Sulfadimidine is widely used for the treatment of coccidiosis in laying hens at the dose of 2 g/l for 6 consecutive days via the drinking water. As a result, there is concern that the residues of this drug may be retained in the eggs and present a potential risk to human health.

The aim of our study was to determine the residue concentrations of sulfadimidine in whole eggs of laying hens during and after its oral administration up to the 15th day of the withdrawal period. The sulfadimidine residues were determined by a gradient high-performance liquid chromatography system with a photo-diode array detector at 275 nm. Our results indicate that the oral administration of sulfadimidine to laying hens produced a rapid and sustained increase in sulfadimidine residues in their eggs. The highest residue concentrations of sulfadimidine were found on the 6th day of administration. After withdrawal, the residues declined rapidly, but they were still detectable on the 10th day of the withdrawal period. On day 11, the residues were below the established MRL (0.1 mg/kg). The limit of quantification (LOQ) for sulfadimidine was 0.3 mg/kg, the detection limit (LOD) was 0.09 mg/kg, and the recovery ranged from 91 to 98%.

Key words: determination, eggs, HPLC, sulfadimidine residues

INTRODUCTION

Sulfonamides are one of the oldest groups of pharmacologically active substances used in veterinary medicine to date. Their discovery, in 1935, signified the beginning of a new era in the treatment of a wide range of bacterial diseases and a number of protozoan infections. In laying hens, sulfonamides are used for the treatment of coccidiosis. As a result, there is concern that the residues of these drugs may be retained in the eggs and present a potential risk to human health (Šutiak et al., 2000; Kožárová et al., 2001; Kožárová et al., 2002).

Direct toxic or allergic reactions have been described after administration of therapeutic doses of sulfonamides to humans (Booth, 1988; Spoo and Riviere, 1995). Besides these reactions other negative effects of sulfonamides or their metabolites on the human body have been reported, particularly after long-term consumption of animal products containing trace amounts. Accumulation of
these trace amounts in animal products has resulted in a build-up of resistance and the development of hypersensitivity to sulfonamides (Agarwal, 1992; Spoo and Riviere, 1995). Sulfonamides are known for their negative effects on the thyroid gland in relation to the development of thyroid gland tumours (Swarm et al., 1973; Ahmed and Ahmed, 1989).

In order to decrease the potential risk to the consumer’s health and ensure the reduction of sulfonamide residues in edible tissues and eggs to an acceptable level, these substances must be administered only in recommended concentrations and their respective withdrawal times must be observed. With regard to the pharmacokinetic properties of sulfonamides and the persistence of their residues in edible tissues and eggs of food-producing animals, the original 7-day withdrawal period has, since 1980, been increased to 15 days (Ausberg, 1989).

The current legislation established the MRL of 0.1 mg kg\(^{-1}\) for sulphonamides (all compounds of the sulphonamide group) in foods of animal origin (Council Regulation (EEC) No. 2377/90; Codex Alimentarius of The Slovak Republic, 1996).

Various methods for the determination of sulfonamide residues have been reported in the literature. These methods differ in terms of degree of sophistication and procedure. In the present two-tier testing system for the monitoring of sulphonamide residues in various biological matrices, microbiological and immunological methodologies were used predominantly as screening methods, and high-performance liquid chromatography (HPLC) as the confirmatory one (Agarwal, 1992; Guggisberg et al., 1992; Kožárová et al., 2001; Kožárová et al., 2002; Pavelka and Golian, 2002).

Sulfadimidine has been observed to be effective against coccidiosis in laying hens when administered orally via the drinking water at the dose of 2 g l\(^{-1}\). This paper describes an HPLC method for determination of sulfadimidine residues in the whole eggs of laying hens. The eggs were examined during and after its oral administration up to the fifteenth day of the withdrawal period. Residue analysis was performed using the methodology described by Furusawa and Mukai (1994), Malisch (1994) and Furusawa (1999).

**MATERIAL AND METHODS**

Animal treatment and processing of egg samples: Twenty-one 40-week-old laying hens (Hisex Brown hybrid) weighing between 1.6 – 1.8 kg were used in the experiment. The laying hens were placed in individual animal-care approved cages with free access to feed and water, and they were randomly divided into two groups, one experimental and one control group. The laying hens were fed with the commercially-produced laying ration, NV-180, \textit{ad libitum}. Sixteen laying hens from the experimental group received sulfadimidine via the drinking water for 6 consecutive days at the dose of 2 g l\(^{-1}\) of water. Medicated drinking water was prepared fresh each day. The remaining five hens served as a sulfadimidine-free control. All eggs laid by the hens during administration and after the withdrawal of
Sulfadimidine were collected daily, weighed, broken, homogenized, and stored in polyethylene bags in a deep-freezer until required for analysis.

**Solvents and reagents:** All solvents and reagents were analytical or HPLC grade. Methanol, acetonitrile, n-hexane, ethyl acetate and acetic acid were obtained from Merck (Darmstadt, Germany), anhydrous sodium sulphate, sodium chloride, and sodium acetate from Lachema (Brno, Czech Republic), and deionized and redistilled water was prepared on Milli-Q Plus (Millipore, France).

