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# Direct Competitive ELISA for Sulfamethazine

Department of Veterinary Medicine Graduate School,
Cheju National University



Eun -A Ko

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**Eun A Ko**
**(Supervised by professor Jun Hong Park)**

**Department of Veterinary Medicine**
**Graduate School, Cheju National University**

## Abstract

Law in most countries has regulated residual sulfamethazine (SMZ) in livestock products. The assay methods for SMZ have some difficulties. To reduce these obstacles, a direct competitive enzyme-linked immunosorbent assay (ELISA) was developed to screen SMZ residues. Polyclonal antiserum to SMZ was raised in rabbit. Immunoaffinity purified immunoglobin G (IgG) was used as a coating capture antibody. SMZ in free form and SMZ- horseradish peroxidase conjugated form were competed. Color reaction was determined at 450nm. The direct competitive ELISA showed linear ranging from 0.01 to 5 $\mu$g/g SMZ. Cross reactivity of the IgG was 89, 62 and 30% at 0.1, 1 and 10$\mu$g/g of sulfamerazine, respectively, but negligible in the other sulfonamides used. The spiked SMZ in serum (0.05 and 0.1$\mu$g/g) was determined, and the recovery was calculated as 99%. SMZ concentration in swine muscles was determined by direct competitive ELISA and compared with HPLC ranging from 0.05 to 1$\mu$g/g. The results of direct competitive ELISA and HPLC were correlated ($r=0.993$, $p<0.01$). These indicate the homemade ELISA would be accurate to HPLC. A stability test, in which, the IgG coated microtiter plate stood at 40℃, showed no difference in B/Bo (%) of SMZ until 14 days. This result indicates the IgG coated microtiter plate would be stable for 2 years. The ELISA method developed in this experiment was simpler, faster, and could be used for a screening tool.

**Key words**: Sulfamethazine, ELISA, Immunoglobulin G, Cross reactivity.

# Contents

# Ⅰ. Introduction

Sulfamethazine (SMZ), one of the most important sulfa antimicrobials, has been identified as the major problem in approximately 95% of all violations in tissue residues involving sulfonamides (Bushway and Fan, 1995). Sulfonamides are used therapeutically to cure infections and prophylactically to control the outbreak of diseases, to improve feed efficiency, and to promote growth. The presence of sulfa drug residues in livestock products has been reported in several investigations (Augsburg , 1989, Stahr *et al*., 1991, Shin and Kim, 1994). SMZ is a suspected carcinogen, which has often been identified as the major problem in tissue residue (Littlefield *et al*., 1990, Sternesjo *et al*., 1995). The U.S. FDA revealed in 1988 that it could promote the development of thyroid cancer and be hazardous to humans. The carcinogenic drug had been in the food supply for about 40 years without opposition (Augsburg, 1989). The FDA established the maximum residue level (MRL) of SMZ in swine tissues (liver, kidney or muscle) to be 0.1ppm. The withdrawal period of SMZ is fifteen days. Now, national screening of SMZ residue in animal products shows over 80,000 cases in 1998 (Korean Ministry of Agriculture and Forestry, 1998).



The current analysis methods for SMZ in tissues and feeds are bioassay (Sternesjo *et al.,* 1995), gas chromatography (Chiavarino *et al*., 1998, Reeves, 1999), liquid chromatography (Hah *et al*., 1994, Tsai and Kondo, 1995, Casetta *et al*., 1996, Liang *et al*., 1996, Maxwell and Lightfield, 1998, Combs and Ashraf-Khorassani, 1999), thin layer

