JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Lateral Flow Colloidal Gold Immunoassay Strip for the Rapid Detection of Enrofloxacin Residues

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A rapid immunochromatographic lateral flow test strip of competitive format has been developed using a gold-conjugated monoclonal antibody for the specific determination of enrofloxacin (ENR) residues in chicken muscles. For this purpose, a specific monoclonal antibody (mAb) for ENR was generated and characterized. The mAb showed negligible cross-reactivity with other related compounds. Using ENR standards prepared in chicken muscle extracts from 0 to 24.3 ng/mL (μg/kg), the method indicated that the detection limit of the test strip, as measured in a strip scanner, was as low as 0.138 μg/kg of ENR and the half-maximal inhibition concentration (IC_{50}) was 0.935 μg/kg. For samples spiked at 10, 20, and 30 μg/kg, the recovery was between 85.3 and 96.1% and the coefficient of variation [CV (%)] was between 4.5 and 7.91%. Parallel analysis of muscle samples from chickens fed ENR showed good comparable results obtained from the test strip and LC-MS. Each test requires 5−10 min. The data indicate that the method has high sensitivity, specificity, and the advantages of simplicity and speed of performance. Therefore, the test strip provides a useful screening method for quantitative, semiquantitative, or qualitative detection of ENR residues in chicken muscles.

KEYWORDS: Enrofloxacin; test strip; colloidal gold; immunoassay; rapid test

INTRODUCTION

Fluoroquinolones are bactericidal antibiotics that have been increasingly used in veterinary medicine to treat microbial infections. Enrofloxacin (ENR) is a synthetic antibacterial agent that belongs to the fluoroquinolone group. They exert their bactericidal effects by inhibiting DNA gyrase within susceptible bacteria (*1*). Enrofloxacin residues in edible animal tissues raise concerns for public health. Cooking procedures do not affect enrofloxacin residues, which remain stable during heating (*2*). Human infections with fluoroquinolone-resistant *Campylobacter* species associated with the consumption of poultry have become increasingly common (*3*−*5*). Because of concerns about drug residues entering the food chain and contributing to bacterial resistance, the European Commission has established the maximum residue limit (MRL) for drugs employed in veterinary medicine. The MRL has been set at 30 μg/kg for the sum of enrofloxacin and its active metabolite (ciprofloxacin) for muscle tissue (*6, 7*).

So far, classical analytical methods have been described for the detection of enrofloxacin in tissues, including high-performance liquid chromatography (HPLC) (*8*−*10*), liquid chromatography−mass spectrometry (LC-MS) (*11*), and LC-MS/MS (*12, 13*). These methods require extensive sample preparation as well as highly trained individuals to operate sophisticated instruments and interpret complicated chromatograms or spectral results. Consequently, these traditional methods, although highly accurate, are time-consuming, costly, and generally not suitable for use in the field (*14*).

To test samples for the presence of these substances under working conditions, rapid and inexpensive screening methods are required. An enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody (mAb) has been successfully developed for the detection of enrofloxacin residues (*7, 15*−*17*). Compared with the ELISA, a one-step strip test has its advantages, such as all of the reagents are included in the strip and results can be obtained within 5−10 min (*18*−*23*). An immunochromatography assay for ofloxacin detection has been described by Sun et al. (*24*), and an immunochromatography strip for the detection of 12 fluoroquinolones was described by Zhu et al. (*25*). However, this paper is different from the above two papers, the aim of this study being to develop an

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10.1021/jf802648z CCC: $40.75
Published on Web 11/18/2008

immunochromatographic lateral-flow test strip for the detectionof enrofloxacin in chicken muscles. The test strip was rapid, simple, and effective and was shown to be suitable for the rapid detection of ENR residues.

MATERIALS AND METHODS

Chemicals and Materials. Enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, norfloxacin, sulfadiazine, chloramphenicol, streptomycin, gentamycin, ampicillin, gold(III) chloride trihydrate, and a mouse monoclonal antibody isotyping kit were purchased from Sigma. BSA and OVA were bought from BDH (VWR International Ltd.), and goat anti-mouse IgG antibody (whole molecule) was from Sino-American Biotechnology Co. (Luoyang, China). FCA, FIA, and EDC were purchased from Pierce, and PEG1500 was from Roche (Mannheim, Germany). Ninety-six-well culture plates were bought from Nunc. RPMI-1640, HAT, and HT medium were purchased from Invitrogen. Protein G affinity columns were bought from Amersham Biosciences. Nitrocellulose membrane, glass fiber, and absorbent pad were purchased from Millipore. Eight-week-old female BALB/c mice were obtained from the Laboratory Animal Center, Zhengzhou University, China, and raised under strictly controlled conditions. Other reagents and solvents were of analytical grade or higher.

