NOTE *Public Health*

Direct Competitive Enzyme-Linked Immunosorbent Assay for Sulfamethazine

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ABSTRACT. A direct competitive enzyme-linked immunosorbent assay (ELISA) for screening sulfamethazine (SMZ) in pork tissues was developed. The assay was made with the affinity-purified polyclonal antibody-coated microtiter plate. A cross reactivity of IgG was observed at 3.5 μg/g of sulfamerazine among nine kinds of sulfonamide tested. Pork tissues fortified with SMZ was mixed with octadecyl silica (C_{18}), and extracted with dichloromethane. The extracted SMZ was measured by homemade ELISA, commercial ELISA, and HPLC. The results were correlated ($r=0.993$, $p<0.01$). The homemade ELISA was sensitive to determine SMZ at the maximum residue level (MRL) as commercial one. During stability test of the IgG coated microtiter plate performed at 40°C for 14 days, no difference in sensitivity was observed. We developed homemade ELISA with a detection limit of 10 *n*g of SMZ per g of pork tissues, and it could be used to screen SMZ in pork tissues.

KEY WORDS: ELISA, pork, sulfamethazine.

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Sulfamethazine (SMZ) is used therapeutically to cure infections and prophylactically to control the outbreak of diseases, to improve feed efficiency, and to promote growth of animals. SMZ is a suspected carcinogen that is frequently found as a major residue in tissues [9, 15]. The FDA set the maximum residue level (MRL) of SMZ in swine tissues (liver, kidney, and muscle) at 0.1 ppm. The period of disposal of SMZ is fifteen days. The current quantitative analysis methods for SMZ in tissues and feeds are gas [4, 11] and liquid [7, 8, 17] chromatography and mass spectrometry [1, 3, 19]. These analytical techniques are expensive and require both specialized equipment and highly trained analysts. Depending on the amount of the sample preparation needed, the analysis may take several days to complete. Therefore, screening large numbers of samples is difficult and substitutional methods are required [18]. Such qualitative analysis methods are bioassay [15] and thin layer chromatography [12, 13, 16]. However, the bioassay has poor sensitivity to sulfonamides. The direct competitive ELISA method is sensitive and has a relatively high specificity for SMZ residue determination [5, 6]. These advantages make ELISA an excellent tool for screening large numbers of samples for residue monitoring. As many as 2,400 samples can be analyzed in 8 hr [2]. The commercially available ELISA kits cost high compared with the bioassay, and this may be one obstacle to use ELISA in field [10]. In this study, we developed a homemade ELISA for quantifying SMZ residues in pork tissues.

A direct competitive ELISA was developed by using affinity-purified antibody, SMZ-bovine serum albumin (BSA) conjugate, and SMZ labelled with horseradish peroxidase (HRP). SMZ was conjugated to BSA, HRP, and gelatin by the glutaraldehyde method [5]. Briefly, BSA, HRP, or gelatin was dissolved 2:1 in 75 m*l* of 0.1 M phosphate buffer; dioxane and 0.35 m*l* of 25% glutaraldehyde were added to the solution. The solution was stirred gently for 3 hr at room temperature and then dialyzed for 6 days at 4°C against 0.1 M phosphate buffer with twice changes a day. An immunogen of SMZ-BSA conjugate in 1 m*l* of 0.1 M phosphate buffer/Freund's complete adjuvant mixture (1:1, vol/vol) was injected subcutaneously into the rabbit. A booster immunization with an immunogen prepared in 1 m*l* of 0.1 M phosphate buffer/Freund's incomplete adjuvant mixture (1:1, vol/vol) was given every two weeks until a satisfactory titer was obtained. Antiserum was purified by immuno-affinity chromatography [14]. The resin, CNBr-activated Sepharose 4B, was preserved in 1 mM HCl, washed with distilled water, and then washed with the coupling buffer. The SMZ-gelatin conjugate was dissolved in the coupling buffer and stirred for 2 hr. To reduce nonspecific absorption of proteins, a 0.2 M glycine solution in phosphate buffer was mixed with the gel. The wash cycle was completed with alternating high and low pH buffer solutions four or five times. The antiserum diluted in the coupling buffer was applied to the gel. The pH was reduced with the glycine/HCl elution buffer to dissociate bound complexes.

