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Enzyme Immunoassay for the Sulfamethazine Residues in Pork Tissue

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ABSTRACT—To control the maximum residue level (MRL) for sulfamethazine (SMZ) residues in pork tissue, a microbial inhibition method is a regulatory screening assay method in Korea. Microwell plate-based competitive enzyme immunoassay (ELISA) kit is available for routine screening of SMZ residues in pork tissue. One ELISA kit is evaluated. Phosphate buffer extracts of samples fortified with SMZ at 0, 1, 5, and 10 ng/g were used in a recovery test of the kit. Market pork samples were assayed by the kit. Recovery of sulfamethazine was 104% at 10 ng/g. Intraassay variations and interassay variations for the kit were 7.70% and 5.76%, respectively. Concentration causing 50% inhibition of color development compared with blanks was 16.4ng. The violative pork samples with over MRL (0.1 μg/g) was 4 of 32 cases (12.5%) by used ELISA kit. This result indicates a possibility of the ELISA kit for screening test of SMZ residues in pork tissue, and still needs a comfirmatory assay for mandatory purposes.

Key words □ Residues, Sulfamethazine, Pork, ELISA, Determination

Sulfamethazine (SMZ) is used in food-animal production for improved growth and therapeutic agents. Because of the long biological half-life of SMZ in swine there is a substantial risk of violative residues in pork tissues if withdrawal time is not obeyed. Legal regulations in the Korea set limits on SMZ residues of 0.1 μg/g in pork.[1] SMZ residues in pork tissue can be determined using bioassay, TLC, and HPLC.[2-5]

Microbial inhibition methods have been used as a routine screening for the detection of SMZ residues in animal products using *Bacillus subtillis* and *Sarcina lutea*. However, non-specific zones of growth inhibition were reported during determination of antibiotic residues in chicken muscle.[6] TLC and HPLC techniques are either tedious or time consuming, or require extreme care and attention to yield good precision. Enzyme-linked immunosorbent assay (ELISA) is available to detect SMZ residues in various food animal tissues.[7] The test may be used in field or regulatory laboratories.

The purpose of present study is to evaluate the performance of available immunoassay for the detection of violative levels of SMZ residues in pork tissues. This study indicated the possible use of one commercial ELISA kit for the detection of SMZ residues at concentration of 1, 5, and 10 ng/g in pork tissues and in market pork samples.

MATERIALS AND METHODS

Pork tissue samples (n=32) were collected in Cheju island and kept frozen (-20°C) until use. A quantitative microwell ELISA for SMZ in pork tissues was performed according to the procedures described in direction included in the kit (Idetek Inc., USA). This test is based on the principle of direct, competitive, solid-phase enzyme immunoassay and compares the relative color development of the sample to a negative control as the measurable endpoint. Briefly phosphate buffer (pH 7.4) extracts (30 m*l*) of pork tissue (3.00 g) fortified with SMZ at 1, 5, and 10 ng/g, or SMZ standards were dispensed (0.1 m*l*) into different microwells. Horseradish conjugated SMZ was added into the microwells. The microwells were incubated, washed out, and colorized. Absorbance was measured at 405 nm using ELISA reader (SLT 400, Austria).

RESULTS AND DISCUSSION

Detection range of SMZ was 1~25 ng/g/well (10~250 ng/g tissue) and detection limit was 10 ng/g tissue using EL-

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ISA. Fifty per cent color development decreased at SMZ 16. 4 ng/g (Fig. 1). Recovery of SMZ was 104~116% at samples fortified with SMZ 1~10 ng/g (Table 1). Intraassay variations and interassay variations for the kit were 7.70% and 5.76%, respectively (Table 2). Color of reaction mixtures and per cent bound (B/Bo) decreased by 10% after 2 hours standing (Fig. 2). Pork samples with SMZ residues over 0.1 μg/g were 4 of 32 cases using ELISA. SMZ residues (less 0.10 μg/g) were traced 11 of 32 cases (Table 3).

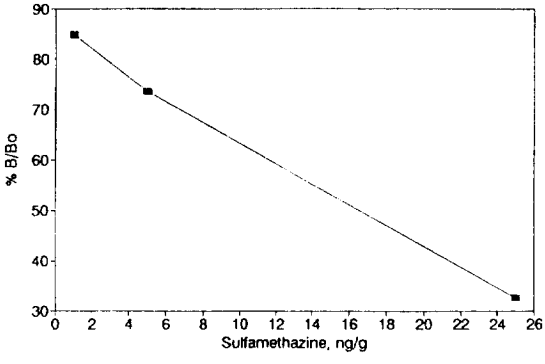

Fig. 1. Standard curve for sulfamethazine.

Table 1. Recovery of sulfamethazine in pork tissue

Fortified (ng/g)	Recovered (ng/g)	Recovery (%)
1.00	1.04	104
5.00	5.80	116
10.00	10.38	104

Table 2. Intraassay and interassay variances of sulfamethazine

Intraassay variance

Intraassay (n=6)	Mean±SD	CV (%)
1	1.37±0.11	4.61
2	1.12±0.09	11.07
3	1.03±0.06	8.39
4	0.45±0.02	6.76

Interassay variance

Intraassay (n=6)	Mean±SD	CV (%)
1	1.21±0.06	4.61
2	1.04±0.13	11.07
3	0.90±0.08	8.39
4	0.76±0.06	6.76

