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AN IMPROVED METHOD FOR THE DETERMINATION OF SULPHACHLOROPYRAZINE IN MEAT AND LIVER OF BROILERS DURING AND AFTER THEIR TREATMENT FOR COCCIDIOSIS

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The paper presents results of the HPLC determination of sulphachloropyrazine residues (active component of the drug "Esb_3 30%") in muscle tissue and liver of broiler chickens inoculated with laboratory-grown coccidium in the course and after treatment with this sulphonamide.

Extraction of sulphachloropyrazine from samples of broiler muscle tissue and liver was carried out with a mixture of solvents dichloromethane-methanol-acetic acid (90:5:5, v/v/v), followed by extract purification by chromatographic separation on a XAD-2 column and elution of sulphachloropyrazine residues with dichloromethane. The HPLC determination of sulphachloropyrazine residues was accomplished on a Bio Sil C-8 HL 5 μm column with a mobile phase consisting of 60% aqueous solution of acetonitrile and NH_3 (pH=9.5), using a UV detector at 254 nm.

The method developed allows quantitative determination of the residues of the anticoccidial agent in broiler tissue samples with a detection limit of 0.02 μg g^{-1}. Recovery of the method for this type of samples with a complex matrix was satisfactory, the results ranging from 79.2±0.6 to 86.7±0.2% for muscle tissue and from 81.7±0.8 to 87.3±0.7% for liver.

Keywords: broiler, HPLC, sulphachloropyrazine, tissues

Coccidiosis is an infective disease of the digestive tract which is most frequent with poultry, causing a decrease in daily increment, prolonged fattening, poorer skin pigmentation, slower feed conversion and increased mortality. The disease is caused by Protozoas from the genera of *Eimeria*, *Isospora* and *Cryptospora*, and it is manifested by damaging the intestine epithelial cells, less frequently the bile duct and renal tubuli (RADOSTITS et al., 1994). If coccidiosis is manifested in its clinical form, the treatment is most often accomplished by sulphonamide-based derivatives, i.e. the derivatives of p-aminobenzolsulphonic acid (KATZUNG, 1995).

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Sulphachloropyrazine, 4-amino-N(chloropyrazinyl)monosodium, monohydrate (active substance of the drug "Esb_3 30%" – Novartis) is a sulphonamide of a wide spectrum of action, capable of stopping the development cycle of coccidia and bacteria and thus their propagation, blocking primarily the synthesis of folic acid (BEVILL, 1988).

As chicken meat plays an important role in human nutrition, it is of essential importance from the health point of view to study the presence of sulphachloropyrazine in muscle tissue and liver of broilers during their treatment and in the post-treatment period. By determining the sulphachloropyrazine residues and establishing the withdrawal time, the risk of introducing this sulphonamide into the organism is diminished.

In the methods for determining sulphonamides in meat and meat products and other biological materials the most important, and at the same time the most difficult, step is the process of extraction and extract clean-up to remove matrix components that could interfere in the sulphonamide determination.

In the last decade or so, a number of instrumental techniques have been introduced for the determination of sulphonamide residues, such as thin-layer chromatography (THOMAS & SOROKA, 1982; HAAGSMA, 1985), immuno-enzymatic method (NOUWS et al., 1985), supercritical fluid chromatography (GUGGISBERG et al., 1992), and gas chromatography coupled with mass spectrometry (STOUT et al., 1984; SIMPSON et al., 1985). However, the most prominent place among them is occupied by high performance liquid chromatography (HPLC). By this method, sulphonamide residues are determined either directly after the separation on a chromatographic column using a UV detector (VAN'T KLOOSTER et al., 1991; KOSTADINOVIĆ, 1998), or indirectly by applying derivatization procedures followed by measurements on a fluorescent detector (TAKEDA & AKIYAMA, 1991; AERTS et al., 1986).

Sulphachloropyrazine is a new-generation sulphonamide which has been introduced in our country relatively recently. As far as we know, there are no reports concerning the determination of this sulphonamide in the muscle tissue of treated broilers. We can only mention the method recommended by the producer of this coccidiocide (ANDERSON et al., 1990). It consists of the extraction of sulphachloropyrazine from tissue samples with a mixture of solvents dichlormethane--methanol-acetic acid (90:5:5, v/v/v), extract cleaning on a cationic exchanger and HPLC determination using the TSK-ODS column with a mixture of 0.25 mol dm^{-3} ammonium acetate (pH=5.2) and acetonitrile (82.5:17.5 v/v) as mobile phase.