**Standard solutions:** Sulphadimidine (Sulfamethazine sodium salt, S 5637), as a standard, was obtained from Sigma Chemical CO. (St. Louis, MO, USA). A stock solution (0.5 mg.ml⁻¹) was prepared by dissolving 25 mg sulfadimidine standard in 50 ml methanol. Working solutions of sulfadimidine were prepared by serial dilutions with methanol. The stock and working solutions were stored in a refrigerator at 4°C.

**High performance liquid chromatography:** Analyses of the standard and extracted sulfadimidine residues were conducting using an HP 1090 liquid chromatograph with a photo-diode array detector (Hewlett-Packard, USA). The separation was performed on a LiChroCART RP-18e column (125 – 4, 5 μm) (Merck, Germany) using acetonitrile-acetate buffer (pH 4.6) (25:75, v/v) as the mobile phase at a flow-rate of 1.0 ml/min at a temperature of 40°C. The injection volume was 25 μl, and the detection was conducted at 275 nm.

**Sample extraction and sample clean-up:** An accurately weighed 50 g aliquot of the homogenized eggs was extracted with 100 ml acetonitrile on a mechanical shaker for 15 min. The extract was passed through a filter paper using a Buchner funnel. The residue on the filter paper was washed with 25 ml acetonitrile and the procedure was repeated once more. The collected filtrates were poured into a separating funnel. After adding 7 g sodium chloride the combined filtrate was left until it separated completely in to two layers. The lower aqueous layer was discharged and the upper acetonitrile layer was dried with anhydrous sodium sulphate followed by filtration. The separated aqueous layer was extracted with 25 ml ethyl acetate. After extraction, both the ethyl acetate and acetonitrile extracts were collected in a 100 ml round-bottom flask and evaporated to dryness in a rotary vacuum evaporator (Laborota 4000, Heidolph, Germany) at 40°C. The residue was redissolved in 1 ml methanol and 1 ml of mobile phase, and transferred to a centrifuge tube. The flask was washed twice with 2 ml n-hexane, and 4 ml n-hexane was added to the centrifuge tube. The mixture was centrifuged twice at 3000 rpm for 10 min (Jouan BR 4l, France). The lower layer was filtered through a disposable syringe filter unit and transferred to a vial. A 25 μl aliquot of the solution was injected into the HPLC system.

**RESULTS**

The mean residue concentrations of sulfadimidine found in the whole eggs of laying hens given this substance through the drinking water for 6 days, are shown in Table 1.
Table 1. Sulfadimidine residues (mg.kg⁻¹) in the whole eggs of laying hens during oral administration for 6 days at 2g l⁻¹ in the drinking water

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg kg⁻¹ ± SD</td>
<td>4.8±0.53</td>
<td>11.6±2.4</td>
<td>25.7±3.2</td>
<td>38.1±4.1</td>
<td>42.6±4.6</td>
<td>43.1±4.8</td>
</tr>
</tbody>
</table>

SD – standard deviation

Table 2. Sulfadimidine residues (mg.kg⁻¹) in the whole eggs of laying hens after its oral administration throughout the 15 days of the withdrawal period

<table>
<thead>
<tr>
<th>Days of the 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</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg kg⁻¹ ± SD</td>
<td>13.1±2.7</td>
<td>5.15±0.71</td>
<td>2.35±0.36</td>
<td>2.01±0.29</td>
<td>1.57±0.24</td>
<td>1.15±0.18</td>
<td>0.85±0.09</td>
<td>0.59±0.021</td>
<td>0.23±0.013</td>
<td>0.12±0.008</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

SD – standard deviation; ND – not detectable
There was a rapid and sustained increase in sulfadimidine residues in the whole eggs. Thus, high levels were observed already on the first day following the beginning of administration. The maximum sulfadimidine concentrations were achieved on the 6th day of oral administration. As soon as the treatment was discontinued, the sulfadimidine residues declined rapidly during the first few days of the withdrawal period. Thus, sulfadimidine residues were detectable in the eggs up to the 10th day of the withdrawal period, and the mean residue concentration was 0.12 mg kg\(^{-1}\). On the 11th day of the withdrawal period, the sulfadimidine residues decreased below the established MRL (0.1 mg kg\(^{-1}\)).

Recoveries of sulfadimidine from spiked tissue samples at the concentrations of 0.1, 0.5, and 1 mg kg\(^{-1}\) ranged between 91 % and 98 %. The LOQ for sulfadimidine was 0.3 mg kg\(^{-1}\), and the LOD was 0.09 mg kg\(^{-1}\).

The absorption spectrum of sulfadimidine was measured by a photo-diode array detector and the wavelength of 275 nm was chosen to monitor the maximum absorbance by sulfadimidine. Sulfadimidine was successfully detected within 19 min, when the flow-rate was 1.0 ml min\(^{-1}\).

Figure 1 shows the chromatogram of the sulfadimidine standard (10 mg kg\(^{-1}\)) obtained with the photo-diode array detector at 275 nm. The retention time was 18.895 min.