Apparatus. Microplate Readers 450/550 were from Bio-Rad (Richmond, CA). Milli-Q water was obtained from Millipore (Bedford, MA). An XYZ Biostrip Dispenser, CM 4000 Cutter, and TSR3000 membrane strip reader were purchased from Bio-Dot.

Preparation of Immunogens and Coating Antigens. BSA−ENR and OVA−ENR conjugates were made using two methods based on the mixed anhydride reaction described by Bucknall et al. (*16*) and an active ester method by Tijissen (*26*), Hammer and Heeschen (*27*), and Watanabe et al. (*7*). The conjugates were purified by dialysis against 2 L of 0.01 M PBS (pH 7.2), with six changes of PBS to remove the uncoupled free hapten and then lyophilized.

Production of mAb Against ENR. *Immunization of Mice.* Twenty BALB/c female mice (8 weeks old) were immunized with ENR−BSA conjugates. The first dose consisted of 50 μg of immunogen for injection subcutaneously as an emulsion of PBS and Freund's complete adjuvant. Three subsequent injections were given at 3-week intervals with the same dosage of immunogen emulsified in Freund's incomplete adjuvant. Antisera were collected 4 weeks after the fourth immunization and were screened for anti-ENR activity by ciELISA. The mouse showing the highest anti-ENR activity received a fifth injection intraperitoneally (ip). Three days later, the spleen of the injected mouse was removed for hybridoma production.

Cell Fusion and Hybridoma Screening. Cell fusion procedures were carried out according to the procedure described by Köhler and Milstein (*28*) and Zhang et al. (*20*). Briefly, the spleen of the immunized mouse was removed, and the splenocytes were isolated and fused with NS0 cells using PEG1500. The fused cells were then distributed into 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium. Ten days after fusion, supernatants of hybridoma colonies were recovered and screened by indirect ELISA for secretion of mAb binding to ENR. Selected clones were subcloned by limiting dilution. Ascites fluids were produced in paraffin-primed BALB/c mice. The mAbs were purified from ascites using a protein G affinity column. The subclass of the isotypes of the purified antibody was determined by using a mouse monoclonal antibody isotyping kit. Measurement of monoclonal antibody affinity (K_a) was carried out according to the procedure described by Batty et al. (*29*).

Preparation of Colloidal Gold. Colloidal gold with a mean particle diameter of 15 nm was produced by reduction of gold chloride with 1% sodium citrate as described previously by Hayat (*30*) and Zhang et al. (*18, 20*). Briefly, 50 mL of 0.01% gold chloride trihydrate solution in superpurified water was heated to boiling, and then 1.5 mL of 1% sodium citrate solution was added with stirring. Gradually, the color changed from light yellow to brilliant red. After the color change, the solution was boiled for another 5 min to complete the reduction of the gold chloride, cooled, and stored at room temperature with 0.05% of added sodium azide.

Preparation of Colloidal Gold Labeled mAb. The colloidal gold labeled anti-ENR mAb was prepared according to the method of Yokota (*31*). Briefly, colloidal gold solution was adjusted to pH 9.0 with 0.2 mol/L sodium carbonate. The optimum protein concentration for labeling was determined by the following steps: 25 μL of anti-ENR mAb solution was 2-fold serially diluted in DDW, and then 25 μL of colloidal gold solution was added. Mixtures were incubated for 15 min at room temperature, and then 100 μL of 10% NaCl solution was added. The color of samples changes from brilliant red to blue as the concentration of mAb decreases. The optimum concentration of mAb for colloidal gold labeling was the lowest concentration of mAb solution that did not change color.

