Acta Veterinaria (Beograd), Vol. 55, No. 5-6, 493-500, 2005.

UDK 619:637.4.04/.07

USE OF PREMI®TEST FOR THE DETECTION OF SULPHONAMIDE RESIDUES IN CHICKEN EGGS

HUSSEIN K*, MARCINČÁK S*, MÁTÉ D*, KOŽÁROVÁ IVONA*, SOKOL J* and ZDOLEC N**

*University of Veterinary Medicine, Košice, The Slovak Republic
**Faculty of Veterinary Medicine, University of Zagreb, Croatia

(Received 15. April 2005)

The presence of sulphadimidine residues in eggs after a peroral administration of Sulfadimidin PG plv. sol. ad us. vet. (120 mg/hen/day) to laying hens was studied. Premi®Test, four-plate microbiological method, and HPLC were used for the detection of sulphadimidine residues. Positive findings of the Four-plate test (FPT) were confirmed by the results of Premi®Test. Using the FPT, the absence of sulphadimidine residues was confirmed 72 hours after the last sulphadimidine administration. The presence of sulphadimidine residues has been detected by Premi®Test within 8 days and by the FPT within 3 days after the last administration. As compared with the results of Premi®Test, the FPT has reported false-negative results for five days (kappa < 0.6). Conformity of results obtained by both Premi®Test and HPLC was confirmed in this study (kappa = 0.6).

Key words: egg, Four-plate test, Premi®Test, sulphadimidine residues

INTRODUCTION

In European countries, coccidiosis is a serious disease caused by parasitic elements. Coccidiosis causes the most serious economical losses under intensive conditions of poultry breeding and rabbit farming. Except mortality, indirect losses (decreases in growth and weight of laying hens) can considerably lower the production of eggs.

Currently, numerous substances with antimicrobial effects are used in veterinary medicine worldwide (Nagy *et al*., 1996). Sulphonamides are reported to be one of the oldest pharmacologically active substances used in veterinary medicine (Braham *et al*., 2001). The discovery of sulphonamides in 1935 started a new era in the therapy of various bacterial diseases and protozoan infections. At present, sulphonamides are seldom used for preventive purposes due to the development of new wide-spectrum antibiotics, as well as due to an increasing resistance of causative agents to them. However, sulphonamides are still effective tools in the elimination of coccidiosis (Kožárová and Máté, 2000).

The presence of inhibitory substances and residues of veterinary drugs in food is permanently monitored in both veterinary and human medicine (Popelka *et al*., 2001). The residues of anticoccidials in foods of animal origin can endanger consumer's health directly or indirectly. Moreover, they show a negative influence on technological processes in the food industry. From the viewpoint of the consumer, antibiotics used in slaughter animals can enter the food chain and invoke the development of resistance or allergy (Salem, 1998; Kožárová and Máté, 2000). The presence of anticoccidials in animal products can also adversely influence the processes of food production. If the raw food materials containing residues of inhibitory substances are used for food production, they can reduce enzymatic activity of desirable micro-organisms and disable the correct course of biotechnological processes (Salem, 1998).

To eliminate health risks to consumers, as well as a negative impact to the environment and the technology of food production, the control of foods of animal origin must become more effective. Therefore, the availability of simple and reliable screening systems for the detection of antibiotics is an essential tool to ensure food safety. Recently, a new broad spectrum screening test for the detection of antimicrobial residues in eggs, the Premi®Test, has been developed (Stead *et al*., 2004; Lohajová *et al*., 2004).

In this study, the presence of sulphonamide residues in eggs of laying hens was detected with the help of Premi®Test. Results have been compared with the FPT and HPLC method.

MATERIAL AND METHODS

Methanol, acetonitrile, n-hexane, ethyl acetate and acetic acid were purchased from Merck company (Darmstadt, Germany). Sulphamethazine sodium salt and trimetoprime were purchased from Sigma company (USA). Anhydrous sodium sulphate, sodium chloride and sodium acetate were from Lachema (Brno, Czech Republic). Deionized water and chemicals have p. a. purity, respectively HPLC grade.

For the detection of sulphonamide residues by the four plate microbial disc assay (Bogaerts and Wolf, 1984), the plates inoculated with *Bacillus subtilis* BGA (pH 7.2) were used. Spore suspension of *Bacillus subtilis* BGA and the test agar (pH 7.2) were purchased from Merck (Darmstadt, Germany).