Microtiter plates were coated with IgG [illegible] incubated for 5 hr at 40°C. The plates were washed four [illegible] with distilled water and patted dry with paper towels. The plates were blocked by adding 200 μl of 1% ovalbumin in 0.1 M PBS. After 30 min of incubation, SMZ standards or samples mixed with a diluted SMZ-HRP conjugate solution (1:1, vol/vol) were added. The plates were incubated for 1 hr at 37°C and washed to remove any unbound SMZ and SMZ-HRP conjugate. Substrate (0.009% 3,3',5,5'-tetramethy-benzine) was added and the reaction was stopped after 20 min with a 2 M H_2SO_4 solution. The absorbances were then read in a dual-wavelength mode (450–650 nm). A calibration curve was obtained by direct ELISA by using serially diluted SMZ standard solutions. The concentrations of the SMZ standards

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used were 0.01, 0.05, 0.1, 0.5, 1 and 5 μg/g. SMZ standard and sample were diluted with PBS containing Tween-20 to reduce nonspecific binding of interrupting substances.

The specificity of IgG was determined with nine kinds of sulfonamide (sulfamerazine, sulfaquinoxaline, sulfanilamide, sulfathiazine, sulfapyridine, sulfamic acid, sulfadimethoxine, sulfisoxaxole, and sulfaguanidine) at concentrations of 0.1, 1, and 10 μg/g. The concentrations of SMZ in pork tissues were determined by homemade ELISA, commercial ELISA (Neogen Co., U.S.A.), and high-performance liquid chromatography (HPLC), and the results were compared. The mobile phase was 20% acetonitrile containing 1% acetic acid. The flow-rate was 1 ml/min. A HPLC equipped with an UV-VIS detector and a Nucleosil 100–5 C_{18} column with an internal diameter × length of 7.6 × 300 mm was used. Duplicate stock solutions of SMZ in pork were prepared at concentrations of 0.05, 0.1, 0.5, and 1 μg/g. Pork tissues (0.5 g) injected with SMZ were mixed with octadecyl silica 15–40 μm (C_{18}, 2 g) and packed into a glass column. Then, the column was eluted with hexane and dichloromethane. The dichloromethane eluate was evaporated, and resolved with the mobile phase. A sample filtered through a 0.45-μm filter was injected into the HPLC system. A 0.1 m*l* aliquot of standard solution of SMZ was added to SMZ free pork tissues (spiked at 0.05 μg/g) in the column. Average SMZ recovery for SMZ-fortified pork tissues was 90%. The matrix effect was minimized by diluting the samples (1:1, vol/vol) before direct competitive ELISA. The stability of the anti-SMZ IgG-coated microtiter plate at elevated temperatures was tested at 40°C for 0, 1, 3, 7, and 14 days.

The results of the direct competitive ELISA produced a linear range from 0.01 to 5 μg/g SMZ (Fig. 1). The detection limit of this assay was 10 *n*g/g SMZ, and it was below the regulatory limit (0.1 μg/g). A cross reaction of the anti-SMZ IgG was observed at 3.5 μg/g of sulfamerazine, which was defined to inhibit 50% antibody binding. The cross reactivity was not observed at 10 μg/g of the other kinds of sulfonamide tested; sulfaquinoxaline, sulfanilamide, sulfathiazine, sulfapyridine, sulfamic acid, sulfadimethoxine, sulfisoxaxole, and sulfaguanidine (Fig. 2). This anti-SMZ IgG was reported not to react with the sulfonamides commonly used except sulfamerazine, or with procaine penicillin, chlortetracycline, or *p*-aminobenzoic acid [6]. The SMZ concentration in pork tissues fortified with 0.05 to 1 μg SMZ/g was determined by homemade ELISA and HPLC. There was agreement between the two analytical methods (Pearson correlation = 0.993, $p<0.01$, Fig. 3). The detection limit was 3 *n*g/g for commercial ELISA and 10 *n*g/g for homemade ELISA. The homemade ELISA was sensitive to determine SMZ at MRL (100 *n*g/g) as was commercial one. The IgG-coated microtiter plate was incubated at 40°C, and subjected to direct ELISA during incubation. No difference in percent binding (B/Bo %) of SMZ was observed for up to 14 days (Fig. 4). However, the color reaction decreased with time. The results of this stability test indicate that IgG-coated microtiter plates would be stable for at least 2 years.

The ELISA method was simpler, and samples could be analyzed faster than by the HPLC method. Because of the possibility of false positive results of ELISA owing to unspecific anti-SMZ IgG reacted with metabolites of SMZ, confirmatory assay is still required for all legal and statutory purposes. However, this ELISA method could exclude negative samples before HPLC assay, and reduce the cost of quantitative assays. This paper presents a homemade direct competitive ELISA to efficiently determine the concentration of SMZ. The affinity-purified anti-SMZ IgG can be immobilized on the membrane or plastic beads [14]. This can be used for 'on-site' residue screening.

