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Validation and Comparative Studies of Four Sulfonamide Immunoassays Based on the Same Generic Polyclonal Antibody

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Abstract Four different immunoassays based on the same generic polyclonal antibody were validated by high performance liquid chromatography, respectively. They also were compared with each other in terms of sensitivity, precision, and accuracy for the quantification or screening of sulfonamide residues in food samples. Correlation studies showed that there was a good correlation between the immunoassays and liquid chromatography data. The conventional plate assay has better precision and the plate-enhanced chemiluminescent assay has higher sensitivity. These two methods all could be used as quantification methods for large numbers of samples and complements of the conventional analytical methods in laboratory. The flow-through strip assay has higher sensitivity and the dip-stick strip assay was less affected by matrix effect. These two methods all could be used as valuable tools for the rapid on-site screening of sulfonamide residues in animal-derived food samples.

Keywords Sulfonamide · Immunoassay · Generic polyclonal antibody · High performance liquid chromatography · Animal-derived food

Introduction

Sulfonamides are widely used for therapeutic and prophylactic purposes in humans and other animals and sometimes as additives in animal feed. Franco et al. [1] reported that widespread use of sulfonamide drugs in factory farming without proper withdrawal periods led to accumulation of sulfonamides in meat, eggs, and milk as well as in fish. In European Union, Canada, and the USA, the maximum residue limit (MRL) of total sulfonamides in

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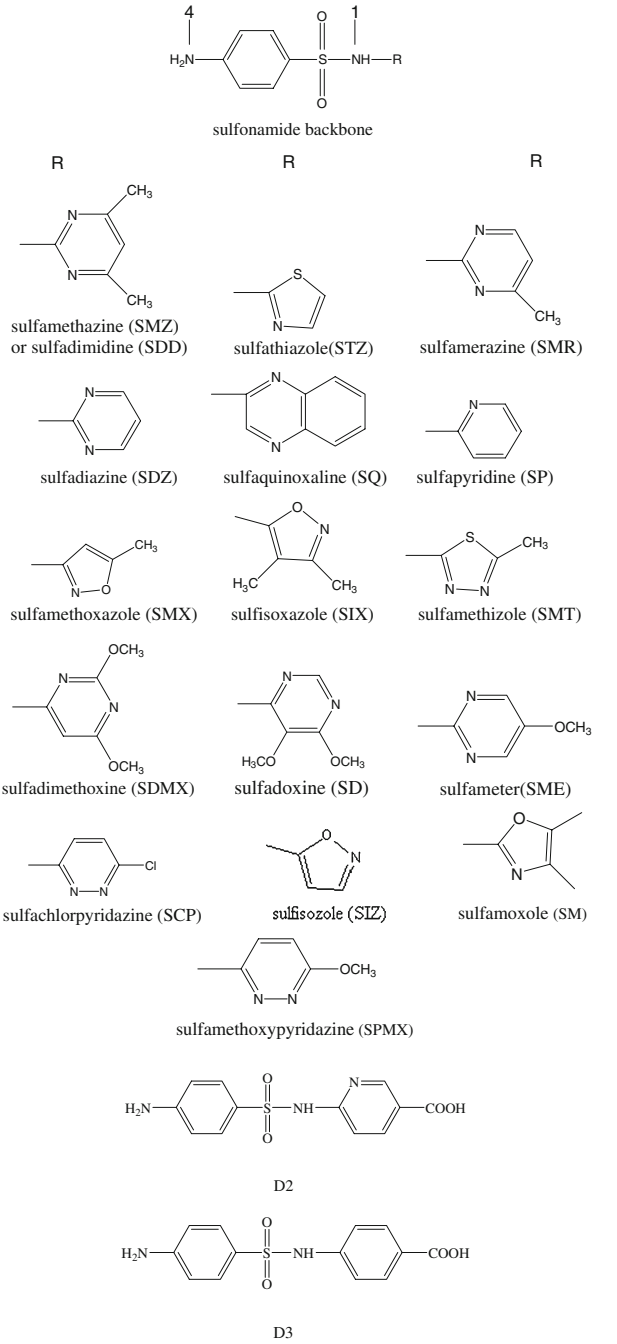
edible tissues is 100 μg kg^{-1}, and 20 μg kg^{-1} in Japan. The backbone and the structures of sulfonamides that appeared in this work are shown in Fig. 1.