Two milliliters of mAb solution, at the optimum concentration of 2 μg/mL, was incubated with 10 mL of colloidal gold solution (pH 9.0) for 30 min at room temperature. After the addition of 1 mL of 10% BSA solution in 20 mmol/L sodium borate (pH 9.0), the mixture was incubated at room temperature for another 10 min, and the labeled mAb washed by repeated centrifugation (25000*g*) at 10 °C for 30 min with 20 mmol/L sodium borate (pH 9.0) containing 1% BSA and 0.1% sodium azide. The precipitate was resuspended in the washing buffer and stored at 4 °C for use.

Preparation of the Conjugate Pad. Conjugate solution was prepared by dilution of the colloidal gold labeled mAb to ENR with 20 mmol/L sodium borate buffer (pH 8.0) containing 8.75% (w/v) sucrose, 8.75% (w/v) BSA, 0.6 mol/L NaCl, 10 mmol/L EDTA, and 0.1% (w/v) NaN_3 to a final concentration of 2 μg/mL. A conjugate pad was made by dipping a 7 × 300 mm glass fiber (Millipore) in the conjugate solution and then drying for 1 h at 56 °C. The pad was stored in a desiccator at room temperature.

Immobilization of Capture Reagents. The ENR−BSA (1 mg/mL) and goat anti-mouse IgG (1 mg/mL) were applied to the nitrocellulose membrane by dispenser as the test and control lines, respectively. The test and control lines were situated 0.5 cm apart at the center of the membrane. These reagents were applied in the form of dots at 50 dots mL^{-1} cm^{-1} on the membrane. After drying for 1 h at 40°, the membrane was blocked with 2% (w/v) BSA and then dried, sealed, and stored under dry conditions.

Preparation of Sample Pad and Absorbent Pad. Sample and absorbent pads of C048 (Millipore) were made from nonwoven, 100% pure cellulose fiber. The sample pad was cut to 15 × 300 mm and saturated with a buffer (pH 8.0) containing 20 mmol/L sodium borate, 2.0% (w/v) sucrose, 2.0% (w/v) BSA, and 0.1% (w/v) NaN_3 and then dried and stored as described above. The absorbent pad was cut to 40 × 300 mm.

Assembly of the Strip. The strip assembly procedure was similar to that described by Zhang et al. (*18, 20*). The sample pad, conjugate pad, blotted membrane, and absorption pad were assembled on the plastic backing support board sequentially with a 1−2 mm overlap and covered by color film at both ends. The master card was cut to 3 mm width strips using a CM 4000 Cutter (Bio-Dot). Strips were then sealed in a plastic bag in the presence of desiccant gel and stored at 4 °C.

Test Procedure and Principle. Eighty microliters of standard solution or sample extract was added onto the sample pad, and the solution migrated toward the absorbent pad; a result could be seen after 5 min. When ENR was absent from the sample, all of the detection reagent would be trapped by capture reagent to form an easily visible test line. When ENR is present in the sample, it competes with the immobilized capture reagent for the limited amount of detection reagent. The more ENR present in the sample, the weaker the test line color. If sufficient ENR is present in the sample, it will completely block the reaction with the capture reagent; thus, there is no visible test line on the nitrocellulose membrane. Provided that the test strip and the test procedure are correct, the control line is always visible. If no colored capture line or only a red line at the test line appears, the testing procedure was likely improper or the strip invalid, and the test should be repeated using a new strip.

Preparation of Spiked Chicken Muscle Samples. A standard stock of enrofloxacin was prepared by diluting the initial solution prepared in HCl (0.03 mol/L) to give a final stock solution at 1 mg/mL. The

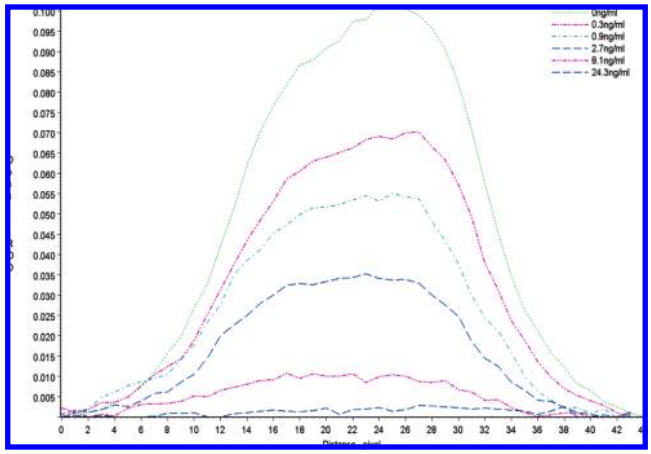

Figure 1. Relative optical density (ROD) curves of standard samples. Standard ENR samples at 0, 0.3, 0.9, 2.7, 8.1, and 24.3 ng/mL were tested using the test strips. Test lines were scanned with a TSR3000 membrane strip reader.

