal antibody for TC and 17 other analogs was calculated using the following equation:

Cross-reactivity (%) = (IC_{50} of TC)/(IC_{50} of other analytes)×100%

CoMFA. The CoMFA calculation was performed as described in our previous report.[31] In brief, the minimum-energy conformations of all TCs were calculated using the Tripos force field, and the atomic point charges were calculated using the Gasteiger-Hückel method. Minimizations were achieved using the Powell method for the maximum 1000 steps until the root mean square (RMS) of the gradient became less than 0.005 kcal/(mol Å). TC was selected as the template molecule to align the other analogs. The regression analysis was performed using the partial least-squares (PLS) method. Regression analysis was performed using the leave-one-out method. The optimum number of components to be used in the conventional analysis was defined as that which yielded the highest cross-validated q^2.

Acknowledgments

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Notes and references

1 A. Önal, Food Chem., 2011, **127**, 197.
2 J. W. Cheng, S. L.Charland, L. M. Shaw, S.Kobrin, S.Goldfarb, E. J.Stanek & S. A.Spinler, *Pharmacotherapy,* 1997, **17**, 584.
3 J. Hu, J. Shi, H. Chang, D. Li, M. Yang, Y. Kamagata, *Environ. Sci. Technol.,* 2008, **42**, 3415.
4 C. E. Tsai & F. Kondo, *Res. Vet. Sci.,* 1994, **56**, 277.
5 N. Wu, M. Qiao, B. Zhang, W. D. Cheng & Y. G. Zhu, *Environ. Sci.Technol.,* 2010, **44**, 6933.
6 European Commission, *Off. J. Eur. Union,* 2002, **L221**, 8.
7 Food and Drug Administration, 2004, Title 21-Food and Drugs, PART 556.
8 Regulation No. 235 Ministry of Agricuture of the People's Republic of China, 2002.
9 W. C. Andersen, J. E. Roybal, S. A. Gonzales, S. B. Turnipseed, A. P. Pfenning & L. R. Kuck, *Anal. Chim. Acta,* 2005, **529**, 145.
10 G. T. Peres, S. Rath & F. G. R. Reyes, *Food Control,* 2010, **21**, 620.
11 J. Kurittu, S. Lonnberg, M. Virta & M. Karp, *J. Agric. Food Chem.,* 2000, **48**, 3372.
12 W. Jiang, Z. Wang, G. Nölke, J. Zhang, L. Niu & J. Shen, *Food Anal. Methods,* 2012, **6**, 764.
13 M. A. Burkin & I. A. Galvidis, *Food Agric. Immunol.,* 2009, **20**, 245.
14 F.Gao, G. X. Zhao, H. C.Zhang, P.Wang & J. P. Wang, *J. Environ. Sci. Health B,* 2013, **48**, 92.
15 J. Adrian, F. Fernández, F. Sánchez-Baeza & M. P. Marco, *J. Agric. Food Chem.,* 2012, **60**, 3837.
16 M. Jeon & I. Rhee Paeng, *Anal. Chim. Acta,* 2008, **626**, 180.
17 N. Pastor-Navarro, A. Maquieira & R. Puchades, *Anal. Bioanal. Chem.,* 2009, **395**, 907.
18 B. A. Faraj & F. M. Ali, *J. Pharmacol. Exp. Ther.,* 1981, **217**, 10.
19 N. Pastor-Navarro, S. Morais, A. Maquieira & R. Puchades, *Anal. Chim. Acta,* 2007, **594**, 211.
20 Y. Zhang, S. Lu, W. Liu, C. Zhao & R. Xi, *J. Agric. Food Chem.,* 2007, **55**, 211.
21 J. P. Goldring, C. Thobakgale, T. Hiltunen & T. H. Coetzer, *Immunol. Invest.,* 2005, **34**, 101.
22 C. B. Zhao, D. P. Peng, Y. L. Wang, L. L. Huang, D. M. Chen, Y. F. Tao & Z. H. Yuan, *Food Agric. Immunol.,* 2008, **19**, 163.
23 Z. Wang, N. Li, S. Zhang, H. Zhang, Y. Sheng & J. Shen, *Food Addit. Contam.,* 2013, **30**, 244.
24 C. Suarez-Pantaleon, J. V. Mercader, C. Agullo, A. Abad-Somovilla & A. Abad-Fuentes, *J. Agric. Food Chem.,* 2010, **58**, 8502.
25 Z. Wang, J. Zhang, S. Zhang & J. Shen, *Food Agric. Immunol.,* 2011, **22**, 1115.
26 P. Cliquet, E. Cox, W. Haasnoot, E. Schacht & B. M. Goddeeris, *J. Agric. Food Chem.,* 2003, **51**, 5835.
27 S. Paula, M. R. Tabet, S. M. Keenan, W. J. Welsh, , & W. J. Jr. Ball, *J. Mol. Biol.,* 2003, **325**, 515.
28 S. Paula, M. R. Tabet, C. D. Farr, A. B. Norman & W. J. Jr. Ball *J. Med. Chem.,* 2004, **47**, 133.
29 Y. F. Zhang, Y. Ma, Z. X. Gao & S. G. Dai, *Anal. Bioanal. Chem.,* 2010, **397**, 2551.
30 Z. Wang, Z. Kai, R. C. Beier, J. Shen & X. Yang, *Int. J. Mol. Sci.,* 2012, **13**, 6334.
31 Z. Wang, Y. Zhu, S. Ding, F. He, R. C. Beier, J. Li, H. Jiang, C. Feng, Y. Wan, S. Zhang, Z. Kai, X. Yang & J. Shen, *Anal. Chem.,* 2007, **79**, 4471.