In general, the most widely used analytical methods for identification and quantification of sulfonamides in animal food are based on chromatographic methods

Fig. 1 Structures of the sulfonamides studied in this work



or bacteriological growth inhibition assays. Among these, the sensitive instrumental methods such as high performance liquid chromatography (HPLC) have been used to detect sulfonamide residues in most cases reported by Wang et al. [2]. However, these methods are laborious, expensive, and more suitable for confirmation but not for screening of large numbers of samples. In recent years, there has been an increase in demand for simple, quick, accurate, and specific methods for the determination of sulfonamides. The application of other less time-consuming and easier analytical techniques such as immunoassays which are capable of detecting low amounts of residues in many samples rapidly is also reported by others [3–5]. Previously, we [6, 7] reported the production of generic polyclonal antibodies and the development of a conventional plate enzyme-linked immunosorbent assay (ELISA) suitable for the determination of 11 structurally different sulfonamides at concentrations less than 100 μg L^{-1} in assay buffer. In the following study, the developed ELISA was applied to determining seven sulfonamides in pig muscle, liver, chicken muscle, egg, milk, and fish at or below the level of MRL (100 μg kg^{-1} or 100 μg L^{-1}). In this paper, validation and comparative studies of four broad-specificity immunoassays (conventional plate ELISA, plate ECL-ELISA, flow-through strip ELISA, and dip-stick strip ELISA) based on the same generic polyclonal antibody were investigated; these methods were validated by conventional HPLC method and compared in terms of sensitivity, precision, accuracy, and some other aspects. The detailed description and comparison maybe could help the successor to better understand and apply these developmental immunoassays.

Experimental

Materials and Instrumentation

Reagents

Sixteen sulfonamides were purchased from Sigma–Aldrich Co. (St. Louis, USA), bovine serum albumin (BSA), horseradish peroxidase (HRP), reagent grade 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, USA). Protein-A Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Purified water was obtained using a Millipore Milli-Q water system from Millipore (MA, USA).

Solutions

Phosphate-buffered saline (PBS, 10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.5), phosphate-buffered saline with 0.05% Tween 20 (PBS-T), coating buffer (CB, 50 mmol L^{-1} sodium carbonate buffer, pH 9.6), TMB substrate solution for conventional plate ELISA (prepared by adding 3.3 mg TMB in 250 μL DMSO to 25 mL of phosphate–citrate buffer (0.1 mol L^{-1} citric acid+0.2 mol L^{-1} Na_2HPO_4, pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution), and luminol substrate solution for ECL-ELISA (1.0 mmol L^{-1} luminol, 1.25 mmol L^{-1} *p*-iodophenol, 1.0 mmol L^{-1} H_2O_2 in 0.1 mol L^{-1} Tris–HCl buffer, pH 8.6) were used. Stock solution of sulfonamides was prepared by dissolving 1.0 mg of single sulfonamide in 1.0 mL of methanol and then kept at −20 °C for further dilution.

Instrumentation

Polystyrene 96-well microwell plates were from Nunc (Roskilde, Denmark), opaque high binding plates for chemiluminescent measurements were from Costar (Cambridge, USA), and the microplate washer was from Bio-Rad (Hercules, CA, USA). Immunoassay absorbance was read with a Multiscan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) in dual-wavelength mode (450–650 nm), and chemiluminescent intensity was measured with a Fluoroskan Ascent FL also purchased from Thermo. Nitro-cellulose membranes with a pore size of 0.45 μm were purchased from Pierce (USA). Semirigid polyethylene sheets and adhesive tape were purchased from local market. Filter paper and analytical grade buffer chemicals were purchased from Hope Biotech Co. Ltd. TEDA (Tianjin, China). Other chemicals were purchased from Sigma. HPLC analysis was performed on a Shimadzu HPLC system (Kyoto, Japan).

Experimental Methods

Hapten Synthesis and Antibody Production

Chemical structures of haptens used in this work are shown in Fig. 2. Hapten synthesis and the preparation of enzyme and protein conjugates were according to Zhang's report [6]. Generic polyclonal antibodies used in this report were produced by haptens D3 coupled to KLH as immunogen. Hapten D2 was coupled to HRP as enzyme tracer. Antibodies were produced in rabbits according to a general immune procedure and IgG from the antisera was purified by protein A-Sepharose 4B affinity chromatography.

