JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an Immunochromatographic Lateral-Flow Test Strip for Rapid Detection of Sulfonamides in Eggs and Chicken Muscles

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A rapid immunochromatographic lateral-flow test strip was developed in the competitive reaction format for the detection of sulfonamides in eggs and chicken muscle. A monoclonal antibody against the common structure of sulfonamides was conjugated to colloidal gold particles as the detection reagent and an *N*-sulfanilyl-4-aminobenzoic acid (SUL)−bovine serum albumin (BSA) conjugate was immobilized to a nitrocellulose membrane as the capture reagent to prepare the test strip. With this method, it required only 15 min to accomplish the semiquantitative or quantitative detection of sulfonamides. The sensitivity to sulfonamides (sulfamonomethoxine, sulfamethoxydiazine, sulfadimethoxine, and sulfadiazine) was at least 10 ng/mL, as determined with an optical density scanner. By eye measurement, the sensitivity was 20 ng/mL for sulfamonomethoxine, sulfamethoxydiazine, and sulfadimethoxine and 40 ng/mL for sulfadiazine. On the basis of a sulfamonomethoxine standard curve, recoveries were from 89.5 to 95.6% for sulfamonomethoxine, from 89.5 to 95.1% for sulfamethoxydiazine, from 85.0 to 95.6% for sulfadimethoxine, and from 44.8 to 60.9% for sulfadiazine in egg and chicken muscle samples. A parallel analysis of 27 egg samples and 28 chicken muscle samples from the animal experiment showed that the differences between test strips and high-performance liquid chromatography (HPLC) were from 0.8 to 11.2% for egg samples and from 2.2 to 34% for chicken muscle samples for the quantitative detection, and the agreement rates between test strips and HPLC were 100%, based on the maximum allowed residue level of sulfadiazine (100 ng/g) established by the European Union and China. In conclusion, the method is rapid and accurate for the detection of sulfonamides in eggs and chicken muscles.

KEYWORDS: Immunochromatography; test strip; sulfonamides; egg; chicken muscle

INTRODUCTION

Sulfonamides are a family of broad-spectrum synthetic bacteriostatic antibiotics given to food animals for prophylactic or therapeutic purposes. As a result, sulfonamides can be present in food products of animal origin. To protect consumers from risks related to sulfonamide residues, the maximum allowed residue level (MRL) of sulfonamides has been established. For instance, in Europe and China, the MRL of sulfonamides in edible tissues is 100 μg/kg (*1*, *2*). For the analysis of sulfonamides, several analytical methods have been developed including high-performance liquid chromatography (HPLC) (*3*, *4*), gas chromatography−mass spectrometry (GC-MS) (*5*), and enzyme-linked immunosorbent assay (ELISA) (*6*). HPLC and GC-MS are sensitive and specific but are laborious and expensive. They are not suitable for screening large numbers of samples. ELISA is an efficient immunoassay that can be used for the analysis of numerous samples, but it requires labor-intensive operations including incubation, washing, and enzymatic reactions during signal generation. More recently, a novel method called immunochromatographic assay, a one-step process, has been used in the detection of sulfamethazine (SMZ) (*7*), but it is only specific for an individual sulfonamide. It would be more efficient to have an immunochromatographic assay with the capacity to detect several sulfonamides.

The sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the N^4-position and differ in the substitution at the N^1-position (**Figure 1**). In the present study, a monoclonal antibody against the common structure of sulfonamides was prepared and conjugated with colloidal gold to develop an immunochromatographic lateral-

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10.1021/jf062523h CCC: $37.00
Published on Web 02/23/2007

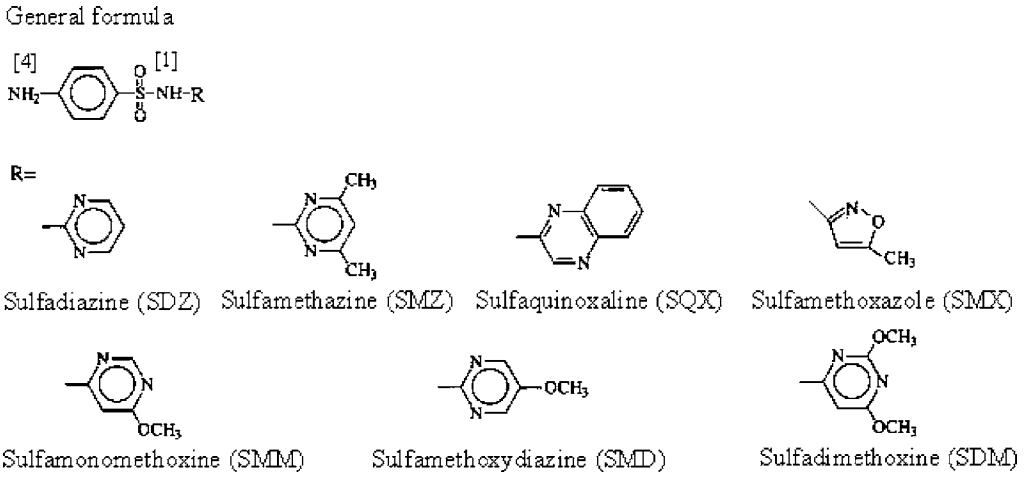

Figure 1. Structures of the compounds studied.

flow test strip for the semiquantitative or quantitative detection of several sulfonamides in eggs and chicken muscles. As confirmed by the results from HPLC analyses, the test strip was rapid, simple, and effective for the detection of four sulfonamides.