In this work we employed the same extraction procedure, whereas in the part of the clean-up and determination we introduced a number of modifications.

1. Materials and methods

1.1. Inoculation of broilers with laboratory-grown coccidia species

Heavy-line broiler chickens Arbor acres of both sexes (70 chicken), 28 days old, with average body weight of 1030 g, were inoculated with laboratory-grown coccidia species by p.o. administration of 1.00 cm^3 of coccidia suspension so that each chick received 2×10^4 oocista *Eimeria* (*E. necatrix*, *E. tenella*, and *E. mitis*). When the first clinical signs of coccidiosis appeared the broilers were treated with the preparation "Esb_3 30%", according to the producer's instructions. The drug was administered through drinking water: 2.00 g of the preparation were dissolved in 1.00 dm^3 of water, and the treatment was carried out following the regime of three-day treatment, two-day break, three-day treatment.

1.2. Taking samples of muscle tissue and liver

Samples of the muscle tissue and liver were taken after broilers sacrifice by randomly chosen 10 chickens, carried out on the third day of treatment, on the first day of the break following the three-day treatment (4th day), on the last day of treatment (8th day), and then on each of the subsequent three days after completing the treatment (9–11th day).

1.3. Preparation of samples

The extraction efficiency and recovery of the HPLC method for the determination of sulphachloropyrazine residues were studied by the standard addition method. To 10 g samples of broiler muscle tissue and liver the amounts of 1.0, 5.0, and 10.0 μg of sulphachloropyrazine standard were added. Three replicates were carried out for each sample with standard addition. Blank probes (samples of muscle tissue and liver of broilers not treated with sulphachloropyrazine) were run in parallel, and all the results were corrected for sulphachloropyrazine content in the blank.

1.3.1. Extraction procedure. Samples of 10 g of minced meat or liver were homogenized on a vibration shaker with 60 cm^3 of the solvent mixture dichlormethane--methanol-acetic acid (90:5:5 v/v/v) for 30 minutes. The extract was decanted and the extraction was repeated two more times, using the same volume of the extractant. The precipitated proteins were removed by filtration through the Whatmann No. 1 filtering paper. The filtrate was heated in a vacuum evaporator to the acetic acid fraction. By adding dropwise a solution of 0.1 mol dm^{-3} NaOH, the pH was adjusted to 7.0.

1.3.2. Clean-up procedure. In our procedure the extract cleaning was accomplished by passing it through a glass column (20×1 cm) filled with the XAD-2 adsorbent (grain size 0.3–1.0 mm; Serva; USA). The effluent was discarded and the sulphachloropyrazine residues were eluted with dichlorometane in 5 cm^3 fractions. It was found that the whole amount of sulphachloropyrazine absorbed is removed with 15 cm^3 of the eluent.

1.4. Calibration diagram

To construct the calibration diagram for the HPLC determination of sulphachloropyrazine 50 µl of its standard solution with the contents of 2, 4, 8, 12, and 16 µg cm^{-3} were taken whereby the obtained signals correspond to the amounts of 0.1, 0.2, 0.4, 0.6, and 0.8 µg of sulphachloropyrazine per probe. On the basis of the obtained chromatographic peaks we constructed the calibration graph presented in Fig. 1 (inset).

1.5. HPLC determination of sulphachloropyrazine residues

Contents of sulphachloropyrazine in the investigated samples were determined by HPLC on a Bio Rad system (Model 2800) with a Bio Rad UV spectrophotometric detector 1801. The column used was a C-8 HL (4.6×200 mm) 5 µm with the mobile phase: 60% acetonitrile in water with pH=9.5 adjusted with NH_3. Before use, the mobile phase was degassed on an ultrasonic bath. The other parameters of the HPLC determination were as follows: injected volume 50 µl; total flow-rate 1 cm^3 min^{-1}; wavelength 254 nm; column temperature 22 °C.