Conventional Plate ELISA Procedure

Direct competitive ELISA was applied in this study to analyze the sulfonamides. The microwell plates were coated with purified antibodies at 1 μg per well in 100 μL CB, incubated overnight at room temperature. Plates were then washed three times with 10 mmol L^{-1} PBS-T and unbound active sites were blocked with 200 μL of 1% BSA in PBS per well for 1 h. After the plates were washed four times, for competitive assays, 100 μL standards in PBS (or diluted sample solution) and 100 μL hapten D2-HRP tracer in PBS were then added to each well and incubated for 1 h at room temperature. Following washing five times, the HRP tracer activity was then measured by adding 150 μL per well of TMB substrate solution. The enzymatic

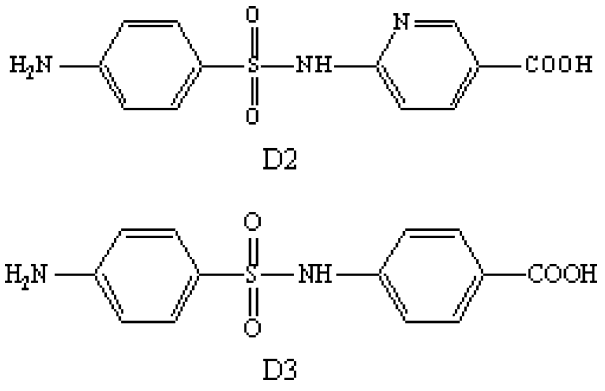


Fig. 2 Chemical structures of haptens studied in this work

reaction was stopped after 30 min by adding 2.5 mol L^{-1} H_2SO_4 (50 μL per well) and the absorbance was then read in dual-wavelength mode (450 nm as test and 650 nm as reference).

Plate ECL-ELISA Procedure

The coating, blocking, and competitive procedure were similar with the conventional plate ELISA. After the competitive reaction completed and the plate was washed, 150 μL of luminol substrate solution was added automatically to each well by a Fluoroskan Ascent FL. The intensity of light emission was measured immediately after the addition of the substrate (30 s) and the results were expressed in relative light units.

Flow-through Strip ELISA

Flow-through device The filter paper was wetted with a stream of distilled water from a wash bottle. After thoroughly shaking off excess water, it was placed between the membrane and the polyethylene card (acts as a supported material only). The air entrapped between the membrane and the absorbent body was removed by repeated rolling (two to three times) with a rimless tube or a glass tube under slight pressure over the membrane strip. Another dry filter paper could be placed at the edges of the device in order to absorb excess water.

Preparation of strips with antibody immobilization on the membrane Nitro-cellulose membrane strips (1.2×5 cm) were marked with a pencil to give four circles (diameter=8 mm). The field of the circle is the reaction zone. Nitro-cellulose membrane strips (1.2×5 cm) were soaked in Tris–HCl buffer (20 mmol L^{-1}, pH 8.0, 0.9% NaCl) for 1 h. The strips were then semi-dried by gently shaking off excess water in the air. Antibody was diluted with PBS and then applied in a volume of 20 μL at the center of each circle (1 μg/spot) with a microliter pipette. Attention should be paid to avoid the liquid reagent lateral spreading. After drying the membrane at room temperature for 15 min, it was further dried by incubating at 37 °C for 30 min. The vacant sites of the membrane were blocked with 1% BSA in PBS, and then washed three times with PBS-T. The strips were dried at 37 °C for 30 min and stored in desiccators at 4 °C.

Flow-through strip ELISA procedure Sulfonamide standards in PBS were mixed with enzyme tracer in PBS buffer. To each antibody spotted zone, 150 μL of mixture was added. After the liquid reagent flowed through the spotted zone, the membrane was thoroughly washed three times with PBS-T. After gently shaking off excess water, the membrane was placed on a glass plate. The substrate solution (1.25 mmol L^{-1} 3,3′,5,5′-tetramethylbenzidine–1.6 mmol L^{-1} hydrogen peroxide in acetate buffer, pH 5.0) was added uniformly over the membrane surface. After incubation for 2 min in substrate solution, the membrane was washed with water to stop the reaction. The color intensity of the test strip was visually compared with that of negative control (sulfonamide concentration is 0 μg L^{-1}) and a control of 5 μg L^{-1} (corresponding to the MRL). The intensity of the color of spot is inverse to the concentration of sulfonamide. The result should be observed immediately.