Classical methods for the SMZ residues in pork tissues are TLC, microbiological method, and HPLC in Korea. These methods have disadvantages in terms of sensitivity, labor, cost, false response, and limited number of samples analysed in a working day. Standard microbiological method is sensitive to detect 3000 μg/ml of raw milk for SMZ residues.[8] To overcome these obstacles fluorescamine was used in spectrofluorometry and TLC,[9,5] and trimethoprim was used in microbiological method.[8]

Using microbiological method, Chang *et al.*[10] found 63 cases of inhibition zones in 220 swine urine samples, and two of them were violative cases by HPLC. Park *et al.*[3,11] did not found a violative case in 177 and 155 pork tissues, and Hur *et al.*[12] found 8 violative cases in 115 pork tissues (7.0%). Using TLC method, Shin *et al.*[5] found one violative case in 34 pork tissues (2.9%). Using HPLC method Park and Lee[13,14] found 5 violative cases in 24 pork tissues in 1989 (20.8%) and 5 cases in 130 pork tissues in 1990 (3. 8%). Park *et al.*[15] found SMZ residues of 17.6% in swine tissues. Park *et al.*[16] did not found violative cases in 10 pork tissues. Laborious extraction step is an obstacle to analyze large samples in a day using HPLC method. Matrix solid-phase dispersion (MSPD) method reduced extraction

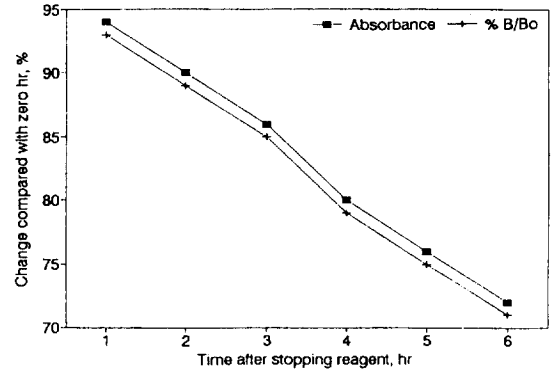

Fig. 2. Color stability of used ELISA

Table 3. Sulfametazine residues in pork tissue

Sulfamamethazine (ug/g)	Case	%
less 0.5	17	53
0.5~1.0	11	34
over 1.0	4	13

time in HPLC method.[15] Sample size used in previous reports was too small to yield national violative rate of SMZ residues in pork tissues.

ELISA has potential for sulfonamide residues in various foods, and provides rapid detection and quantitation in SMZ screening assay.[17,6] Application of ELISA in SMZ residues in pork tissues is slow to develope. Obstacles in ELISA application is instrumentation, lack of availability of commercial test reagents, and the difficulty of extracting desired materials from sample. National program to reduce residue problems in animal products has been progressed. HPLC and ELISA reader are supplied in local public health and environmental institutes and veterinary medicine institutes. The recent commercial availability of several ELISA-based tests for SMZ allows their use to detect SMZ residues in animal tissues. Disadvantage of commercial ELISA is a quality variation in each brand (i.e., antibody and enzyme conjugate) and analysis cost. However, ELISA is used widely in clinical chemistry and residue analysis. Home made ELISA for SMZ residues was reported.[18] Commercial ELISA kit does not provide performance data such as recovery, variance, and interferance, and needs to be evaluated for the detection of SMZ residues in animal tissue.

Recovery of SMZ was 104~116% at samples fortified with SMZ 1~10 ng/g (Table 1). This recovery is comparable with 69.6~83.5% in liquid extraction and 84.0~96.2% in MSPD.[15] Four enzyme immunoassay kits were evaluated in channel catfish muscle.[19] Intraassay variations for two microwell-based assays (SIGNAL and IDS One-Step ELISA) were 5.6 and 7.7%. Interassay variations for these tests were 7.9 and 16.6%. Similar variations were observed in present study. Intraassay variations and interassay variations for the used kit (Idetek Inc.) were 7.70% and 5.76%, respectively (Table 2). We determined SMZ residues over 0.1 μg/g in 4 of 32 cases. SMZ residues (less 0.10 μg/g) were traced in 11 of 32 cases using ELISA (Table 3).

We observed changes in absorbance and % B/Bo decreased coincidentally by about 10% after 2 hours standing (Fig. 2). This indicates % B/Bo of batch samples maintained in a trial. The present results obtained with fortified samples and market pork tissues indicate that ELISA can be used in the test of SMZ residues in pork tissues and SMZ residues in pork tissue over 0.05 μg/g can be detected using ELISA.

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국문요약

돈육중의 설파메타진 잔류량은 미생물발육억제법으로 스크리닝 분석되고 있다. 돈육중의 설파메타진 잔류량을 측정하는 마이크로플레이트를 이용한 경쟁적 효소면역법 키트가 상품화되어 있어,본 실험에서는 효소면역법 키트를 평가하고, 돈육중의 설파메타진 잔류량을 효소면역법으로 측정하였다. 회수율 측정시에는 돈육 시료에 설파메타진 표준품을 1, 5, 10 ng/g 되도록 첨가한 후 인산완충액으로 추출하여 사용하였다. 회수율은 설파메타진 10 ng/g인 경우에 104%였다. 분석내 분산과 분석간 분산은 각각 7.70%와 5.76%였다. 발색반응을 50% 억제하는 설파메타진 농도는 16.4 ng/g이었다. 돈육시료 32점중 효소면역법으로는 4 점에서(12.5%) 0.1 μg/g 이상의 잔류가 검출되었다. 이 실험의 결과 효소면역법은 돈육중의 설파메타진 잔류량 측정시 스크리닝 목적으로 사용될 수 있을 것으로 생각된다.

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