MATERIALS AND METHODS

Equipment. *N*-Sulfanilyl-4-aminobenzoic acid (SUL) was analyzed with an Avatar 360 FTIR Spectrometer (Nicolet Instrument Corp., Verona Road, Madison, WI) and a Mercury-plus 400 Nuclear Magnetic Resonance spectroscopy system (Varian Corp., Palo Alto, CA). CB CO_2 incubators from Binder (Tuttlingen, Germany) were used for cell cultivation. An ELX800 Universal Microplate Reader (BIO-TEK Instruments Inc., Winooski, VT) was used to measure the optical density. Transmission electron microscopy (TEM) images were recorded with a Hitachi H600 transmission electron microscope (Hitachi Instrument Co., Tokyo, Japan). UV−visible spectra were obtained by using an 8453 UV/Visible Spectrophotometer (Aligent Technologies, Palo Alto, CA).

A ZX1000 Dispensing Platform and a CM4000 Guillotine Cutter (BioDot, Irvine, CA) were used to prepare test strips. A TSR3000 Test Strip Reader (BioDot) was used to analyze the intensity of test strips. The samples were evaluated with a 2695 Alliance HPLC System (Waters Co., Milford, MA).

Chemicals and Reagents. 4-Acetylsulfanilyl chloride was purchased from Aldrich (Milwaukee, WI), and methyl 4-aminobenzoate was obtained from Beijing Huateng Chemical Ltd. (Beijing, China). Gold chloride ($HAuCl_4 \cdot 3H_2O$), sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), bovine serum albumin (BSA), human serum albumin (HSA), polyvinylpyrrolidone K30, sulfamonomethoxine (SMM), sulfamethoxydiazine (SMD), sulfadimethoxine (SDM), sulfadiazine (SDZ), sulfaquinoxaline (SQX), SMZ, sulfamethoxazole (SMX), and trimethoprim (TMP) were purchased from Sigma (St. Louis., MO). The goat anti-mouse antibody was obtained from Sino-American Biotechnology Co. (Luoyang, China). Nitrocellulose membranes, glass fibers, and absorbent paper were purchased from Millipore Corp. (Bedford, MA). All solvents were of analytical grade or higher.

Sample Materials. *Experimental Egg Samples.* The hens (White Leghorns) were randomly divided into control and test groups. The test group ($n = 5$) was treated with feeds containing SMM (100 mg/kg; purity, more than 98%) for five consecutive days (*8*) and then maintained without SMM treatment for 10 days; the control group ($n = 2$) was treated with the similar feeds not containing SMM. The eggs from both the control and the test groups were collected at days 2, 4, 6, and 8 after the withdrawal of the SMM feeds, and the eggs collected from the control group were used as control samples. All egg samples were subjected to test strips and HPLC for SMM analysis.

Experimental Chicken Muscle Samples. The broilers were randomly divided into control and test groups. The control group ($n = 8$) was not treated with SMM; the test group ($n = 20$) was treated with feeds containing SMM (100 mg/kg; purity, more than 98%) for 5 days (*8*), which were then maintained without SMM treatment. Five broilers from the test group and two broilers from the control group were slaughtered at days 1, 2, 3, and 4 after the withdrawal of the SMM feeds. The muscle samples (100 g per sample) were collected and used for SMM analyses by test strips and HPLC.

Samples from Marketed Eggs and Chicken Muscle. One hundred forty egg samples and 120 chicken muscle samples were randomly collected from markets in March 2006 and tested by test strips developed in this study.

Sample Pretreatment for Test Strip Assay. The sample pretreatment was accomplished by sample homogenization, extraction, and drying. Each muscle or egg sample was homogenized in ethyl acetate (2 mL/g wet weight). The homogenate was vortexed for 3 min and then centrifuged at 2000*g* for 10 min. The resultant supernatant (300 μL) was evaporated to dryness by heating at 60 °C or in a 60 °C water bath under a gentle flow of nitrogen. The residue was resuspended in 150 μL of phosphate buffer (pH 7.4) and mixed thoroughly before analysis.