Dip-stick ELISA

Preparation of membrane strip for dip-stick ELISA The membrane was cut into sections (1.5×1.0 cm). Portions (1 μg/section) of antibody were spotted on the membrane with CAMAG Linomat 5 automatic TLC sampler. The test strips were dried at 37 °C for 30 min.

The remaining protein binding sites of the membrane were blocked by immersing the strips in 1% BSA in PBS. The strips were washed and dried, and then stored at 4 °C.

Dip-stick ELISA procedure The prepared test strips (antibody-coated) were mounted onto an inert plastic using double-sided adhesive tape. The antibody-coated strips were immersed in a mixture of sulfonamide standard solution and enzyme tracer. After incubation for 10 min (immersing strips in the mixture), the test strips were washed with 0.05% Tween 20 in PBS three times. Finally, the strips were immersed in a test tube containing 3 mL of substrate solution (H_2O_2–TMB) for 2 min. The assay was stopped by immersion in distilled water. The color intensity of the test strip was visually compared with that of negative control and a control of 5 μg L^{-1}. Since the color was not stable for long, the reading had to be done immediately.

Instrumentation for HPLC analysis

The test samples were verified using a Shimadzu HPLC equipped with a LC-10AT vp pump with Hamilton injector (25 μL loop), a DGU-12A online degasser, and a CTO-10AS vp column oven. A C_{18} reversed-phase column (25 cm×4.6 mm I.D., 5 μm) was used. The analysis was performed at 270 nm and the mobile phase was methanol–water (28:72) (water pH value was adjusted to 3.2–3.3 before mixed with methanol) at a flow-rate of 1.0 mL min^{-1}. The temperature of the column oven was 35 °C.

Sample Preparation

Four different matrices such as pig muscle, chicken muscle, egg, and fish samples were chosen according to several criteria, one of which is their presence in the table of established MRLs. These samples were bought from local markets. The sample tissues were minced with a conventional kitchen mixer. Before the spiking and recovery studies, each test sample was verified to contain sulfonamides less than 5 μg kg^{-1} by HPLC.

Sample preparation for ELISA For a spiking study of pig muscle, chicken muscle, egg, and fish, three different sulfonamides (the more, middle, and less sensitive sulfonamides of all the seven detectable sulfonamides in the real sample extracts) were individually spiked at three different levels (50, 100, and 200 μg kg^{-1}) in 4 g samples, 20 mL PBS was added, thoroughly mixed for 2 min, and then filtered using filter paper or centrifuged at 4,000×*g* for 10 min. The extracts were diluted 4-fold with PBS for pig muscle and chicken muscle, and 4-fold with 1% BSA–PBS for fish. The tubes containing egg samples were gently mixed for 30 min to avoid foam and emulsion firstly and then followed the above procedure. The extracts were diluted 4-fold with PBS before ELISA analysis. Three samples were prepared per concentration and the extracts were analyzed in triplicate.

Sample preparation for HPLC Three different sulfonamides were individually spiked at three different levels (50, 100, and 200 μg kg^{-1}) in 4 g samples; 10 g anhydrous $NaSO_4$ was mixed with the sample to absorb water. Twenty milliliters of acetonitrile was added, and then thoroughly mixed for 2 min, centrifuged at 4,000×*g* for 10 min. The residue was extracted again using 20 mL acetonitrile. The extracts were incorporated and the solvent of the upper liquid was removed under reduced pressure. The residues were dissolved in 2 mL mobile phase and then filtered with 0.45 μm filter membrane before HPLC analysis.

Results and Discussion

The immunoassays have been used since the 1980s, and was accepted as one of three primary methods (GC, HPLC, and immunoassay) for residue determination of some kinds of food sample by the Association of Analytical Communities; however, it is still necessary to evaluate their efficiency in different foodstuffs and to correlate their results with other accepted analytical methods such as HPLC.