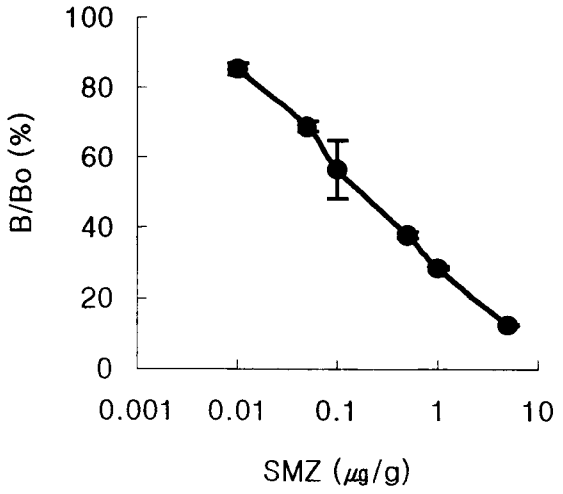

Fig. 1. Standard curve of SMZ obtained by ELISA. The error bar indicates standard deviation. B: Absorbance of SMZ standard, Bo: Absorbance of blank solution.

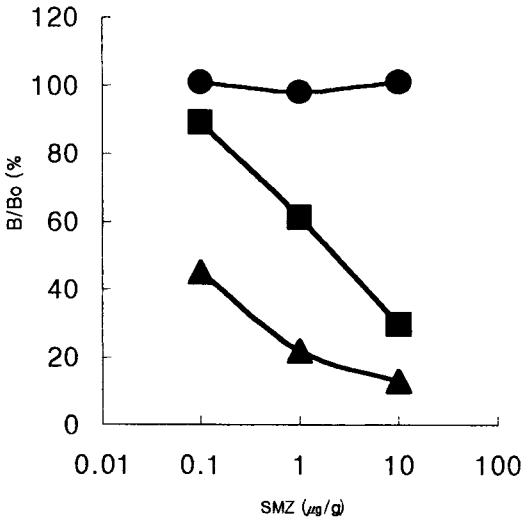

Fig. 2. Cross reactivity of anti-SMZ IgG. SMZ (▲), sulfamerazine (■), other sulfonamides* (●). *sulfaquinoxaline, sulfanilamide, sulfathiazine, sulfapyridine, sulfamic acid, sulfadimethoxine, sulfisoxaxole, and sulfaguanidine

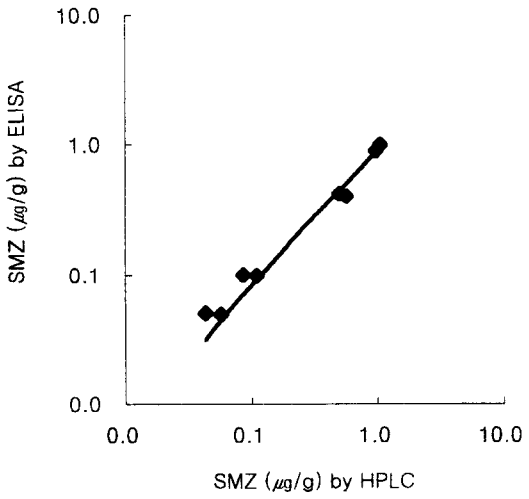

Fig. 3. Comparison of SMZ concentrations determined by ELISA and HPLC in pork samples (n=8) (r=0.993, p<0.01). SMZ was added into the samples.

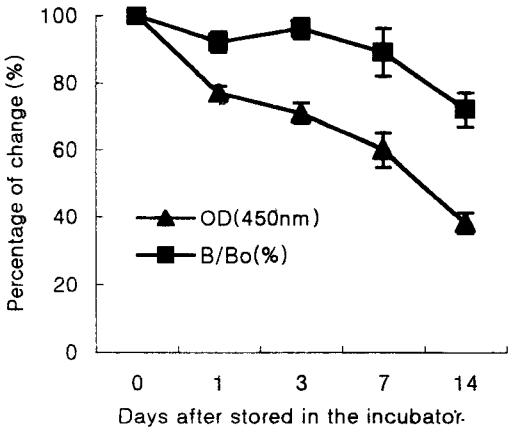

Fig. 4. Effects of the storage period on the stability of IgG-coated microtiter plates at 40°C.

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