chromatography (Reimer *et al.*, 1991, Shearanm *et al.*, 1994, Szabo and Winefordner, 1997) and mass spectrometry (Boison and Keng, 1994, Casetta *et al.*, 1996, Volmer, 1996). These analytical techniques are expensive, and they require specialized equipment, and a highly trained analyst. Depending on the amount of sample preparation needed, the analysis can take several days to complete. As a result, the screening of large numbers of samples has been limited and supplementary methods are required (Unruh *et al.*, 1993). For this reason, direct competitive ELISA was developed. This method provides sensitivity, and a relatively high degree of specificity for SMZ residue determination (Dixon-Holland and Kats, 1988, Dixon-Holland and Kats, 1991). These advantages also make ELISA excellent tools for screening of large numbers of samples for residue monitoring. As many as 2,400 samples could be analyzed in 8 hours (Bushway and Fan, 1995). Some ELISAs for SMZ were reported (Ashworth *et al.*, 1985, Holland *et al.*, 1988, Ram *et al.*, 1991, Stahr *et al.*, 1991, Lim and Kim, 1996, Haasnoot, 1996) and there are many different types of ELISAs in principles. (Thorell and Larson, 1978, Voller *et al.*, 1979, Ausubel *et al.*, 1995, Venkatesh and Murthy, 1996): (1) Antibody binds to antigen in the solid phase and is subsequently detected with an enzyme-labelled second antibody (indirect or sandwich ELISA). (2) Antibody can be measured by competition with enzyme-labelled antibody for antigen on the solid phase (competitive ELISA for detection antibody). (3) Competition between labelled and un-labelled antigen for binding to antibody on the solid phase can be used for measurement of antigen (competitive antigen capture ELISA).

This method offers an easy, rapid way of measuring antigen (Yolken and Leister, 1981).

We developed a direct competitive ELISA for the quantitation of SMZ residues in swine muscle and blood.

## Ⅱ.Materials and Methods

Freund's adjuvants, horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin, glutaraldehyde, 3,3', 5,5'-tetramethyl-benzidine (TMB), SMZ, 2-2'-azino-di-3-ethyl-benzthiazoline sulfonic acid (ABTS), $C_{18}$ (Octadecylsilyl-derivatized silica) were purchased from Sigma Chemical Co., in the USA. CNBr-activated Sepharose 4B were purchased from Pharmacia Biotech.in Sweden. 96 well microtiter plates were purchased from Costar Co., in the USA. Absorbances for ELISAs were measured with a microplate reader (Columbus, SLT-LAB instrument, Austria) in dual-wavelength mode (450-650nm). High-performance liquid chromatography (HPLC) equipped with UV-VIS detector (UV1000, Thermo Separation Production, USA) and Nucleosil 100-5 $C_{18}$ column with a length x internal diameter of 7.6 x 300mm was used.



SMZ was conjugated to BSA, HRP, or gelatin by glutaraldehyde method (Dixon-Holland and Kats, 1988). Briefly, BSA (600 ㎎), HRP (10 ㎎), or gelatin (600 ㎎) was dissolved in 75 ㎖ of 2:1 solution of 0.1M phosphate buffer (pH 7.2), dioxane and 0.35 ㎖ of 25% glutaraldehyde was added to the solution. The solution was mixed gently for 3 hours at room temperature. The solution was dialyzed for 6 days at 4℃ against 0.1 M phosphate buffer (pH 7.0) by changing twice a day.

Anti-SMZ was raised in a rabbit. Immunogen (100 ㎍) of SMZ-BSA conjugate in 1㎖ of 0.1 M phosphate buffer (pH7.2) /Freund's complete adjuvant

mixture (1:1, v:v) was injected into a rabbit subcutaneously (8-week-old female New Zealand White). Booster immunization of immunogen prepared in 1㎖ of a 0.1 M phosphate buffer (pH 7.2) /Freund's incomplete adjuvant mixture (1:1, v/v) were injected every two weeks (Thorell and Larson, 1978, Harlow and Lane, 1988). The rabbit was bled periodically through the ear vein to measure antibody titers. The rabbit was boosted until a satisfactory titer was obtained.

Antiserum titer was determined by an indirect competitive ELISA. Binding of the antiserum onto the wells coated with SMZ-gelatin conjugate (SMZ-Gel) was determined by reaction with HRP labeled protein A (protein A -HRP) and enzyme substrate (0.1% ABTS). Color reaction was determined at 405nm.

Antiserum was purified by immunoaffinity chromatography (Scopes, 1984, Ferencik, 1993, Staak *et al.*, 1996). The resin, CNBr-activated sepharose 4B, was preserved in 1mM HCl, washed with D.W. and then washed with the coupling buffer (0.1M $NaHCO_3$ buffer, pH8.3 containing NaCl). SMZ-gelatin conjugate was dissolved in coupling buffer. The solution was mixed with the gel for 2 hours. To reduce nonspecific absorption of proteins, a solution of 0.2M glycine in phosphate buffer was mixed with the gel. A wash cycle was completed by high and low pH buffer solutions four or five times. Antiserum diluted in coupling buffer was applied to the gel. Bound complexes were disrupted by a reduction on pH using glycine/HCl elution buffer.