Validation Studies of Two Plate ELISA Methods by HPLC

Validation studies of the conventional plate ELISA and the plate ECL-ELISA methods by HPLC were carried out by spiking experiments. Five sulfonamides were selected to be spiked in different animal food samples and then analyzed by two plate ELISA methods and HPLC, respectively. Results of validation studies of the conventional plate ELISA, plate ECL-ELISA, and HPLC were found to be statistically different, whereas there were no significant differences between them. Simple linear regression analysis between the results of conventional plate ELISA and HPLC or plate ECL-ELISA and HPLC for each sample are presented in Fig. 3. The correlation coefficients (R^2) of the conventional plate ELISA and HPLC data are 0.95 for pig muscle, 0.96 for chicken muscle, 0.93 for fish, and 0.93 for egg. The correlation coefficients (R^2) of the plate ECL-ELISA and HPLC data are 0.92 for chicken muscle and 0.93 for pig muscle. In summary, there are good correlations between the two plate ELISA methods and HPLC data for these samples, respectively. The slopes were all greater than 1.0 and indicated a high positive bias for the ELISA relative to the HPLC methods. This was possibly due to the loss of sulfonamides incurred as a consequence of sample cleanup and evaporation steps for HPLC analysis or the matrix effects of ELISA could not be avoided completely. Hongyo et al. [8] compared the monoclonal antibody-based ELISA with thin layer chromatography and HPLC for aflatoxin B1 determination in naturally contaminated corn and mixed feed and suggested using the same extraction solvent to increase the correlation between ELISA and HPLC methods. In addition, the same extraction procedure allows measuring only the effect of detection variances. The lower regression for HPLC–ELISA methods may be related to the absence of a cleanup step in the ELISA. Similarly, if the same cleanup procedure was performed in both methods, the regression obtained for HPLC and ELISA may be higher.

Validation Studies of Two Strip ELISA Methods by HPLC

Three sulfonamides were selected to be spiked in pig muscle sample and then analyzed by two strip ELISA methods and HPLC, respectively. The values of single sulfonamide spiked in pig muscle determined by the two strip ELISA methods were validated by HPLC. Table 1 gives an overview of the results obtained by flow-through and dip-stick ELISA compared to the values determined by HPLC. The two assays have demonstrated its accurateness: results of visual evaluation were in agreement with HPLC results on the whole. When analyzing the real samples, the results of ± (positive/negative, sulfonamide concentration was around MRL) should be further validated by instrumental methods such as HPLC. These rapid tests could offer simple, rapid, and cost-effective on-site screening tools to detect sulfonamides in animal food.