Sample Pretreatment and Analytical Procedure for HPLC Analysis. The methods were described elsewhere for egg samples (*9*) and chicken muscle samples (*10*). Briefly, 5 g of the samples was placed in a 50 mL centrifuge tube, to which 25 mL of acetonitrile and 4 g of anhydrous Na_2SO_4 were added and the extraction of sulfonamides was achieved by shaking for 5 min. After centrifugation, the supernatant was transferred into a 250 mL conical flask. The extraction procedure was repeated once, and the extracts were combined and evaporated. The residue was suspended in 3 mL of acetonitrile/water (95:5, v/v) and purified on an alkaline Al_2O_3 SPE column. For chicken muscle samples, sulfonamides were eluted with 5 mL of acetonitrile−water (75:25, v/v), and the final volume was adjusted to 10 mL with 0.017 mol/L of phosphoric acid. For egg samples, the sulfonamides were eluted with 10 mL of acetonitrile−water (70:30, v/v). The eluate was evaporated, and the residue was dissolved in 2 mL of acetonitrile−methanol−water−acetic acid (2:2:9:0.2, v/v). The resultant solution was filtered through a 0.45 μm disposable syringe filter, and 20 μL of the filtrates for egg samples and 50 μL of the filtrates for chicken muscle samples were injected onto a 2695 Alliance HPLC System. The chromatographic separation was accomplished in 30 min with a gradient elution on a C_{18} analytical column (Waters Co.). The mobile phases, which were 0.017 mol/L phosphoric acid−acetonitrile (80:20, v/v) for chicken muscle samples and acetonitrile−methanol−water−acetic acid (2:2:9:0.2,v/v) for egg samples, were pumped at a flow rate of 1.0 mL/min. The UV detector was set at 270 nm. The results for all of the samples were calculated based on a standard curve prepared by using sulfonamides standard solutions.

Hapten Synthesis. The hapten (SUL) has the N^4-sulfonamide ring system, which is the common structure of all sulfonamides. The method used for synthesis was adapted as described previously (*11*).

Preparation of SUL−Protein Conjugates. The mixed anhydride coupling method (*12*) was adapted to prepare SUL−protein conjugates

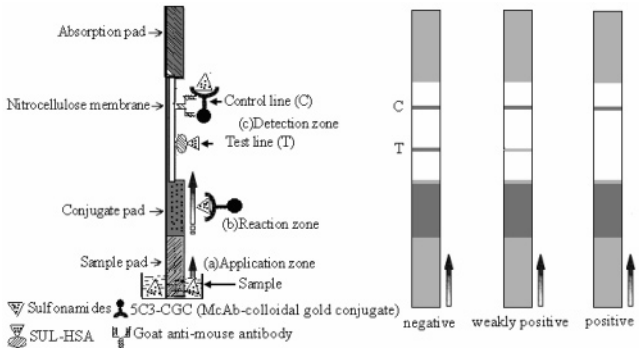

Figure 2. Schematic diagram for the immunochromatographic lateral-flow test strip for sulfonamides. (**a**) Application zone, (**b**) reaction zone, and (**c**) detection zone. The strip consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorption pad. The conjugate pad contains 5C3-CGC (McAb-CGC). In the detection zone, the nitrocellulose membrane is used as a chromatographic support on which SUL-HSA and goat anti-mouse antibody are immobilized. By visualization, negative, weakly positive, and positive samples can be distinguished.

as follows: 5.84 mg of SUL was dissolved in 1 mL of N,N-dimethylformamide (DMF) followed by the addition of 6.8 μL of tributylamine and 3.8 μL of isobutyl chlorocarbonate. After the mixture was stirred for 25 min at 4 °C, 67 mg of HSA or BSA in 10 mL of phosphate buffer (PBS, 0.01 M, pH 7.4) was added. The mixture was adjusted to pH 8.0 and stirred at 4 °C for an additional 4 h. After the mixture was dialyzed against 2 mM phosphate buffer (pH 7.4) at 4 °C for 2 days, it was stored at −26 °C.

Preparation of Monoclonal Antibody. Monoclonal antibody 5C3 (IgG2b/κ) specific for the common structure of SUL and sulfonamides was obtained by immunizing mice with HSA−SUL and selected with SUL and sulfonamides (SMM, SMD, SDM, and SDZ) as described previously (*11*). The 5C3 was purified from mouse ascitic fluid by using caprylic acid and ammonium sulfate (*13*, *14*) and dialyzed against 2 mM phosphate buffer (pH 7.4) at 4 °C for 2 days.

Synthesis of Colloidal Gold. Colloidal gold was prepared by using a previously reported method (*15*), with slight modifications. In a 500 mL round-bottom flask, 200 mL of 0.01% (w/w) $HAuCl_4$ in twice distilled water was brought to a boil with vigorous stirring, and 4 mL of 1% trisodium citrate was added to the solution. The solution turned deep blue within 20 s, and the color changed to wine-red 60 s later. After continued boiling for an additional 10 min, the heating source was removed and the colloid was stirred for 15 min. The colloidal gold solution was stored at 4 °C in a dark-colored glass bottle until use.