The Premi®Test was purchased from DSM (Netherlands) and the Thermoblock (Biotech, The Slovak republic) was used as a block heater for Premi®Test ampule incubation. Premi®Test ampule method for the detection of antibiotic residues utilizes a culture medium containing *Bacillus stearothermophilus* var. *calidolactis*. Premi®Test combines the principle of agar diffusion test with the change in colour caused by metabolism of the test-microorganism. Homogenized liquid egg sample in the amount of 100 μl was transferred onto the agar in the ampule, incubated for twenty minutes at room temperature (pre-diffusion) and than removed. Ampules were then placed into the water bath at a temperature of 80 °C for 10 minutes. After this heat pre-treatment

the ampules were incubated for 3 hours at 64 ± 1 °C and the change in colour was evaluated.

A liquid chromatography method (Sokol, 2001) with UV detection at 265 nm was used for the determination of sulphadimidine residues in eggs.

Twenty laying hens (ISA Brown) in the 35th week of laying period, bred under permanent veterinary supervision, have been involved in this experiment. Laying hens were bred separately in cages. An antibiotic-free feeding mixture HYD–10 (Tajba, Čaňa, The Slovak Republic) was fed *ad libitum*. SULFADIMIDIN PG pl. sol. (PharmaGal, Nitra, The Slovak Republic) was administered to laying hens within 3 days with the oesophageal probe in an individual daily dose of 120 mg per kg of body weight. A break for 3 days was then followed by the second drug administration for another 3 days. Six laying hens were not administered and were used as a control group.

Eggs were collected, signed and stored from the first to the last day of drug administration and also within 15 days of withdrawal period for SULFADIMIDIN PG pl. sol.

Statistical analysis was performed with the help of statistical program Graph Pad Prism version 3.0 (2000). Results were expressed as arithmetic mean ± standard deviation (X±SD). Individual methods used for the determination of sulphadimidine residues were analysed statistically by the Student's paired t-test ($P<0.05$). Methods were then compared and analysed for their conformity using the Win Episcope 2.0 test and the kappa value was calculated.

RESULTS

Based upon the results shown in Tables 1, 2 and 3, the administration of SULFADIMIDIN PG pl. sol. to laying hens in a dose of 120 $mgkg^{-1}$ with the oesophageal probe (in accordance with recommendations by the producer) has been followed by a rapid occurrence of drug residues in the egg contents.

As to results of four-plate test (FPT), the presence of residues was manifested by the formation of a clear zone of inhibition at least 2 mm in size. Positive findings were recorded from the first day of administration up to the second day of break. Positive results were found again after the fifth sulphadimidine administration (Table 1). Within 15 days of withdrawal period (set by the producer), a rapid decrease in size of inhibition zones in all egg samples was observed ($P<0.05$). FPT was not able to detect the presence of sulphadimidine residues from the third day of withdrawal period (Table 2).

The presence of sulfadimidine residues in egg samples determined with the help of Premi®Test after administration of SULFADIMIDIN PG pl. sol. to laying hens is shown in Table 1. As the level of drug residues in eggs was less than the detection limit of Premi®Test (0.05 $mg.kg^{-1}$), negative results have been obtained on the first day of drug administration. Starting with the second day of administration, the egg samples showed the presence of residues up to the end of the period of drug administration (the occurrence of sulphadimidine residues exceeded the above-mentioned detection limit). Sulphadimidine residues have

also been found within the 3-days-break, when the drug was not administered to laying hens.

Table 1. Determination of sulphonamide residues in eggs by FPT and Premi®Test during drug administration