Table 1. G/Peak and G/D × Area of the Relative Optical Density (ROD) of Test Lines of the Standard ENR Samples[a]

ENR concn (ng/mL)	G/D × area-ROD (pixel)	G/peak-ROD (pixel)
0	118.2183	0.1011
0.3	81.2751	0.0701
0.9	59.3692	0.055
2.7	41.1353	0.0352
8.1	12.7417	0.0107
24.3	2.8493	0.0028

[a] Standard ENR samples were tested using the test strips and the test lines scanned with a TSR3000 membrane strip reader. G/D × area, mean density value of the sampled line points multiplied by area of the sampling window on the image. G/peak, peak value of the ROD points of the scanned line.

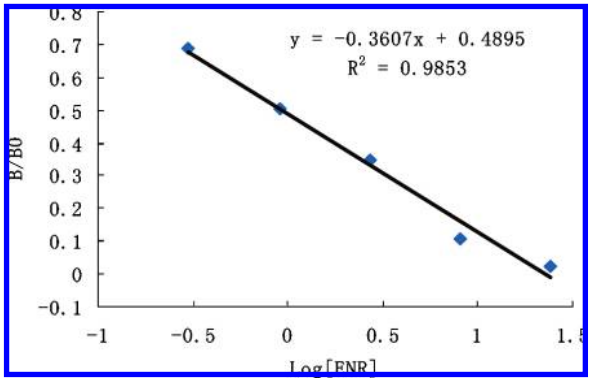

Figure 2. Standard curve for ENR using test strip detection. The *X*-axis is expressed as log concentration. B/B_0 represents the percentage of relative optical density (ROD) of standards divided by that of the ROD at 0 ng/mL. The linear regression correlation coefficient (R^2) is 0.9853, and the IC_{50} was calculated as 0.935 ng/mL.

stock solution was serially diluted with PBS to give the working standard solutions (0, 0.3, 0.9, 2.7, 8.1, 24.3 ng/mL) to be used for spiking 1 g of normal minced chicken muscle extracts, respectively.

Sample Pretreatment for Strip Test. Briefly, 1 g of minced chicken muscle was homogenized with 1 mL of PBS (pH 7.4). The homogenates were mixed on a vortex mixer for 10 min and then centrifuged at 3000*g* for 10 min at room temperature. The supernatants were analyzed either directly or following further dilution as required by the strip test.

RESULTS AND DISCUSSION

Hapten Conjugation. Enrofloxacin is a small molecule with a molecular weight of 359.40. To prepare the immunogen and

Figure 3. Standard ENR samples were tested using the test strips and the test lines with the naked eye.

the coating antigen, this molecule was conjugated to a protein carrier molecule. BSA and OVA were used as carriers. Enrofloxacin was coupled via its carboxyl group to the free amino groups of the protein carriers, resulting in the immunogen (BSA−ENR) and the coating antigen (OVA−ENR). By ultraviolet absorbance spectral curve and SDS-PAGE electrophoresis, hapten enrofloxacin was coupled successfully to carrier protein, The conjugation ratio by mixed anhydride method is 1:29 and by active ester method is 1:16. To immunize BALB/c mice using different immunogens, the titer and inhibition level of antisera were also different. Although all of the mice produced titer anti-ENR, the mouse immunized with immunogen (BSA−ENR) by active ester method and the coating antigen (OVA−ENR) by mixed anhydride method showed higher titer (1:51200) and lower IC_{50} values (11 ng/mL); thus, it was chosen for subsequent experiments to cell fusion for the monoclonal antibody preparation. Optimum hapten−carrier molecular ratio in artificial antigens is helpful to improve immune response, but the optimum ratio was a controversial problem. Wust thought the higher ratio was better (*32*), Schneider thought the optimum ratio was 10−20:1 (*33*), and Eilange thought the optimum ratio was 5−25:1 (*34*). In this research the conjugation ratios of ENR to BSA were 29:1 and 16:1; moreover, the ratio of 16:1 was better than 29:1 in immune response.