A direct competitive ELISA was developed using the affinity purified antibody, SMZ-BSA conjugate and SMZ labelled with HRP. Microtiter

plates were coated with IgG (1:160, v/v) and allowed to incubate for 5 hours at 40℃. The plates were washed four times with D.W. and patted dry on paper towels. Microtiter plates were blocked by adding 200 ㎕ of 1% ovalbumin in 0.1 M PBS (w/v). After 30 minutes of incubation, standards of SMZ or samples mixed (1:1, v/v) with diluted SMZ-HRP conjugate solution (1:200, v/v) were added. The plates were incubated for 1 hour at 37℃ and washed to remove any unbound SMZ and SMZ-HRP conjugate. Substrates (0.009% TMB) were added and the reaction was stopped after 20 min with 2M $H_2SO_4$ solution. The absorbances were then read in a dual-wavelength mode (450-650nm). The calibration curve was obtained by the direct ELISA established using serially diluted SMZ standard solutions and SMZ concentrations in samples.

PBS-T and PBS were used for diluting the sample solution to characterize the effect of detergent (Tween 20). The optimal dilution of IgG necessary for the ELISA was determined by the checkerboard test. Variously diluted IgG (1:20, 1:40, 1:80, 1:160, 1:360, v/v) was incubated with SMZ-HRP conjugate solution (1:200, v/v). The concentrations of SMZ standards used were 0.01, 0.05, 0.1, 0.5, 1, and 5 ㎍/g. SMZ standard and sample were diluted with PBS containing Tween-20 to reduce nonspecific binding of interrupting substances. Recovery tests of SMZ (0.05 and 0.1㎍/g) in swine serum were carried out. The swine serum was diluted 1:40 (v/v) in PBS-T to reduce nonspecific absorption of proteins before the recovery test. The specificity of IgG was determined using 9 sulfonamides (sulfamerazine, sulfaquinoxaline, sulfa-nilamide, sulfathiazine, sulfapyridine, sulfamic acid, sulfadimethoxine,

sulfisoxaxole, and sulfaguanidine) at the concentrations of 0.1, 1, and 10 ㎍/g. The concentrations of SMZ spiked in pork tissues were determined by the direct competitive ELISA and HPLC, which compared the results. Pork tissue (0.5 g) was mixed with $C_{18}$ (2 g) and packed at glass column. Then, the column followed by hexane and dichloromethane. The solution was transferred to the reservoir and a vacuum. Residual dichloromethane was removed by evaporation and then added mobile phase prior to HPLC analysis. The mobile phase was a 20% acetonitrile containing 1% acetic acid. The flow-rate was 1㎖ /min. Stock solutions of SMZ were prepared at concentrations of 0.05, 0.1, 0.5 and 1㎍/g each in PBS-T. The matrix effect was minimized by diluting the samples (1:1, v/v) before direct competitive ELISA. To estimate the coupling capacity of gel for antiserum, samples of the coupling buffer diluted IgG (0.5:1, 1:1, 2:1, v/v) were taken before addition to the gel. The eluated purified antibody was tested in a direct competitive ELISA system for it's specific reactivity. The stability of IgG stored at elevated temperatures with IgG was tested at 40℃ for 0, 1, 3, 7, and 14 days, respectively.