Under the given determination conditions the retention time for sulphachloropyrazine was 11.41 min.

2. Results and discussion

2.1. Determination of the relationship between chromatographic peak area and sulphachloropyrazine concentration

On the basis of the calibration diagram for the HPLC determination of sulphachloropyrazine (Fig. 1 (inset)), it is evident that a successful quantification of this coccidiocide is possible in the concentration range from 2 to 16 µg cm^{-3}, as the correlation coefficient for the linear relationship between the peak area and concentration is very good (r=0.999). In case of the determination of higher concentrations it would be necessary to change the measurement conditions. Such new conditions would allow the determination of higher concentration but the detection limit would then be higher.

2.2. Recovery

The extraction efficiency and the recovery achieved by the HPLC procedure for the determination of sulphachloropyrazine residues in muscle tissue and liver samples were tested by standard addition method, using the sulphachloropyrazine spikes of 0.1, 0.5, and 1.0 μg/g.

In Fig. 1 the chromatograms obtained for the extract of broiler muscle tissue with 0.1 μg/g of sulphachloropyrazine standard addition (a), for the muscle tissue of broilers receiving no sulphachloropyrazine (b), and for the muscle tissue of after the three-day treatment and one-day break (c) are presented.

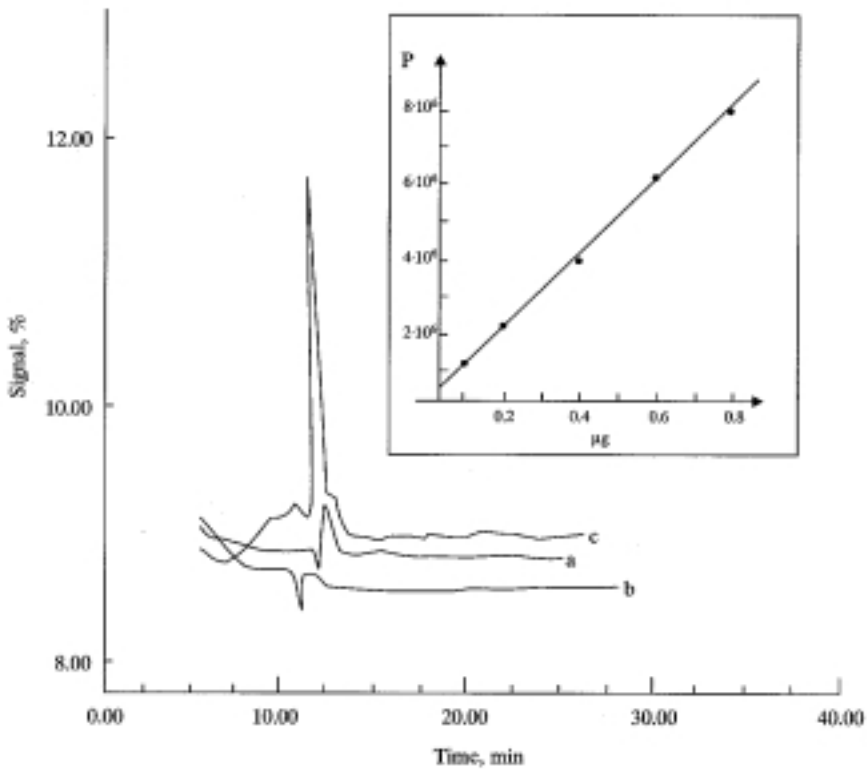

Fig. 1. Chromatograms of the broiler muscle tissue with standard addition 0.1 μg/g of sulphachloropyrazine (a), muscle tissue of broilers receiving no sulphachloropyrazine (b), and muscle tissue of broilers on the first day of the break following the three-day treatment (c). Inset: calibration graph

Using standard addition method it was found that the detection limit for sulphachloropyrazine was 0.02 μg/g.

The results of studying the efficiency and recovery of the applied HPLC procedure for the determination of sulphachloropyrazine in the investigated biological samples with addition of sulphachloropyrazine are presented in Table 1.

The results demonstrate that the developed procedures for extraction and HPLC determination can be successfully used to determine sulphachloropyrazine residues in the samples of muscle tissue and liver of broilers. The recovery of the method was in the range from 79.3±0.6 to 86.7±0.2% for muscle tissue and from 81.7±0.8 to 87.3±0.7% for liver samples. Having in mind the complexity of the matrix, these recovery values should be considered as satisfactory.