Preparation of Colloidal Gold-McAb Conjugate. The purified McAb 5C3 was conjugated with colloidal gold to generate 5C3-CGC (colloidal gold conjugate). Briefly, 60 μg of the purified McAb 5C3 in 0.5 mL of distilled water was added to 10 mL of gold colloid solution (adjusted to pH 8.0), and the mixture was stirred vigorously for 30 min. For this preparation, 2.5 mL of 5% (w/v) BSA aqueous solution was added to block excess reactivity of the gold colloid, followed by stirring for 30 min. After centrifugation at 12000*g* at 4 °C for 30 min, the supernatant was removed by aspiration, and the resultant 5C3-CGC pellet was suspended in 2 mM borax buffer (pH 9.0) containing 0.1% (w/v) PEG-20000 and washed twice with the same buffer. The final pellet was resuspended in 1 mL of the same buffer.

Preparation of the Immunochromatography Strip. A schematic diagram for the preparation of the immunochromatography lateral-flow test strip is shown in **Figure 2**. The sample (absorbent paper) and the conjugate pads (glass-fiber membrane) were treated with 20 mM phosphate buffer containing 2% BSA, 2.5% sucrose, 0.3% polyvinylpyrrolidone K30, and 0.02% sodium azide, pH 7.4, and dried at 37 °C. Subsequently, SUL−HSA (0.15 mg/mL) and goat anti-mouse antibody (1 mg/mL) were applied to the nitrocellulose membrane to the test and control lines by using the BioDot XYZ Platform at a jetting rate of 0.75 μL/cm and then dried at 37 °C. The absorption pad (absorbent paper) was used without treatment. The 5C3-CGC was applied to the treated conjugate pad at a jetting rate of 9 μL/cm and lyophilized to dryness. The absorption pad, the nitrocellulose membrane, the pretreated conjugate pad, and the sample pad were assembled as the test strip and attached to a plastic scaleboard. Test strips with 65 mm in length and 3 mm width were prepared. The application zone of the test strips was put in a 120 μL sample for analysis.

Principle of Immunochromatographic Lateral-Flow Test Strip and Quantification of Sulfonamides. The assay was based on the competitive reaction theory (**Figure 2**). When a standard sample was applied to the sample pad, it rapidly wet through to the conjugate pad, and the colloid gold-labeled McAb (5C3-CGC) was then solubilized. The 5C3-CGC began to migrate along with the sample flow front up to the nitrocellulose membrane. The 5C3-CGC was trapped by the SUL−HSA immobilized on the membrane, forming a clear red test line. The excess 5C3-CGC migrated farther and was trapped by the goat anti-mouse antibodies to form the control line. If the sulfonamide was present in the sample, it competed with the immobilized SUL−HSA on the test line to bind the limited amount of 5C3-CGC. The more sulfonamide present in the sample, the weaker was the test line. As determined by visualization, the result was negative (−), weakly positive (±), or positive (+) and the intensity of the test line was in proportion to the amount of sulfonamide present in the samples. Several concentrations of SMM (10, 20, 40, 80, and 100 ng/mL) and the negative control were included in the preparation of standard curves. The intensity of their test lines was obtained by using the test strip reader, and the quantitative values were expressed as G/Peak-relative optical density (G/Peak-ROD). The standard curves were constructed by plotting the G/Peak-ROD values obtained from each reference standard against corresponding concentrations in ng/mL. The G/Peak-ROD value was used to determine the concentration of the test samples from the standard curve. It required 15 min to complete a test.

RESULTS

Characterization of the Colloidal Gold Particles. The diameter of colloidal gold was obtained by UV−vis measurements and examination under TEM (**Figure 3**). The UV−vis spectroscopic analysis showed a maximum absorbance at 520 nm, and the TEM images of the gold particles revealed that the average diameter of the colloidal gold particles was 18.6 ± 1.2 nm (n = 100). These results indicated that the preparation of colloidal gold particles met the requirements for the preparation of CGC and signal generation in test strips.

Specificity of the Test Strips. Each standard sample containing various concentrations of SMM (10, 20, 40, 80, and 100 ng/mL) and negative control samples were assayed eight times by the procedures described previously. The color intensity of the test line was quantified by the test strip reader. The results were expressed as G/Peak-ROD. A dose−response curve was obtained for reference SMM standards ranging from 0 to 100 ng/mL (**Figure 4**). The 50% inhibition (IC_{50}) of SMM was 18.3 ng/mL. The IC_{50} values for other sulfonamides were also measured. On the basis of SMM, the cross-reactivity rate with McAb (5C3) was 100%. The cross-reactivity with major sulfonamides was established (**Table 1**).