## Ⅲ. Results

A typical titration curve for the antiserum against SMZ in a rabbit immunized with antigen (SMZ-BSA) was obtained. High antibody titers were obtained from a rabbit after a 3rd boosting immunization. Antibody titer was determined by a direct competitive ELISA in the assay system to compete the binding of SMZ-gelatin coated to the solid phase with the antiserum (Figure 1). Even though the wells were blocked with ovalbumin, SMZ-HRP was bound to the well surface. This nonspecific binding could be overcome by diluting it in PBS containing tween-20 (PBS-T) (Figure 2). Optimal diluting of affinity purified anti-SMZ antibody was determined by ELISA using variously diluted IgG. The optimal dilution of IgG was reached with the 1:160 (Figure 3). This concentration was chosen for the subsequent experiments. The direct competitive ELISA shows linear range from 0.01 to 5$\mu$g/g SMZ (Figure 4). The detection limit of this assay was below the regulatory limits (0.01$\mu$g/g). The cross reactivity of the anti-SMZ IgG was 89, 62 and 30% at 0.1, 1 and 10$\mu$g/g of sulfamerazine, respectively, but neglectible against sulfaquinoxaline, sulfanilamide, sulfathiazine, sulfapyridine, sulfamic acid, sulfadimethoxine, sulfisoxaxole, and sulfaguanidine (Figure 5). Recoveries of SMZ 0.05$\mu$g/g and 0.1$\mu$g/g in spiked swine sera ranged from 96% to 102% (Table 1). SMZ concentration in swine muscles, which were fortified with SMZ ranging from 0.05 to 1$\mu$g/g, was determined by ELISA and HPLC. These results indicate the agreement between the two analytical methods (Pearson correlation = 0.993, $p<0.01$, Figure 6). The ELISA method was simpler, and samples could be analyzed fast when

compared to the HPLC methods. Figure 7 shows those protein concentrations increased when the dilutions of antiserum for affinity column decreased. Figure 8 shows those higher dilutions of antiserum and a smaller amount of specific eluate was observed. The IgG coated microtiter plate stood at 40℃ incubator, and subjected to direct ELISA during incubation. No difference in percent binding (B/Bo %) of SMZ was observed until 14 days (Figure 9). However, color reation decreased over time.

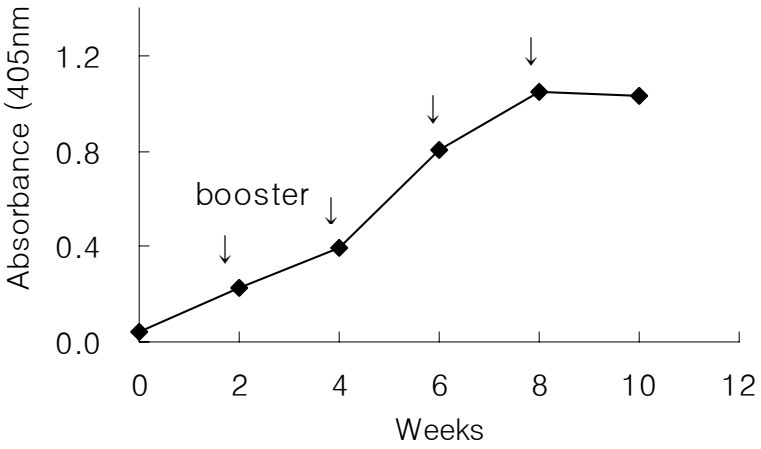


Figure 1. Antiserum titer of immunized rabbit. Each well was coated with 100$\mu\ell$ of SMZ-gelatin (1:20,000), and the coating antigen was reacted with antiserum against SMZ (1:800) and then with protein A conjugated to HRP (1:5,000).

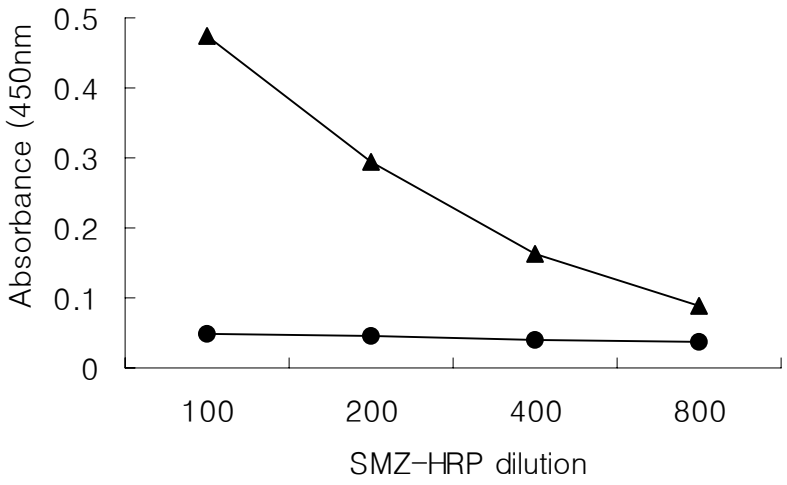


Figure 2. Effect of detergent (Tween 20) on nonspecific binding SMZ-HRP to plate well. The well was coated with affinity purified antibody, blocked with ovalbumin, SMZ-HRP reagent, substrate and stop solution, were added sequentially. SMZ-HRP diluted in PBS containing Tween 20 (●). SMZ-HRP diluted in PBS (▲).