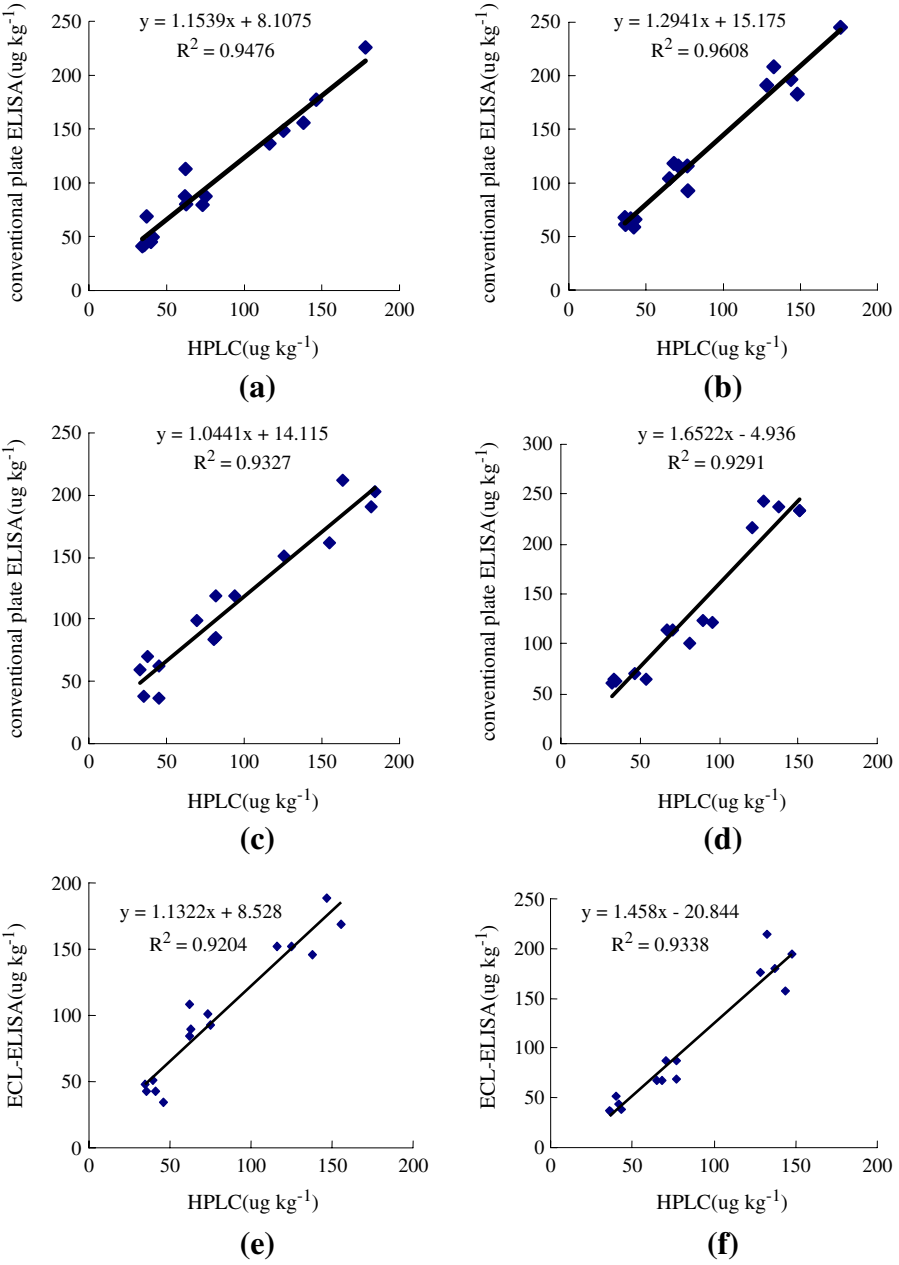


Fig. 3 Correlation between the two ELISA methods and HPLC results for each animal tissue spiked with single sulfonamide. Conventional plate ELISA: **a** pig muscle: R^2=0.95. **b** Chicken muscle: R^2=0.96. **c** Fish: R^2=0.93. **d** Egg: R^2=0.93. Plate ECL-ELISA: **e** Pig muscle: R^2=0.92. **f** Chicken muscle: R^2=0.93. *x* and *y* represent sulfonamide concentration (μg kg^{-1}) determined independently by HPLC and ELISA

Table 1 Comparison of results obtained by flow-through, dip-stick ELISA, and HPLC for pig muscle sample.

Analytes	Spiked level (μg kg^{-1})	Results of flow-through assay[a] (n=3)	Results of dip-stick assay[a] (n=3)	Results of HPLC validation (μg kg^{-1}) (n=3)
Sufameter	50	−, −, −	−, −, −	34.5
	100	±, ±, ±	±, ±, ±	62.6
	200	+, +, +	+, +, +	141.8
Sulfapyridine	50	−, −, −	−, −, ±	35.6
	100	±, ±, −	±, ±, ±	75.2
	200	+, +, +	+, +, +	146.4
Sulfamethoxypyridazine	50	−, −, −	−, −, −	41.2
	100	±, ±, −	±, ±, −	73.3
	200	+, +, +	+, +, +	128.9

[a] + Positive, sulfonamide concentration was more than MRL; − negative, sulfonamide concentration was less than MRL; ± positive/negative, sulfonamide concentration was around MRL

Table 2 Assay sensitivities of four assays for 16 sulfonamides.

Analytes	Conventional plate ELISA		Plate ECL-ELISA		Flow-through strip ELISA		Dip-stick ELISA	
	LOD[b] (μg L^{-1})	IC_{50}[a] (μg L^{-1})	LOD[b] (μg L^{-1})	IC_{50}[a] (μg L^{-1})	LOD[c] (μg L^{-1})	SCC[d] (μg L^{-1})	LOD[c] (μg L^{-1})	SCC[d] (μg L^{-1})
Sulfisozole	0.1	1.3	0.1	0.73	1	20	5	50
Sulfathiazole	0.2	2	0.14	0.76	1	20	5	50
Sufameter	0.4	3.2	0.21	1.25	1	20	5	50
Sulfamethoxypyridazine	1.5	8.4	0.43	2.73	1	50	5	50
Sulfapyridine	1.6	10	0.39	2.92	1	50	5	50
Sulfamethizole	2.1	12.8	0.55	10.4	5	50	10	100
Sulfachlorpyridazine	3.0	14	6	30	5	100	10	100
Sulfamerazine	6.2	19	5.1	15.6	10	100	10	200
Sulfadoxine	6.8	31	9.5	20.5	20	500	20	250
Sulfadiazine	7.0	32	6	32	20	500	50	500
Sulfaquinoxaline	10	90	3.8	75	50	1,000	50	500
Sulfamethazine	22	130	6.2	140	100	1,000	100	1,000
Sulfamethoxazole	23	135	20.5	108	100	1,000	150	2,000
Sulfamoxole	27	150	31	67	150	2,000	200	5,000
Sulfadimethoxine	27	190	6.5	180	200	5,000	250	5,000
Sulfisoxazole	80	>200	47	230	500	5,000	500	10,000