Test Sensitivity. Each standard sample containing various concentrations of SMM, SMD, and SDM (10, 20, 40, 80, and 100 ng/mL), SDZ (10, 20, 40, 80, 100, and 160 ng/mL), and each negative control sample was assayed eight times by using the methods as described previously. The color intensity of the test line was confirmed by direct visualization and quantified by the test strip reader. The results were expressed as G/Peak-ROD. Data are presented as mean values ± standard deviations (SDs). Student's t test was used for determining the difference between the groups. SPSS software 12.5 for Windows (SPSS Inc., Chicago, IL) was used for data analysis. A P value of less than 0.05 was considered statistically significant. In the analyses, the samples containing 10 ng/mL or more of SMM, SMD, SDM, or SDZ displayed positive signals ($P < 0.01$) (**Figure 4**). As

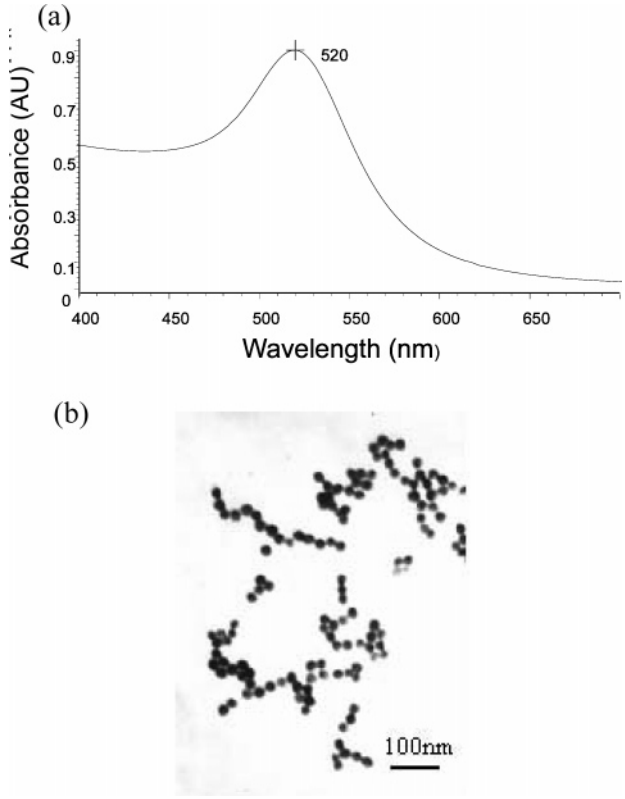

Figure 3. UV–visible spectra of colloidal gold solution (**a**) and TEM image of colloidal gold particles (**b**). The maximum absorbance of the colloidal gold solution is 520 nm, and the size of the colloidal gold particles is 18.6 ± 1.2 nm (100 particles sampled).

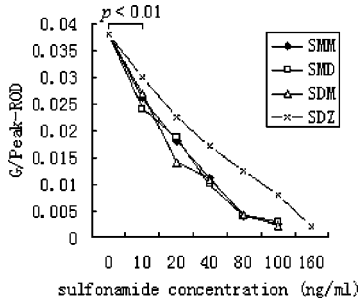

Figure 4. Standard curve of sulfonamides by test strips. Each negative control sample and standard sample of sulfonamides was assayed in test strips eight times. The test line color intensity in strip was expressed as G/Peak-ROD. Data are presented as the mean of eight measurements. The coefficient variations (CVs) of test line color intensity were from 8.05 to 14.3% for SMM standard samples, from 7.05 to 14.3% for SMD standard samples, from 2.16 to 13.6% for SDM standard samples, from 6.98 to 14.3% for SDZ standard samples, and 5.28 for negative control samples, respectively.

determined visually, if one band with red color similar to the color of the negative control sample was present at the test line, the sample was considered to be negative (−); if one band with red color was weaker than the red band of the negative control sample, the sample was considered to be weakly positive (±). This occurred when the concentration of SMM, SMD, or SDM was in the range of 20−100 ng/mL or when the concentration of SDZ was in the range of 40−160 ng/mL. When there was no band at the test line, the sample was positive (+); this occurred when the concentration of SMM, SMD, or SDM was 100 ng/mL or greater or the concentration of SDZ was 160 ng/mL or greater. These results indicate that our test strip detects

Table 1. Cross-Reactivity Data for the Monoclonal Antibody (5C3)[a]

compounds	IC_{50} (ng/mL)	cross-reactivity (%)
SMM	18.3	100
SMD	18.1	101
SDM	16	114
SDZ	28.6	64
SQX	342.3	5.3
SMZ	938.5	1.9
SMX	268.5	6.8
TMP	379.5	4.8

[a] IC_{50} was the concentration giving 50% inhibition of McAb 5C3-colloid gold conjugate trapped on the test line. Cross-reactivity was expressed as the percentage of IC_{50} concentrations of sulfonamides divided by that of SMM.