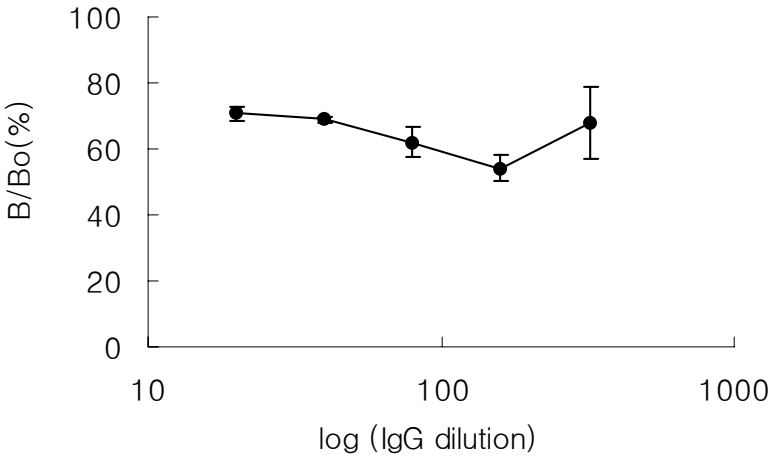


Figure 3. Determination of the optimal dilutes of anti-SMZ antibody. Each well was coated with varying dilutions of immunoaffinity purified IgG. SMZ (0.1$\mu$g/g) and SMZ-HRP (1:200) in PBS-tween 20 solution were added. B/Bo (%) were calculated according to the following formula: B/Bo (%) = (absorbance at 0.1$\mu$g/g of SMZ/absorbance at 0$\mu$g/g of SMZ) ×100. The error bar indicates standard deviation.

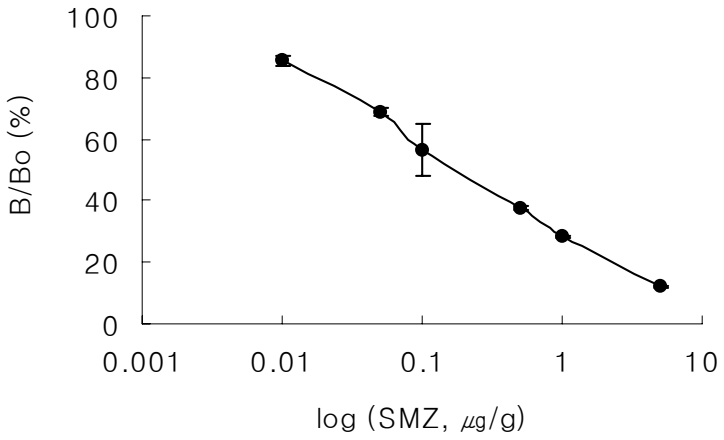


Figure 4. Standard curve of SMZ obtained by the direct competitive ELISA. Each well was coated with anti-SMZ IgG (1:160). SMZ standard and SMZ-HRP conjugate (1:200) solution were reacted. B: Absorbance of SMZ standard, Bo: Absorbance of blank solution.

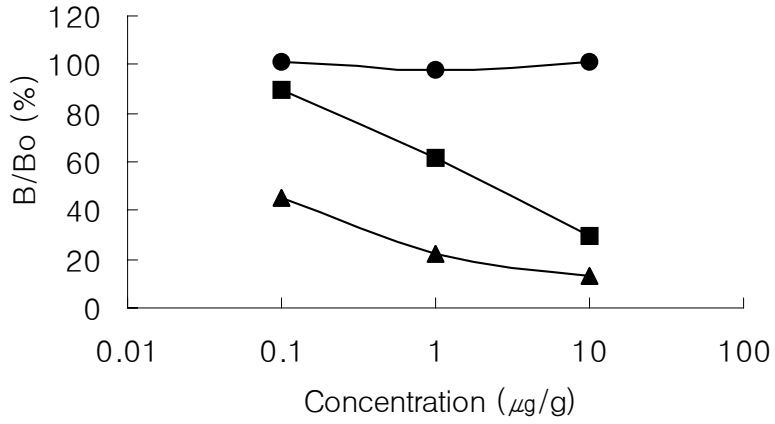


Figure 5. Cross reactivity of anti-SMZ IgG to SMZ ( ▲), sulfamerazine ( ■) and other sulfonamides ( ●).