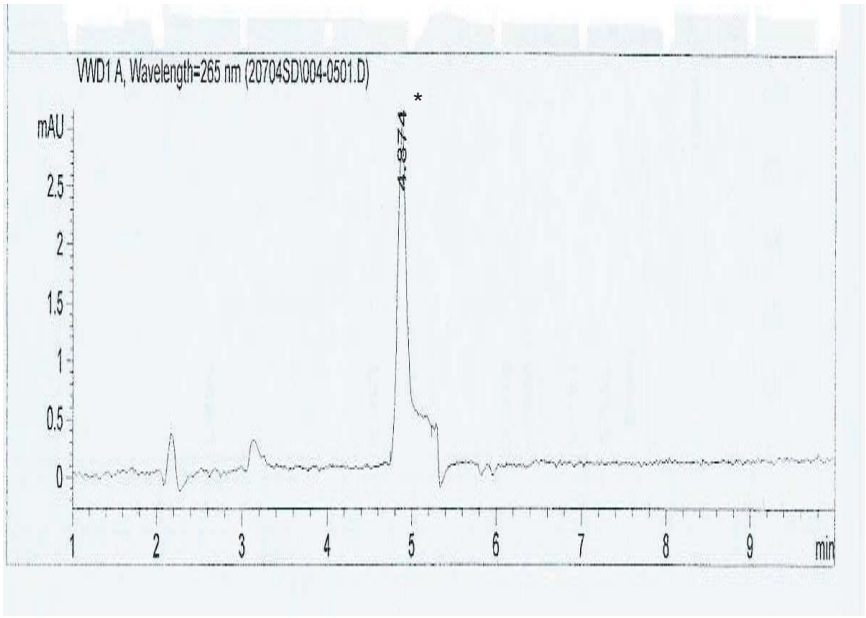
	Four plate test Inhibition zone (mm)	Premi®Test
1. administration	0	–
2. administration	2	+
3. administration	3	+
1. pause	6	+
2. pause	2	+
3. pause	0	+
4. administration	0	+
5. administration	5	+
6. administration	7	+

Table 2. Determination of sulphonamide residues in eggs by FPT and Premi®Test within a withdrawal period for sulphadimidine (15 days)

Withdrawal period (days)	Four-plate test inhibition zone (mm)		Premi®Test	
	sample	control	sample	control
1.	8	0	+	–
2.	3	0	+	–
3.	0	0	+	–
4.	0	0	+	–
5.	0	0	+	–
6.	0	0	+	–
7.	0	0	+	–
8.	0	0	+	–
9. -15.	0	0	–	–

The occurrence of sulphadimidine residues by Premi®Test within the whole withdrawal period of SULFADIMIDIN PG pl. sol. is recorded in Table 2. For the first 8 days of the withdrawal period, sulphadimidine residues exceeded the detection limit of Premi®Test giving positive results. From the ninth day of withdrawal period, negative results have been obtained in all inspected samples.

The result of chromatographic detection (HPLC) of sulphadimidine residues in eggs at the first day of the withdrawal period is recorded in Figure 1. Sulphadimidine was detected by an isocratic system in 4.9-5 minutes. A chromatographic column Phenomenex RP C_{18} (150 x 4.6.5 μm) was used. Mobile phase [acetonitrile/acetate buffer (pH 4.6); 25/75; v/v] was used for the elution of sulphadimidine at 265 nm wavelength, where the maximum absorbance of sulphadimidine has been observed.



*sulphadimidine

Figure 1. Chromatographic determination of sulphadimidine residues in eggs (the first day of withdrawal period). HPLC conditions: Chromatographic column: Phenomenex RP, C_{18}, 150 – 4.6 mm (5 μm), flow rate: 1.0 ml. min^{-1}, UV detection: 265 nm, volume: 20 μl

The results of detection of sulphadimidine residues within the withdrawal period by HPLC are shown in Table 3. On the first and second day after drug administration was stoped, high residual drug concentrations in the eggs have been determined (33.84 ± 3.25, and 31.86 ± 2.95 $mg.kg^{-1}$ respectively). On the third day of the withdrawal period, a significant decrease in sulphadimidine residues in eggs was noticed (1.72 ± 0.33 $mg.kg^{-1}$; $p<0.05$). All these results were above the level of maximal residual limit (MRL).

A significant statistical decrease in sulphadimidine residual concentrations has been found from the fourth to the seventh day of withdrawal period ($p<0.05$). On the seventh day of withdrawal period, the concentrations of sulphadimidine residues in all samples reached a value of 0.110 ± 0.10 $mg.kg^{-1}$. This value was

still above the MRL (0.10 mg.kg^{-1}) set by Codex Alimentarius of The Slovak Republic (1996). From the eighth day of withdrawal period, the concentrations of sulphadimidine in eggs were below the MRL.