[a] IC_{50} was defined as the concentration of analytes causing 50% inhibition of color development for conventional plate ELISA and plate ECL-ELISA

[b] LOD was defined as the concentration of analytes causing 15% inhibition of color development for conventional plate ELISA and plate ECL-ELISA

[c] LOD was defined as the concentration of visual detection limit for flow-through strip ELISA and dip-stick ELISA

[d] SCC was defined as the concentration of suppressing the color development completely for flow-through strip ELISA

Comparative Studies of Four ELISA Methods

The sensitivity of different methods was compared in Table 2. The sensitivity (IC_{50}) of plate ECL-ELISA was about two to three times higher than conventional plate ELISA and the limits of detection (LOD, IC_{15}) are lower than the latter. However, the precision of the conventional plate ELISA was better than plate ECL-ELISA. These two methods all could be used as quantification methods. The results of the flow-through and dip-stick strip ELISA were read by eye and the plate ELISA methods by multi-scan spectrum. So the strip ELISA was obviously less sensitive than the plate ELISA. However, these two strip methods for the detection of sulfonamides in animal food samples use limited operational steps, providing a yes or no response indicating whether or not sulfonamides are above the MRL (100 μg kg^{-1}) and could be used as on-site fast screening methods. Under identical conditions, it was found that flow-through assay has a higher sensitivity than dip-stick assay and the dip-stick assay was less affected by matrix effect than flow-through assay. This may be because the dip-stick ELISA is a specific immunoadsorption assay and the flow-through ELISA is an immunoconcentration assay.

The intra-assay and inter-assay reproducibility was determined to study the assay precision of conventional plate ELISA and plate ECL-ELISA. The variations of percent inhibition for 20, 10, 5, 2.5, 1.25, 0.63, and 0.31 μg L^{-1} of sulfapyridine tested three times on the same day were 3.8, 3.1, 4.2, 7.5, 11.8, 11.4, and 14.7% for the conventional plate ELISA and 5.9, 6.2, 10.7, 15.1, 17.8, and 20.4% for the plate ECL-ELISA, respectively. The assay of the same spiked material run over 5 days gave deviations from the means of 4.2, 5.6, 8.3, 10.9, 13.7, 15.5, and 18.2% for the conventional plate ELISA and 7.2, 9.0, 13.2, 15.4, 19.3, and 22.8% for the plate ECL-ELISA, respectively. To investigate the reproducibility of flow-through and dip-stick strip ELISA, four different concentrations (0, 1, 5, and 10 μg L^{-1}) of sulfapyridine were spotted to the same antibody-coated strip and observed if the color was deeper with the concentration increased, namely, the color has obvious grads. Results showed that eight times there have been obvious grads of flow-through ELISA and nine times there have been obvious grads of dip-stick ELISA for all ten experiments.

Conclusions

Immunoassays have the benefits of being inexpensive and rapid and can be performed on simple, inexpensive equipment. In addition, immunoassays are able to simultaneously analyze a large number of samples with minimal sample cleanup. Immunoassays provide simple, sensitive, cost-effective, and efficient methods for the analysis of animal food samples potentially contaminated with sulfonamides. At the same time, further analysis must be carried out using HPLC method to quantify accurately the content of sulfonamide in the positive samples. Studies should be extended to the analysis of other substrates that may be predisposed to sulfonamide contamination, taking into account the matrix interferences in ELISA assays. The conventional plate ELISA and plate ECL-ELISA could be used as quantification methods for large numbers of samples and complements of the conventional analytical methods in the laboratory. The flow-through and dip-stick strip ELISA using limited operational steps and not needing any instrumental equipment for the detection of sulfonamides can provide a yes/no response indicating whether or not sulfonamides are above the MRL and could be used as valuable tools for the rapid on-site screening of sulfonamide residues in animal food samples.

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