Table 1. Recovery of SMZ spiked in swine serum (n=3).

| No | SMZ ($\mu$g) | OD (450)nm | B/Bo (%) | Recovery (%) |
|---|---|---|---|---|
| | 0 | 2.751(0.07 | 100 | 100 |
| 1 | 0.05 | 2.058(0.07 | 75 | 96 |
| | 0.1 | 1.745(0.04 | 63 | 102 |
| | 0 | 2.444(0.03 | 100 | 100 |
| 2 | 0.05 | 1.766(0.02 | 72 | 100 |
| | 0.1 | 1.528(0.01 | 63 | 102 |

SMZ was fortified to swine serum and diluted with PBS containing Tween 20 (1:40).

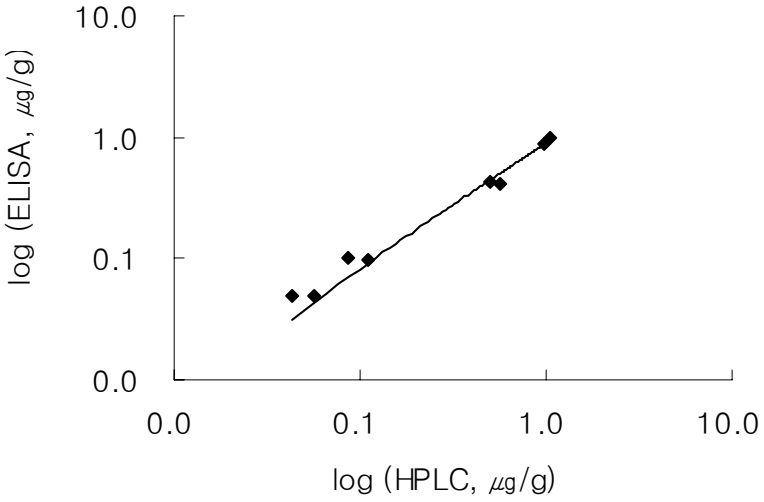


Figure 6. Correlation of SMZ determination between ELISA and HPLC. Each point represents SMZ concentrations obtained by ELISA and HPLC from the pork meat samples spiked with SMZ (◆, n=8). (Pearson correlation = 0.993, $p<0.01$)

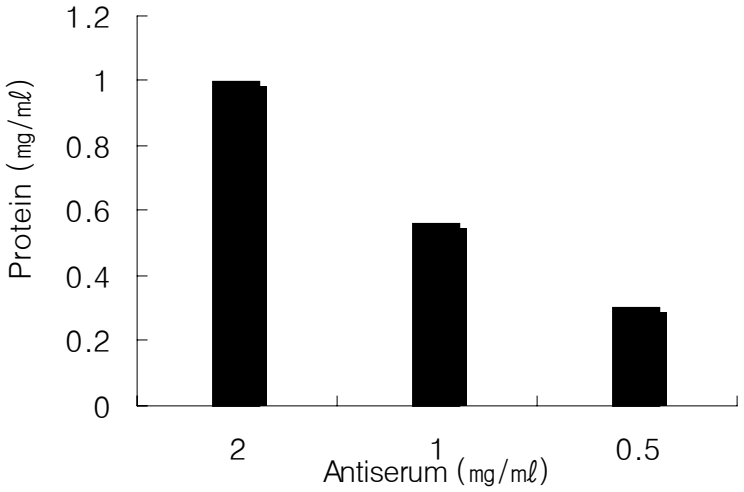


Figure 7. Protein concentrations data of glycine/HCl eluated antiserum ('specific eluate') after affinity chromatography.
Protein concentration (㎎ Protein/㎖) = 280nm ×Correction Factor (280/260nm)