Table 3. Average concentrations of sulphadimidine residues (SD, mgkg^{-1}) detected by HPLC within a withdrawal period for sulphadimidine (15 days)

SD mg.kg^{-1}	Days of withdrawal period								
	1.	2.	3.	4.	5.	6.	7.	8.	9.-15.
x ± sd	33,84 ± 3,25	31,86 ± 2,95	1,72* ± 0,33	1,55 ± 0,15	1,11¤ ± 0,10	0,55¤ ± 0,05	0,11* ± 0,01	0,096 ± 0,01	0

*significant difference ($p<0,05$)

A comparison among FPT as a standard test, the Premi®Test and HPLC showed that the FPT is less sensitive, primarily at low concentrations of sulphadimidine residues (kappa < 0.6). On the other hand, a high correlation between the results of Premi®Test and the results of HPLC method has been confirmed (kappa = 0.6). The last FPT positive results were recorded 48 hours after finishing SULFADIMIDIN PG pl. sol. administration. The Premi®Test showed the last positive results on the eighth day after the last drug administration. As follows from these findings, the FPT showed false-negative results for 6 days. The same results have been obtained by HPLC method with positive findings up to the eighth day after the last administration of the drug.

DISCUSSION

To solve the problems related to the occurrence of inhibitory substances in food, attention must be paid to the control measures. This process requires increased responsibility in the evidence of animals treated within the period of breeding, as well as to comply to withdrawal periods set by the valid food legislation for each individual drug. Foods with a content of inhibitory substances in an amount exceeding the limits must be condemned (Pipová *et al.*, 1995). Therefore, a correct use of screening methods used for both the control and the identification of inhibitory substances in food is of great importance. The use of Premi®Test contributes to a significant decrease in the number of positive animals and their products at the beginning of the food chain and reduces considerably health risks to the consumer (Popelka *et al.*, 2003).

According to recent knowledge, the use of FPT (Bogaerts and Wolf, 1980) suits well for the detection of sulphonamide residues. A combination of *Bacillus subtilis* BGA (pH 7.2) as a test-microorganism and the addition of trimetoprime (in a concentration of 0.05 μg per 1 ml of agar) showed the highest sensitivity to the presence of sulphonamide residues in food. Trimetoprime is a chemical substance used in therapy because of its inhibitory effect against bacterial enzymes (Braham *et al*., 2001). Microbial four-plate test should be able to detect

the presence of sulphonamide residues at the level of MRL (0.1 mg.kg^{-1}). The use of FPT is approved by the valid food legislation. Numerous references report that the sensitivity of FPT differs significantly among various substances in the sulfonamide group (Koenen-Dierick *et al.* 1990, Currie *et al.*, 1998).

Based upon the results obtained, it is possible to state that the FPT without any modification is not able to detect sulphonamide residues at the level of MRL (Council directive EEC No. 2377/90; Codex Alimentarius of The Slovak Republic, 1996**)**.

Premi®Test integrates the strategy of detection of antibacterial substances at the level or below the level of MRL in a wide spectrum of biological samples including eggs. Conventional tests (FPT, New Dutch Kidney Test) require an overnight incubation. On the other hand, the Premi®Test provides reliable results within 3 hours of incubation (Stead et al., 2004, Lohajová *et al.*, 2004). The test principle is based on a growth inhibition of the test-microorganism *Bacillus stearothermophilus*, and the change in colour of the culture medium when the sample is negative (the colour of medium is not changed in the presence of residues).

As follows from Table 2, the determination of sulphonamide residues in eggs within a withdrawal period for 15 days showed positive results for the first 8 days. Sulphadimidine residues exceeded the detection limit of Premi®Test (0.05μg.kg^{-1}) in all inspected samples. The results obtained by Premi®Test were confirmed by HPLC method. Both reliability and sensitivity of Premi®Test for the detection of sulphonamide residues in food have also been reported by Popelka *et al*., (2003), and Stead *et al.* (2004).

The Premi®Test introduces an important tool for monitoring the residues of inhibitory substances in a concentration exceeding the limits. Based upon the results of this study, the detection limit of Premi®Test for sulphonamides ranges from 0.01 to 0.05 mg.kg^{-1} and the test sensitivity meets the requirements of the European legislation.

ACKNOWLEDGEMENT:
This study was supported by the grant VEGA No 1/0617/03.