2.3. Results of the determination of sulphachloropyrazine residues

Using the above procedure we determined the contents of sulphachloropyrazine in samples of the muscle tissue and liver of coccidia-inoculated broilers and in those treated with a therapeutic dose of sulphachloropyrazine. The treatment with sulphachloropyrazine was carried out according to the regime: three-day treatment, two-day break, three-day treatment, samples for the analysis were taken on the third day of treatment, on the first day of the break after the three-day treatment (4th day), on the last day of treatment (8th day), and on the subsequent three days after completing the treatment (9–11th day). The results are presented in Table 2.

Table 1

Results of assessing the efficiency of the determination of sulphachloropyrazine in samples of broiler muscle tissue and liver

Sample	Added (μg/g)	Found ± SD (μg/g)	Recovery ± SD (%)
Muscle tissue	0.1	0.087 ± 0.002	86.7 ± 0.2
Muscle tissue	0.5	0.39 ± 0.06	79.3 ± 0.6
Muscle tissue	1.0	0.83 ± 0.05	83.3 ± 0.5
Liver	0.1	0.087 ± 0.065	87.3 ± 0.7
Liver	0.5	0.409 ± 0.078	81.7 ± 0.8
Liver	1.0	0.825 ± 0.005	82.5 ± 0.5

SD: standard deviation

Table 2

Results of the determination of contents of sulphachloropyrazine residues in muscle tissue and liver of treated broilers

Sample	Sampling day	Found[a] µg/g±SD
Muscle tissue	3rd	22.3 ± 5.4
	4th	0.79 ± 0.24
	8th	2.57 ± 0.02
	9th	0.29 ± 0.03
	10th	0.07 ± 0.01
	11th	0.02 ± 0.02
Liver	3rd	32.4 ± 6.2
	4th	2.00 ± 0.09
	8th	2.33 ± 0.01
	9th	0.06 ± 0.01
	10th	ND
	11th	ND

ND: not detected

[a] results were not corrected for the % of recovery

The presented results indicate that there is a minimal risk of the appearance of sulphachloropyrazine residues in tissues of the broilers treated with this coccidiocide. On the first day of the break following the complete treatment, the content of sulphachloropyrazine residues in the muscle tissue was three times higher than the maximal allowed content of 0.1 µg/g given in the pertinent REGULATIONS (1992), and on the subsequent day it was already below this value.

On the first day of the break after the completed treatment (9th day from the beginning of treatment) the content of sulphachloropyrazine residues was already below the tolerated level, and on the third day of the break (11th day) it was below the detection limit of the HPLC method employed in the liver samples of the sulphachloropyrazine treated broilers.

The obtained results confirmed that sulphachloropyrazine has a short withdrawal time, which for broiler meat is one day. In this respect it is advantageous over other sulphonamide agents used to treat coccidiosis, for which this period is 5 to 6 days. Our method of the extract cleaning-up and detection of sulphachloropyrazine enables a simple, selective and reproducible determination of this coccidiocide in samples with a complex matrix. Compared to the original method (ANDERSON et al., 1990), the determination is simplified, the analysis time is shortened and the potential losses due to transferring the extract to alkaline pH range are eliminated.

3. Conclusions

The developed HPLC method enables successful quantitative determination of sulphachloropyrazine residues in samples of muscle tissue and liver of broilers. The detection limit of 0.02 μg/g and the recovery ranging from 79.2±0.6 to 86.7±0.2% for the muscle tissue and 81.7±0.8 to 87.3±0.7% for the liver samples confirm the applicability of the method.

By determining content of sulphachloropyrazine in the coccidia-inoculated broilers treated with therapeutic doses of this anticoccidial agent it was established that there is a minimal risk of the appearance of its residues in their meat and liver. On the first day of the break following the complete treatment, the content of sulphachloropyrazine residues in broiler muscle tissue was three times higher than the tolerable level of 0.1 μg/g given by the pertaining regulations, which confirms the necessity of establishing the withdrawal period for sulphachloropyrazine, which in case of broiler meat is one day.

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