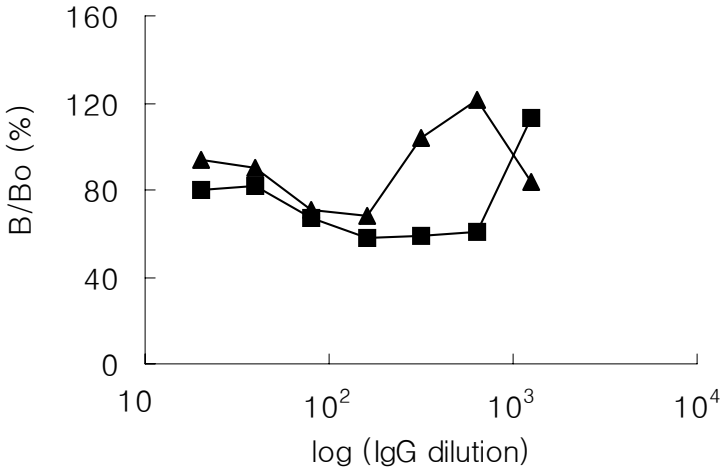


Figure 8. The eluated purified antibody was tested in a direct competitive ELISA system. Antiserum: coupling buffer = 2:1 ( ■), antiserum: coupling buffer = 1:1 ( ▲)

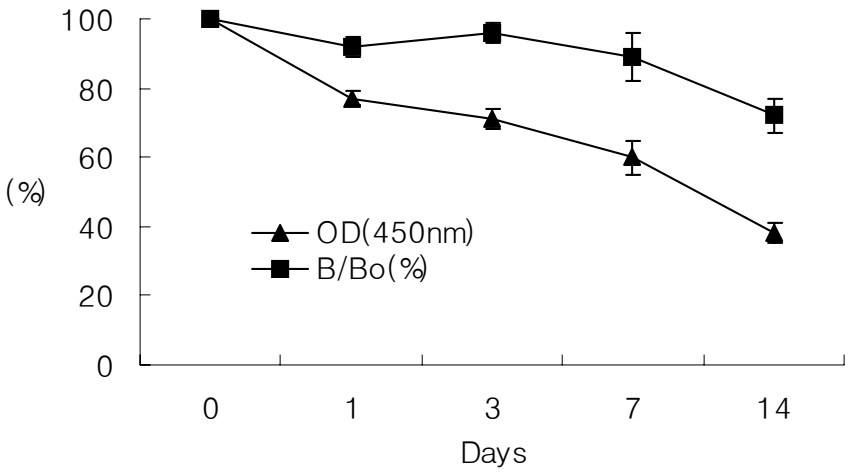


Figure 9. Stability of microtiter plates coated with IgG. The plates were incubated at 40℃ and subjected to ELISA at the time indicated in the figure.

## Ⅳ. Discussion

In analysis of SMZ residue, rapid, sensitive, and reliable analytical methods are needed. Therefore, in recent years numerous immunoassays have been developed or improved for the monitoring of SMZ residues in livestock products. These immunoassays, including commercially available kits, need to be standardized (Park, 1999). The cost of ELISA is high compared to HPLC. We need the basic technology to produce a domestic ELISA kit instead of buying imported ones.

Tween 20 decreases nonspecific reactions (Figure 2). The absorbance in the dilution of ovalbumin with 0.05% (v/v) Tween 20 was stable, but not stable without Tween 20. This condition might be related to nonspecific hydrophobic interactions between the detergent and nonpolar small organic molecules in an aqueous environment (Sugawara *et al.*, 1998). An optimal IgG dilution was chosen based on the B/Bo (%). IgG up to dilutions of 1:160 decreased the B/Bo (%) in the assay, but more diluted than dilutions of 1:160 increased the B/Bo (%). Immunoaffinity chromatography (IC) techniques have been used widely for the purification of antiserum. IC is based on the highly selective interaction of antigens with their antibodies. Since antibody-antigen interactions are so selective, only the antibody, which produces the immune response, or very closely related molecules, will be able to bind to the antigen (Nunes and Barceli, 1999).

The utility and applicability of an analytical method depend in great part on the absence of matrix interferences. The tissue extract contained blood, serum, and some fat, all of which can cause problems with binding.