Establishment of Hybridoma. Spleen cells from the mice immunized with ENR−BSA were fused with NS0 myeloma cells, and the resulting hybridomas were selected in HAT medium. One or more growing hybridomas were observed in almost all wells at 7 days. The supernatants from each well were screened for antibodies against ENR by a direct ELISA using microtiter plates coated with ENR−OVA conjugate. The cells from the wells showing the strongest response (OD > 2.0) were tested again in an indirect competitive ELISA for their ability to recognize free ENR. Two selected hybridomas were screened and further cloned by limiting dilution. After culture and further screening, the hybridomas 4G1-G1 and 4G1-B3 were intraperitoneally injected into mice to produce ascites fluid (mAb).

Characterization of Monoclonal Antibody. The mAb was purified from the ascites fluid. The titers of the purified ascites fluid were 5.12×10^{-5} (clone 4G1-G1) and 1.024×10^{-6} (clone 4G1-B3). Both of these mAbs had high affinity constants (K_a) with 4.5×10^{10} L/mol (4G1-G1) and 9×10^{10} L/mol (4G1-B3). The subclass of the mAb was identified as IgG1. The antibody is highly specific for ENR, and the most sensitive hybridoma named 4G1-

Table 2. Recovery and Intra- and Interassay Precision of the Test Strips for ENR Spiked in Chicken Muscle[a]

	intra-assay			interassay		
spiked ENR (μg/kg)	mean ± SD (μg/kg)	recovery (%)	CV (%)	mean ± SD (μg/kg)	recovery (%)	CV (%)
10	9.57 ± 0.49	95.7 ± 4.90	5.12	9.61 ± 0.76	96.1 ± 7.60	7.91
20	18.08 ± 0.87	90.4 ± 4.35	4.81	17.90 ± 1.22	89.5 ± 6.10	6.82
30	26.45 ± 1.19	88.2 ± 3.97	4.50	25.60 ± 1.20	85.3 ± 4.00	4.69

[a] Extracts of chicken muscle were spiked with ENR at 10, 20, and 30 μg/kg. Intra-assay precision was estimated by using one batch of the test strips ($n = 6$). For interassay precision, three batches of the test strips were used to detect the given samples. The recovery and coefficient of variation (CV, %) are calculated from triplicate assays in all cases.

Table 3. Correlations between Test Strips[y] and LC-MS[x] of Enrofloxacin Value[a]

time (days)	test strips[y] (μg/kg)	LC-MS[x] (μg/kg)	regression eq	R^2	N
3	95.4 ± 7.5	99.35 ± 8.7	$Y = 0.9624x + 0.2134$	0.9976	6
6	35.7 ± 2.4	34.61 ± 3.1			
9	16.5 ± 0.9	18.74 ± 1.2			

[a] Chickens were given enrofloxacin at 5 mg/kg in feed for 3 days, and chicken muscle samples were collected and tested at 3, 6, and 9 days after the final feeding. The samples were analyzed using a test strip and LC-MS methods. (LC-MS provided by the Supervision and Verification Center of Ministry of Agriculture, Zhengzhou, China.).

B3 showed an IC_{50} value of 1.13 μg/L. This means that the sensitivity of mAb increased approximately 8-fold in comparison to that of the antiserum tested above. The results of cross-reactivity demonstrate that this mAb has negligible cross-reactivity with other compounds (<0.01%), that is, ciprofloxacin, danofloxacin, difloxacin, norfloxacin, sulfadiazine, chloramphenicol, streptomycin, gentamycin, and ampicillin. The sensitivity and specificity of obtained monoclonal antibodies were similar to those described by Watanabe et al. (*7*).