Table 2. Recovery of Sulfonamides in Egg and Chicken Muscle Samples[a]

sample	added (ng/g)	found (ng/g) mean ± SD	recovery (%)
egg	SMM 20	19.1 ± 1.5	95.5
	SMM 100	93.1 ± 4.5	93.1
egg	SMD 20	17.9 ± 1.7	89.5
	SMD 100	90.6 ± 4.4	90.6
egg	SDM 20	17.9 ± 2.2	89.5
	SDM 100	95.6 ± 4.3	95.6
egg	SDZ 40	23.7 ± 2.2	59.2
	SDZ 160	97.5 ± 3.0	60.9
muscle	SMM 20	17.9 ± 2.1	89.5
	SMM 100	95.6 ± 3.4	95.6
muscle	SMD 20	18.9 ± 2.3	94.5
	SMD 100	95.1 ± 5.4	95.1
muscle	SDM 20	17.0 ± 2.0	85.0
	SDM 100	92.2 ± 4.7	92.2
muscle	SDZ 40	17.9 ± 2.3	44.8
	SDZ 160	94.6 ± 2.3	59.1

[a] Recoveries were calculated based on SMM standard curve. Mean ± SD ($n = 8$).

sulfonamides with a sensitivity of at least 10 ng/mL, as determined by the strip reader. As determined visually, the sensitivity was 20 ng/mL for SMM, SMD, and SDM and 40 ng/mL for SDZ.

Recovery of Sulfonamides in Egg and Chicken Muscle Samples. Egg and muscle samples determined by HPLC to be sulfonamide-free were spiked separately with SMM, SMD, and SDM at concentrations of 20 and 100 ng/g and with SDZ at concentrations at 40 and 160 ng/g. Each spiked sample was assayed eight times by using test strips. The color intensity of the test band was analyzed by the test strip reader, and the values of samples were obtained based on SMM standard curve as described above. Recoveries were from 89.5 to 95.6% for SMM, from 89.5 to 95.1% for SMD, from 85.0 to 95.6% for SDM, and from 44.8 to 60.9% for SDZ in egg and muscle samples (**Table 2**).

Comparison of Test Strips with HPLC. *Detection of Sulfonamides in Egg Samples.* A comparison between the test strips and the HPLC was performed with 27 SMM egg samples from the animal experiment (**Table 3**). On the basis of the SMM standard curve, the concentrations of samples were calculated for the quantitative detection of SMM. The differences between test strips and HPLC were from 0.8 to 11.2%. For semiquantitative detection, two cutoff levels for the contents of SMM in the samples were set at 20 and 100 ng/g. By visualization as described above, samples were determined to be negative (−, less than 20 ng/g), weakly positive (±, 20−100 ng/g), or positive (+, greater than 100 ng/g), respectively. The results

Table 3. Comparison of Test Strip with HPLC for Detection of Egg Samples[a]

hens no.	day[b]	test strips (ng/mL)	HPLC (ng/mL)	difference between test strips and HPLC (%)	hens no.	day	test strips (ng/mL)	HPLC (ng/mL)	difference between test strips and HPLC (%)
1 (T)[b]	2	574.9 (+)	583.8 (+)	–1.5	1 (T)	6	25.6 (±)	ND (–)	
2 (T)	2	763.1 (+)	750.6 (+)	1.7	2 (T)	6	23.8 (±)	ND (–)	
3 (T)	2	417.8 (+)	402.2 (+)	3.9	3 (T)	6	49.8 (±)	52.7 (±)	–5.5
4 (T)	2	456.7 (+)	433.9 (+)	5.3	4 (T)	6	52.1 (±)	58.6 (±)	–11.1
5 (T)	2	666.8 (+)	672.3 (+)	–0.8	5 (T)	6	44.4 (±)	50.0 (±)	–11.2
1 (C)[b]	2	ND (–)	ND[b] (–)		1 (C)	6	ND (–)	ND (–)	
2 (C)	2	ND (–)	ND (–)		2 (C)	6	ND (–)	ND (–)	
1 (T)	4	236.3 (+)	223.9 (+)	5.5	1 (T)	8	ND (–)	ND (–)	
2 (T)	4	145.6 (+)	155.7 (+)	–6.5	2 (T)	8	ND (–)	ND (–)	
3 (T)	4	270.6 (+)	278.8 (+)	–2.9	3 (T)	8	no egg	no egg	
4 (T)	4	328.1 (+)	300.3 (+)	9.3	4 (T)	8	10.7 (–)	ND (–)	
5 (T)	4	456.2 (+)	442.0 (+)	3.2	5 (T)	8	15.1 (–)	ND (–)	
1 (C)	4	ND (–)	ND (–)		1 (C)	8	ND (–)	ND (–)	
2 (C)	4	ND (–)	ND (–)		2 (C)	8	ND (–)	ND (–)	

[a] HPLC method came from Chinese Profession Standard NY 5039-2001 (Non-environmental Damage Food—Egg), and the detection limit was 50 ng/g; the sensitivity of test strip was 10 ng/g. By visualization, negative samples (–) showed that the concentration of SMM was less than 20 ng/g; weakly positive (PM±) showed that was 20–100 ng/g; and positive (+) showed that was more than100 ng/g. The positive agreement rate was 100%, the weakly positive agreement rate was 60%, and the negative agreement rate was 85.7%. [b] Day, day after withdrawal of SMM; ND, no detection; T, test group; and C, control group.