In this regard, ELISA is similar to other detection techniques, and sample preparation prior to the analysis is still critical for SMZ residue determination. As in chromatographic techniques, extraction of samples is usually complicated. A competitive ELISA was developed by Bushway et al (1994) for the quantification of methyl 2-benzimidazole carbamate in fruit juices. The matrix effect was minimized by diluting the samples before ELISA. Dixon-Holland and Katz (1991) evaluated rapid methods based on HCl extraction for screening SMZ in feed samples by ELISA. Levels of SMZ as low as 0.004㎍ SMZ/g feed were detected. They also reported that undiluted urine and a phosphate-buffered saline extract of swine muscle were measured. Levels of SMZ as low as 0.02 ㎍ SMZ/g muscle tissue and 0.01 ㎍ SMZ/㎖ swine urine were detected.



Recently, a number of publications on SMZ residue analysis employing ELISA techniques have increased significantly compared to other techniques such as chromatography. In general, development of an ELISA method involves three phases: (1) Reagent preparation phase, consisting of the purification and modification of specific antibodies or analyses to be utilized in the final assay format. In this step, plate coating parameters and antibody concentrations will be assessed in order to attain the desired sensitivity. (2) Assay optimization phase, consisting of the development of a functional standard curve as well as selection of the proper conjugate and sample diluents. These diluents will be prepared to approximate, and be compatible with, the sample matrix composition. (3) Assay validation phase, consisting of defining and optimizing the essential assay parameters, including sensitivity,

recovery, linearity, and precision (Nunes *et al.*, 1998). Further development of the assay is followed by a stability test. The results of this experiment on stability test indicate the IgG coated microtiter plate would be stable for 2 years.

This study aims to develop an analysis tool for SMZ. We need to make a requirement for ELISA kits to be used as a screening or confirmatory assay method. Rapid screening or monitoring, quantitative analysis, and confirmation analysis will be significantly improved by the application of immunochemical assays. Quality assurance procedure is essential to develop an ELISA kit.

The assay illustrated the ability to predict concentration of SMZ efficiently, and it demonstrated the utility of the ELISA technique as a screening tool for the determination of SMZ. We are preparing a home-made ELISA kit to reduce assay cost.

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# 초 록

## 설파메타진 분석을 위한 직접 경쟁적 ELISA 개발

고은아

(지도교수: 박전홍)

제주대학교 대학원 수의학과

축산물중의 설파메타진 잔류량은 여러 나라에서 법으로 규제하고 있다. 현재 설파메타진을 분석하는 데 많은 어려움이 있으므로 설파메타진 분석방법으로 직접 경쟁적 ELISA를 개발하였다.

토끼에서 설파메타진 항혈청을 생산하여, 정제된 immunoglobulin G (IgG)를 plate에 고정하였다. 설파메타진과 접합된 horseradish peroxidase와 설파메타진을 경쟁적으로 반응시키고 3,3',5,5'-tetramethyl benzidine로 발색하여 450nm에서 흡광도를 관찰하였다. 표준곡선은 0.01 과 5$\mu$g/g사이의 농도에서 직선을 보였다. 설파메라진과 설파메타진에 대한 IgG는 0.1, 1, 10$\mu$g/g 농도에서 89, 62, 30%의 교차반응을 보였지만, 다른 설폰아마이드계에서는 교차반응을 보이지 않았다. 돼지근육에 설파메타진을 첨가하여 (0.05, 0.01$\mu$g/g) 측정하였을 때 회수율은 99%를 보였다. 돼지근육에 설파메타진을 첨가하여 직접 경쟁적 ELISA 분석방법과 HPLC 분석방법으로 측정하여 비교하였다. 두 분석법간의 상관관계는 0.993 이었다 ($p<0.01$). IgG로 코팅된 플레이트를 시간을 달리하여 40℃에서 보관하였을 때 B/Bo (%) 는 14일까지 큰 변화를 보이지 않았다. 이러한 결과는 IgG가 흡착된 플레이트를 약 2년 동안 보관할 수 있음을 나타낸다.

ELISA 분석방법은 비교적 간편하며 시료를 빨리 분석할 수 있다. 설파메타진을 분석하기 위한 직접 경쟁적 ELISA를 개발하였으며, 앞으로 잔류물질을 검사하는데 사용될 수 있을 것으로 생각된다.

중심어: 설파메타진, ELISA, immunoglobulin G, 교차반응.