Similar results were obtained with the whole eggs of laying hens examined during the experimental period. A chromatogram of sulfadimidine extracted from whole eggs on the 3rd day of the withdrawal period, is shown in Figure 2. The retention time was 18.911 min. On this day, the residue concentration of sulfadimidine was 2.35 mg kg\(^{-1}\).
DISCUSSION

High-performance liquid chromatography has become the most widely used confirmatory technique for the determination of sulphonamide residues in foods of animal origin. The principal approach of the HPLC analysis involves extraction, sample clean-up and HPLC analysis (Agarwal, 1992; Guggisberg et al., 1992).

Traditionally, the extraction of sulphonamide from biological matrices, such as meat, milk, and eggs, has been done with organic solvents (Agarwal, 1992; Guggisberg et al., 1992). Sulfadimidine was extracted from the whole eggs laid during this experiment with acetonitrile and ethyl acetate. Some organic solvents also denature the sample proteins, which result in cleaner extracts, and also help to extract the drug residues bound to proteins (Malisch, 1994). For effective deproteinization, a combination of acetonitrile and acetate buffer as the mobile phase was employed here. To minimize the fat content, n-hexane was applied. After the rather time-consuming processing of samples, the analysis was finally achieved utilising HPLC with photo-diode array detection.

The HPLC system equipped with a photo-diode array detector can detect a wide range of molecules and ensure the identification of the target compound. The absorption spectrum and the retention time provided strong evidence of its identity (Kishida and Furusawa, 2001).

Using the photo-diode array detector, the absorption spectrum of sulfadimidine standard in the mobile phase was measured in order to select the HPLC monitoring wavelength. The measurement was conducted at 275 nm, which gave an average maximum absorbance for sulfadimidine. The spectrum of sulfadimidine obtained from the samples was practically identical to that of the standard.

The retention time of sulfadimidine decreased with increasing pH value of the acetate buffer and the concentration of acetonitrile in the mobile phase. The best separation of sulfadimidine was achieved using an acetonitrile-acetate buffer.
(pH 4.6) (25:75, v/v) as the mobile phase. Sulfadimidine was successfully detected within 19 min, when the flow-rate was 1.0 ml/min. The minimum detectable amount (signal-to-noise ratio > 5) was 0.09 mg/kg⁻¹.

Although the LOD of this method was 0.09 mg/kg⁻¹, sulfadimidine residues in the whole eggs of laying hens were detectable only up to the 10th day of the withdrawal period. From the foregoing data it can be concluded that the 15-day withdrawal period recommended for sulfonamides since 1980 should be sufficient to ensure the reduction of sulfadimidine residues in the whole eggs of laying eggs to the acceptable level of 0.1 mg/kg⁻¹.

While sulfadimidine was continuously administered through the drinking water, a decrease in both water intake and egg production was observed. The decrease in water intake for sulfadimidine at the dose of 2g/l⁻¹ is because of the bitterness of the water (Puyt, 1995). Similar results were recorded by Roudaut and Garnier (2002).

The monitoring of sulfadimidine residues in foods of animal origin is a critical point in the protection of the food chain against the penetration of residues of this drug from the aspect of hygiene and public health. The development of individual analytical methods is related to the specific needs of the final consumer and analytical technologies available at the time. The choice of methods used in the laboratory will often be dictated by the availability of suitable expertise, facilities and equipment. The development of individual analytical methods with appropriately low detection limits, complying with complex and mutually interlinked requirements for screening procedures (rapid, reliable, broad-spectrum, sensitive, low-cost) constitute an impressive challenge (Šutiak et al., 2000; Kožárová et al., 2001; Kožárová et al., 2002).

ACKNOWLEDGEMENT
This study was supported by the grant VEGA SR No. 1/7027/20.

Address for correspondence:
Kožárová Ivona,
University of Veterinary Medicine,
Komenského 73, 041 81 Košice,
Slovak Republic

REFERENCES
Sulfonamidi se obično primjenjuju u tretmanu kokcidioze nosilja u dozi od 2 g/L vode za piće tokom 6 dana. Smatra se da rezidue ovih lekova mogu ostati u jajima i predstavljati potencijalni rizik za zdravlje ljudi.

Cilj ovog rada je bio da se utvrdi koncentracije sulfonamida u jajima tokom i posle p/o primene sulfonamida, kao i 15 dana od prestanka uzimanja leka. Rezidue sulfonamida su određivane na gradijentu visokih performansi u sistemu za tečnu hromatografiju sa foto-diodnim ARRAY detektorom na 275 nm. Naši rezultati ukazuju da davanje sulfonamida nosiljama dovodi do naglog i stalnog povećanja rezidua sulfonamida u jajima. Najveća koncentracija rezidua sulfonamida je utvrđena 6. dana pri p/o davanju. Nakon prestanka primene leka, koncentracija rezidua naglo pada, ali se one mogu detektovati i 10. dana od prestanka davanja. Jedanaestog dana koncentracije su bile ispod utvrđenog MRL nivoa (0.1 mg kg^{-1}). Granična kvantifikacija (LOQ) sulfonamida je iznosila 0.3 mg kg^{-1} a detektovana granica je bila 0.09 mg kg^{-1}.