Address for correspondence:
Slavomír Marcinčák, D.V.M., PhD.
Department of Food Hygiene and Technology
University of Veterinary Medicine
Komenského 73
041 81 Košice
The Slovak Republic
e-mail: marcincak@lycos.com

REFERENCES

1. *Bogaerts R, Wolf F*, 1980, A standardized method for the detection of residues of anti-bacterial substances in fresh meat, *Fleischwirtschaft*, 60, 672-3.
2. *Braham R, Black WD, Claxton J, Yee J*, 2001, A rapid assay for detecting sulphonamides in tissues of slaughtered animals, *J Food Protection*, 64, 1565-73.
3. *Codex Alimentarius of the Slovak republic,* June 1996, *Vestník MP SR*, 28, part 14, 271-95.

4. *Council Regulation (EEC) No. 2377/90* of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, *Off J Eur Comm*, L 224, 1-8
5. *Currie D, Lynas L, Kennedy DG, McCaughey J*, 1998, Evaluation of a modified EC four-plate method to detect antimicrobial drugs, *Food Addit Contam*, 15, 651-60.
6. *Hrdlička J*, 1990, Residues of inhibitory agents in tissues of slaughterhouse animals – comparison of microbiological methods of agar diffusion, *Vet Med (Czech)*, 35, 411-18.
7. *Koenen-Dierik K, De Beer JO*, 1990, Optimization of an antibiotic residue screening test, based on inhibition of *Bacillus subtilis* BGA, with experimental design, *Food Addit Contam*, 15, 528-34.
8. *Kožárová I, Máté D*, 2001, Evaluation of the sensitivity of individual test organisms to residual concentration of selected types of anticoccidial drug, *Bull Vet Inst Pulawy*, 44, 187-92.
9. *Lohajová L, Nagy J, Popelka P,* 2004, In vitro studies for the application of the Premi®Test for the detection of antibiotic residues in chicken eggs - short communication, *Nutrition*, 28, 503-05.
10. *Nagy J, Cabadaj R, Turek P, Pipová M*, 1996, Residues of pharmacologically active substances in food and foodstuff of animal origin, *Slovak Vet J*, 21, 85- 89.
11. *Pipová M, Cabadaj R, Nagy J*, 1995, Hygiene of poultry, eggs, fish and game. DataHelp, Košice, 110 pp.
12. *Popelka P, Cabadaj R, Nagy J, 2001*, Residues of antibiotics in foodstuffs and raw materials of animal origin, *Slovak Vet J*, 26, 1, 20-4.
13. *Popelka P, Nagy J, Popelka P, Marcinčák S, Jevinová P, Hussein K*, 2003, Comparison of BsDA and Premi®test sensitivity to penicillin standards in poultry meat and after administration of Amuril plv. sol, *Folia Veterinaria,* 47, 139-41.
14. *Salem DA*, 1998, Estamination of antibiotics, sulphonamides and nitrofurans residues in chicken meat, *Assiut Vet Med J*, 39, 192-00.
15. *Sokol J,* 2001, Methods of determination of inhibitory substances in foodstuff of animal origin, food, feed and water. Special part. State veterinary and food administration SR, Bratislava.
16. *Stead S, Sharman JA, Tarbin JA, Gibson E, Richmond S, Stark J*, 2004, Meeting maximum residue limits: an improved screening technique for rapid detection of antimicrobial residues in animal food products, *Food addit Contam*, 21, 216-21.

UPOTREBA PREMI® TESTA ZA DETEKCIJU REZIDUA SULFONAMIDA U KOKOŠIJIM JAJIMA

HUSSEIN K, MARCINČÁK S, MÁTÉ D, KOŽÁROVÁ IVONA, SOKOL J i ZDOLEC N

SADRŽAJ

U ovom radu su izneti rezultati dokazivanja rezidua sulfadimidina nakon peroralne administracije (120 mg/grlu/24h) preparata Sulfadimidin PG pulv. kokama nosiljama. Korišćena su tri testa: mikrobiološki, Premi®Test i HPLC. Mikrobiološkim testom, rezidue su dokazivane tri dana nakon administracije a Premi®Testom i nakon 8. dana. Mikrobiološki test je davao lažno negativne rezultate u periodu od pet dana. Potvrđena je podudarnost rezultata dobijenih metodom HPLC i Premi®Testom.