Table 4. Comparison of Test Strip with HPLC for Detection of Chicken Muscle Samples[a]

broiler no.	day[b] HT	test strips (ng/mL)	HPLC (ng/mL)	difference between test strips and HPLC (%)	broiler no.	day	test strips (ng/mL)	HPLC (ng/mL)	difference between test strips and HPLC (%)
1 (T)[b]	1	595.5 (+)	610 (+)	–2.4	11 (T)	3	10.2 (–)	12 (–)	–15
2 (T)	1	409 (+)	418 (+)	–2.2	12 (T)	3	ND (–)	ND (–)	
3 (T)	1	409.1 (+)	388 (+)	5.4	13 (T)	3	23.3 (±)	19 (–)	22.6
4 (T)	1	386.9 (+)	405 (+)	–4.5	14 (T)	3	20.1 (±)	15 (–)	34
5 (T)	1	512.8 (+)	521 (+)	–1.6	15 (T)	3	21.9 (±)	17 (–)	28.8
1 (C)[b]	1	ND (–)	ND[b] (–)		5 (C)	3	ND (–)	ND (–)	
2 (C)	1	ND (–)	ND (–)		6 (C)	3	ND (–)	ND (–)	
6 (T)	2	26.5 (±)	23.2 (±)	14.2	16 (T)	4	ND (–)	ND (–)	
7 (T)	2	23.8 (±)	27.8 (±)	–14.4	17 (T)	4	ND (–)	ND (–)	
8 (T)	2	51.6 (±)	64 (±)	–19.4	18 (T)	4	ND (–)	ND (–)	
9 (T)	2	69.7 (±)	78 (±)	–10.6	19 (T)	4	ND (–)	ND (–)	
10 (T)	2	47.7 (±)	57 (±)	–16.3	20 (T)	4	ND (–)	ND (–)	
3 (C)	2	ND (–)	ND (–)		7 (C)	4	ND (–)	ND (–)	
4 (C)	2	ND (–)	ND (–)		8 (C)	4	ND (–)	ND (–)	

[a] HPLC method came from Document of Chinese Ministry of Agriculture (nongmufa [2001] No. 38); the detection limit was 10 ng/g. The sensitivity of the test strip was 10 ng/g. By visualization, negative sample (–) showed that the concentration of SMM was less than 20 ng/g; weakly positive (±) showed that the concentration was 20–100 ng/g; and positive (+) showed that the concentration was more than 100 ng/g. The positive agreement rate was 100%, the weakly positive agreement rate was 62.5%, and the negative agreement rate was 83.3%. [b] Day, day after withdrawal of SMM; ND, no detection; T, test group; and C, control group.

calculated from the SMM standard curve were essentially the same as those obtained by visualization for test strips. The negative agreement rate was 85.7%, the weakly positive agreement rate was 60%, and the positive agreement rate was 100%. On the basis of the MRL of SDZ (100 ng/g) established by the European Union (EU) and China, the agreement rate between test strips and HPLC was 100%.

Detection of Sulfonamides in Muscle Samples. Similarly, a parallel comparison between test strips and HPLC was performed with 28 SMM muscle samples from the animal experiment (**Table 4**). On the basis of the SMM standard curve, the concentrations of samples were calculated for quantitative detection. The differences between test strips and HPLC were from 2.2 to 34%. For semiquantitative detection, two cutoff levels for the content of SMM in the samples were set at 20 and 100 ng/g. The results based on the SMM standard curve were essentially the same as those obtained by visualization for test strips. The negative agreement rate was 83.3%, the weakly positive agreement rate was 62.5%, and the positive agreement rate was 100%. On the basis of the MRL of SDZ (100 ng/g) established by the EU and China, the agreement rate between test strips and HPLC was 100%.

Application of Test Strip in Analysis of Samples with Unknown Concentrations of Sulfonamides. Among the 140 egg and 120 chicken muscle samples collected from markets, three egg samples and three chicken muscle samples were shown to contain sulfonamide residues, as tested with the test strips. On the basis of the SMM standard curve, the concentrations were 137.4, 76.6, and 82.3 ng/g in the three egg samples and 38.1, 92.5, and 75 ng/g in the muscle samples. By visualization, only one sample was positive (+) and five were weakly positive (±). As determined by HPLC, the concentrations of sulfonamide were 229 (SDZ), 112 (SDZ), and 125 ng/g (SDZ) in the three egg samples and 56 (SDZ), 138 (SDZ), and 80 ng/g (SMM) in the muscle samples.