Sensitivity of the Test Strip. The sensitivity of the test strip was determined by measuring the responses to ENR standard samples. Test lines were scanned with a Bio-Dot TSR3000 membrane strip reader. G/peak and G/D × A (area) of the relative optical density (ROD) decreased as the ENR concentration in the standard samples increased (**Figure 1** and **Table 1**). The concentration of standard ENR and the G/D × A ROD produced a sigmoidal dose−response curve, which gave good linearity within the range of 0.038−22.75 ng/mL. The equation was $Y = -0.3607x + 0.4895$ ($R^2 = 0.9853$) (**Figure 2**). The lower detection limit (LDL) using the scanner was quantitatively defined here as the amount of ENR in the standard sample solution that gave a 20% decrease of the values produced by the blank sample. The LDL by naked eye was set at the amount of ENR causing a 50% decrease of the G/D × area as this difference was clearly visible (*18, 20*). In the present study the LDL of the test strip was calculated to be 0.138 ng/mL ($y = 80\%$) with the scanner and 0.935 ng/mL ($y = 50\%$) by eye. If no scanner is used, the LDL with the naked eye was set at the amount of ENR producing a clearly visible difference in intensity of the test strip in comparison with that when no ENR was added in the sample as a negative control, so it was about 0.9 ng/mL, and this result was basically identical with the above 0.935 ng/mL (**Figure 3**).

For the quantitative assay, the optical density of a test line can be measured with a test strip reader, and according to the regression equation from the standard curve, the level of the ENR residue can be calculated. For semiquantitative and qualitative detection, the color of the test line can be evaluated directly by visual assessment. If the color of the test line was similar to that of the negative control sample, the sample was considered to be negative. If the color of the test line was in the midst of the two definite control samples, the ENR concentration was considered to lie between the two control samples. When the concentration of the ENR residue was greater and there was essentially no color observable at the test line, the sample should be judged as overproofed.

Specificity of the Test Strips. The test strip was developed using the specific mAb (4G1-B3), and this mAb was shown not to cross-react with related compounds, as above. According to the standard curve of the test strip for ENR detection, the IC_{50} of the test strips was calculated to be 0.935 ng/mL (μg/kg), and when competitors at 10000 ng/mL (μg/kg) were tested, the color of test line is the same as that of the negative control sample. All competitors have the same %CR < 0.01. Therefore, the test strip for ENR was highly specific and showed negligible cross-reactivity to the ciprofloxacin, danofloxacin, difloxacin, norfloxacin, sulfadiazine, chloramphenicol, streptomycin, gentamycin, and ampicillin.

Recovery of ENR in Chicken Muscle Samples. To determine the accuracy, chicken muscle extracts containing 10, 20, and 30 μg/kg of ENR were tested. The test was carried out in triplicate with a single batch of test strips and the optical density of the test line measured using the test strip reader and sample values calculated from the standard curve. Recoveries were from 88.2 to 95.7%, and the highest relative standard deviation was 5.12%. For interassay reproducibility, three different batches of the test strips were used for triplicate measurements of the samples. Recoveries were from 85.3 to 96.1%. The highest relative standard deviation was 7.91%. The results are shown in **Table 2**.

Comparative Studies between Test Strips and LC-MS. To further evaluate the validity of the quantitative response of the strip, 30 experimental chickens were analyzed by strip and LC-MS. The 30 chickens were randomly divided into control and test groups. The control group ($n = 8$) was not treated with ENR; the test group ($n = 22$) was treated with feeds containing ENR (5 mg/kg) for 3 days and then maintained without further treatment. Six chickens from the test group and two chickens from the control group were slaughtered at days 3, 6, and 9 after the withdrawal of the ENR feeds. The muscle samples were collected and used for ENR analyses by test strips and LC-MS. The results from the two analysis methods showed good correspondence as shown in **Table 3**.

According to document data, when ENR is given to chickens by oral administration, ENR is partially converted to ciprofloxacin (CIP), and the ratio of CIP/ENR was on average 63% (0.63 ± 0.19) (*35*). Then the parent drug, ENR, on average accounts for 61% of the total. The MRL has been set by the European Commission at 30 μg/kg for the sum of ENR and its active metabolite (CIP) for muscle tissue. Therefore, the estimated MRL of ENR was 18.4 μg/kg (16.5−20.8 μg/kg). The LDL by naked eye was set at the amount of ENR causing a 50% decrease of the G/D × area as this difference was clearly visible. In the present study the LDL of the test strip was calculated to be 0.935 μg/kg by the naked eye. The results of this study demonstrate that the one-step strip test could detect ENR residues in chicken muscle.

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Received for review August 28, 2008. Revised manuscript received November 3, 2008. Accepted November 4, 2008.

JF802648Z