In European countries, coccidiosis belongs to serious diseases caused by parasitic elements. Coccidiosis causes the most serious economical losses under intensive conditions of poultry breeding and rabbit farming (6). Except mortality, indirect losses (decreases in growth and weight of laying hens) can considerably lower the production of eggs.

Currently, numerous substances with antimicrobial effect are used in veterinary medicine worldwide (11). Sulphonamides are reported to be one of the oldest pharmacologically active substances used in veterinary medicine (2, 9). The discovery of sulphonamides in 1935 started a new period 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 (8).

The presence of inhibitory substances and residues of veterinary drugs in food is permanently monitored in both veterinary and human medicine (13). Sulphamethazine sodium salt (as a standard of sulphadimidine) and trimethoprime were taken from Sigma company (USA). Anhydrous sodium sulphate, sodium chloride and sodium acetate were derived from Lachema (Brno, Czech Republic). Deionized water and chemicals have p. a. purity, respectively HPLC grade.

For the detection of sulphadimidine residues by the four plate test – microbial disc assay (1), 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 ampoule incubation. Premi®Test ampoule method for the detection of antibiotic residues utilizes of the desirable micro-organisms and disable the correct course of biotechnological processes (15).

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 much more effective. Therefore, the availability of simple and reliable screening systems for the detection of antibiotics is an essential tool to ensure the food safety. Recently, a new broad spectrum screening test for the detection of antimicrobial residues in eggs, the Premi®Test, has been developed (10, 17).

In this study, the presence of sulphadimidine residues in eggs of laying hens was detected with the help of Premi®Test. Results have been compared with the four-plate test (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 (as a standard of sulphadimidine) and trimethoprime were taken from Sigma company (USA). Anhydrous sodium sulphate, sodium chloride and sodium acetate were derived from Lachema (Brno, Czech Republic). Deionized water and chemicals have p. a. purity, respectively HPLC grade.

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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 ampoule, incubated for twenty minutes at room temperature (pre-diffusion) and then removed. Ampoules were then placed into the water bath with a temperature of 80°C for 10 minutes. After this heat pre-treatment the ampoules were incubated for 3 hours at 64 ± 1°C and the change in colour was evaluated.

A liquid chromatography method (15) with UV detection at 265 nm was used for the determination of sulphadimidine residues in eggs. Sulphadimidine was detected by an isocratic system in 4.9-5 minutes. A chromatographic column Phenomenex RP C3(150 × 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.

Twenty laying hens (ISA Brown) in the 35th week of age, bred under permanent veterinary supervision, have been involved to 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 to them. SULFADIMIDIN PG pl. sol. (PharmaGal, Nitra, The Slovak Republic) was administered to laying hens within 3 days with the oesophageal probe in 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 antibiotic-free laying hens 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 (1999). Results were expressed as arithmetic mean ± SD. Individual methods used for the determination of sulphadimidine residues were analysed statistically by the Student pair 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 and discussion

Based upon the results shown in tab. 1, 2 and 3, the administration of SULFADIMIDIN PG pl. sol. to laying hens in a dose of 120 mg × kg−1 with the oesophageal probe (in accordance with recommendations of the producer) has been followed by a rapid occurrence of drug residues in the egg contents.

As to the 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 (tab. 1). Within 15 days of withdrawal period (set by the producer), a rapid decrease in size of inhibition zones in all samples of eggs was observed (P < 0.05). FPT was not able to detect the presence of sulphadimidine residues from the third day of withdrawal period (tab. 2).

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

<table>
<thead>
<tr>
<th>Administration of drug</th>
<th>Four plate test inhibition zone (mm)</th>
<th>Premi® Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. administration</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>2. administration</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3. administration</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>1. pause</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>2. pause</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3. pause</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>4. administration</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>5. administration</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6. administration</td>
<td>7</td>
<td>+</td>
</tr>
</tbody>
</table>

The presence of sulphadimidine residues in egg samples determined with the help of Premi® Test after administration of SULFADIMIDIN PG pl. sol. to laying hens is shown in tab. 1. As the level of drug residues in eggs was lower 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 drug administration (the occurrence of sulphadimidine residues exceeded the above-mentioned detection limit). Sulphadimidine residues have also been found within a 3-days-break, when the drug was not administered to laying hens. The occurrence of sulphadimidine residues by Premi® Test within the whole withdrawal period of SULFADIMIDIN PG pl. sol. is recorded in tab. 2. For the first 8 days of withdrawal period, sulphadimidine residues exceeded the detection limit of Premi® Test giving the positive

Tab. 3. Average concentrations of sulphadimidine residues (mg × kg−1) detected by HPLC within a withdrawal period for sulphadimidine (15 days) (mean ± SD)

<table>
<thead>
<tr>
<th>Days of withdrawal period</th>
<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
<th>6.</th>
<th>7.</th>
<th>8.</th>
<th>9.-15.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33.84 ± 3.25</td>
<td>31.86 ± 2.95</td>
<td>1.72* ± 0.33</td>
<td>1.55 ± 0.15</td>
<td>1.11* ± 0.10</td>
<td>0.55* ± 0.05</td>
<td>0.11* ± 0.01</td>
<td>0.096 ± 0.01</td>
<td>0</td>
</tr>
</tbody>
</table>

Explanations: *significant difference (p < 0.05)
findings. From the ninth day of withdrawal period, negative results have been obtained in all samples inspected.

The results of sulphadimidine residues detection within a withdrawal period by HPLC are shown in tab. 3. On the first and second day after finishing the drug administration, high residual drug concentrations in eggs have been determined (33.84 ± 3.25, and 31.86 ± 2.95 mg × kg⁻¹ respectively). On the third day of withdrawal period, a significant decrease in sulphadimidine residues in eggs was noticed (1.72 ± 0.33 mg × kg⁻¹; p < 0.05). However, 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 (0.1 mg × kg⁻¹) set by Codex Alimentarius of the Slovak Republic (3). From the eighth day of withdrawal period, the concentrations of sulphadimidine in eggs were below the MRL.

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 of 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 drug of administration.

To solve the problems related to the occurrence of inhibitory substances in food, better attention must be paid to the control measures. This process requires to increase responsibility in the evidence of animals treated within the period of breeding, as well as to keep 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 (12). 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 food chain and reduces considerably health risks to the consumer (14).

According to recent knowledge, the use of FPT (1) 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 trimetoprim (in a concentration of 0.05 μg per 1 ml of agar) showed the highest sensitivity to the presence of sulphonamide residues in food. Trimetoprim is a chemical substance used in therapy because of its inhibitory effect against bacterial enzymes (2). 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 sulphonamide group (5, 7).

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 (3, 4).

Premi® Test integrates the strategy of the detection of antibacterial substances at the level or below the level of MRL in a wide spectrum of biological samples including the 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 (10, 17). 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 (0.05 mg × kg⁻¹) in all samples inspected. 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 Stead et al. (17).

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 (4).

References

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