DISCUSSION

In the present study, McAb 5C3 specific for the common structure of SUL and sulfonamides was prepared, since the sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the N^4-position and differ only in substitutions at the N^1-position. However, because of their different structures (*8*), the IC_{50} values for sulfonamides with 5C3 were different (**Table 1**). On the basis of the IC_{50} values

and the MRL of sulfonamides (100 μg/kg) established by the EU and China, an immunochromatographic lateral-flow test strip was developed using the McAb 5C3 to detect four sulfonamides (SMM, SMD, SDM, and SDZ).

For the quantitative analysis of SMM, SMD, SDM, and SDZ, it requires a reading device, which is the same with a previously developed test strip (*16*). To simplify the procedure, we developed a novel method to differentiate the results (negative, weakly positive, and positive) and to perform semiquantitative analysis by visualization. When a red band similar to that of the negative control sample was present at the test line, the sample was considered to be negative (−) and the concentration of SMM, SMD, or SDM was less than 20 ng/g. When that band was weaker than the band of the negative control sample, the sample was considered to be weakly positive (±) and the concentration of SMM, SMD, or SDM was 20−100 ng/g. When there was no band at the test line, the sample was considered to be positive (+) and the concentration of SMM, SMD, or SDM was 100 ng/g or greater. The procedure can be easily implemented to follow government regulations on the MRL of sulfonamides (100 ng/g).

Because of the differences in the IC_{50} values of SMM (18.3 ng/mL), SMD (18.1 ng/mL), SDM (16 ng/mL), and SDZ (28.6 ng/mL), the quantitative and semiquantitative analyses can only be performed for single sulfonamide or a mixture including SMM, SMD, and SDM that have a similar IC_{50} value. Nevertheless, test strips do not identify what the sulfonamide is or which sulfonamides are in the mixture. Therefore, if there is a positive indication using this screening method, further analyses by HPLC or other instrumental method should be performed in order to identify and quantify individual sulfonamides.

It is laborious to prepare samples for an accurate analysis by HPLC and GC-MS. Here, we used a simple method to extract sulfonamides (SMM, SMD, SDM, and SDZ) from egg and muscle samples for immunochromatographic analysis. With this method, recoveries were from 89.5 to 95.6% for SMM, from 89.5 to 95.1% for SMD, from 85.0 to 95.6% for SDM, and from 44.8 to 60.9% for SDZ in egg and muscle samples, based on the SMM standard curve. Furthermore, for bovine milk, porcine muscle, and porcine liver, we determined that the recoveries were from 89.0 to 98.1% for SMM, from 82.5 to 103.3% for SMD, from 79.0 to 101.2% for SDM, and from 49.0 to 61.9% for SDZ (data not shown). These results are yet to be confirmed by animal experiments.

With the test strips, results were determined based on the SMM standard curve. The difference of IC_{50} between SMM (18.3 ng/mL) and SDZ (28.6 ng/mL) resulted in a low recovery for SDZ and caused the SDZ concentrations tested by test strips to be lower than those tested by HPLC. For the detection of unknown samples from markets, five samples contained SDZ residues by HPLC, which may count for a greater difference in results between test strips and HPLC. In the comparison of test strip results with HPLC results, all HPLC procedures were performed applying Chinese standard methods (*13*, *14*). As applied to chicken muscle samples, the detection limit was 10 ng/g; for egg samples, the limit was 50 ng/g. Nevertheless, because the sensitivity was 10 ng/g in egg and chicken muscles, there was little difference in results derived by the test strip and with HPLC (**Table 3**).

In conclusion, we developed an immunochromatographic lateral-flow test strip for quantitative detection of four sulfonamides in eggs and chicken muscles. It is likely that an immunochromatographic lateral-flow test strip that efficiently and accurately detects additional sulfonamides or a set of test strips that can detect and identify sulfonamides in food products of animal origin can be developed in the future.

ABBREVIATIONS USED

SUL, N-sulfanilyl-4-aminobenzoic acid; BSA, bovine serum albumin; HSA, human serum albumin; MRL, maximum allowed residue level; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography−mass spectrometry; ELISA, enzyme-linked immunosorbent assay; TEM, transmission electron microscopy; SMM, sulfamonomethoxine; SMD, sulfamethoxydiazine; SDM, sulfadimethoxine; SDZ, sulfadiazine; SQX, sulfaquinoxaline; SMZ, sulfamethazine; TMP, trimethoprim; SMX, sulfamethoxazole; CGC, colloidal gold conjugate; DMF, N,N-dimethylformamide; G/Peak-ROD, G/Peak-relative optical density; SD, standard deviation; IC_{50}, 50% inhibition.

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Received for review September 3, 2006. Revised manuscript received January 20, 2007. Accepted January 23, 2007. This project was supported by a grant from the key programs of Wuhan City (20022002062).

JF062523H