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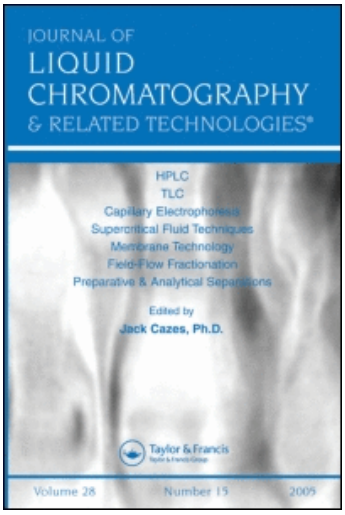

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RAPID ASSAY FOR THE DETERMINATION OF RESIDUES OF AMPROLIUM AND ETHOPABATE IN CHICKEN MEAT BY HPLC

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ABSTRACT

A simple, rapid and sensitive HPLC method for the determination of amprolium and ethopabate in chicken meat, is presented. The samples were extracted with acetone, the organic layer then being separated and evaporated to dryness (ethopabate analyse). Employing traditional liquid-liquid extraction, a clean extract was obtained from the water phase (amprolium analyse). The limits of quantification were 5 and 1 ng/g for amprolium and ethopabate, respectively.

INTRODUCTION

The combination of ethopabate (ETB) and amprolium (AMP) is frequently used in the prophylaxis and treatment of coccidiosis and leukocytozoonosis in chickens.[1,12]

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Several analytical methods for the determination of AMP and ETB in different biological materials based on colorimetry,[2] gas chromatography,[3,4,5] and high performance liquid chromatography with ultraviolet and fluorescence detection[6,7,8,9,10,11,12] have been published. The methods are, however, time-consuming and require the use of large quantities of chemical reagents.

The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method, for the determination of AMP and ETB, which required only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of chicken meat were used.

All chemicals and solvents were of analytical or HPLC grade. AMP was supplied by Sigma Co. (St. Louis, MO, USA), and ETB by Merck Frosst Canada, Pointe - Claire, Dorval, Quebec. AMP stock solution (1mg/mL) and working standards were prepared by dilution with 0.02 M KH_2PO_4. The ETB stock solution (1mg/mL) was made by dissolving 25 mg ETB in 5 mL acetone and diluting to 25 mL with water. The working standards were prepared by dilution with distilled water. The standards solution were stored in the refrigerator.

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Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LC 240 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at an excitation wavelength of 365 nm and emission wavelength of 470 nm for AMP, and an excitation wavelength of 300 nm and emission wavelength of 350 for ETB, with a response of 5 and a factor of 256. The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 15 cm x 4.6 mm I. D.), operated at room temperature and guard column (stainless steel, 2 cm x 4.6 mm I. D.) were packed with 5-μm particles of Supelcosil LC-ABZ, (Supelco) for AMP. The analytical column for ETB (stainless steel, 25 cm x 4.6 mm I. D.), operated at

room temperature, and guard column (stainless steel, 2 cm x 4.6 mm I. D.), were packed with 5-μm particles of Supelcosil LC-ABZ+Plus (Supelco, Bellefonte, PA, USA). The guard column was connected to a A.318 precolumn filters with a A-102 frits (Upchurch Scientific, USA).

The mobile phase for AMP was a mixture of the solutions A and B (90 : 10). Solution A was 0.2 M KH_2PO_4, 0.005 M hexane sulfonic acid Na salt, made by dissolving 27.2 g/L KH_2PO_4 and 0.94 g/L hexane sulfonic acid Na salt in ca. 750 mL water when making 1 litre of solution. The solution was made up to volume with water . Solution B was acetonitrile. The flow rate was 0.8 mL/min. The column effluent was introduced into a vortex mixer (a low dead volume tee (1.2μL)) from a system for HPLC post column reactions (PCRS 520 - Kratos). A Series 10 Liquid Chromatograf (Perkin-Elmer), (fitted with an extra pulse-dampened pump) was used, with a mobile phase of 1.25 M NaOH, 0.025 M $K_3Fe(CN)_6$ made by dissolving 50 g/L NaOH in ca. 75 mL water, adding ca. 700 mL water (waiting until the solution had achieved room temperature), and then adding and dissolving 0.8 g/L potassium ferricyanide. The solution was made up to volume with water. The mobile phase had a flow rate of 0.7 mL/min., and was coupled to the vortex mixer, and a reaction coil (Beam Boost Photochemical Reactor Unit, PTFE 10 m x 0.3 mm I. D. - ITC Dandelsgesellschaft m.b.H.Frankfurt, Germany), operated at room temperature. The solvent stream was then coupled to the fluorescence detector. The system was equilibrated with the mobile phase and the derivating reagents for about 20 min. (0.8 and 0.7 mL/min. respectively) prior to injecting the sample into the HPLC.

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The mobile phase for ETB was a mixture of water - acetronitrile (65 : 35). The flow rate was 0.8 mL/ min.

Sample Pretreatment

The sample, (3g) was weighed into a 50 mL centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 1 mL water (or standard) and 4 mL acetone were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.), and then centrifuged for 3 min. (5000 rpm). Four mL of the supernatant (corresponding to 1.5 g) were transferred into a glass-stoppered centrifuge tube. A volume of 5 mL CH2Cl2 was added and mixed for approx. 5 sec. After centrifugation for 3 min. (3000 rpm), the upper (water) layer was transferred to another glass-stoppered tube (AMP analysis). The organic layer dichloromethane-acetone for ETB analysis was transferred to another glass-stoppered tube, possible water residues in the tube being transferred back to the water phase tube (AMP analysis). The organic layer was evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 60°C and

Reacti-Vap evaporating unit (Pierce, Rockford, IL, USA). The dry residue was dissolved in 500 µl of a mixture of two solutions A and B (70 : 30). Solution A was methanol and B was 0.02 M 1- heptane sulfonic acid sodium salt (Supelco, USA) - 0.01 M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany), made by dissolving 4.45 g/L heptane sulphonate and 1.8 g/L di-sodium hydrogen phosphate 2-hydrate in ca. 750 mL of water when preparing 1 litre of solution. The pH was then adjusted to ca. 6.3 with 5 M H_3PO_4 and to 6.0 with 1 M H_3PO_4, and the solution made up to volume (1 L) with water, the pH again being adjusted to 6.0 with 1 M H_3PO_4. The dissolved residue was kept in a freezer (-20°C) for 5 min. The methanol-based phase was filtered through a Costar Spin-X centrifuge filter unit with 0.2 µm nylon membrane, and centrifuged for 3 min. at 10000 rpm. (5600g). Aliquots of 20 µL were injected into the HPLC at intervals of 10 min. for the determination of ETB.

1g NaCl, 3mL CH3CN and 1 mL 0.3 M NaOH were added to the water phase. The sample was shaken vigorously for 20 sec., and centrifuged for 2 min. at 3000 rpm. The upper layer (CH_3CN) was transferred to another glass-stoppered tube. The sample was extracted twice with 3 mL CH_3CN, and the water layer discarded. The CH_3CN was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 500 µL 0.02 M KH_2PO_4 and filtered through a Spin-X by centrifugation for 3 min. at 10000 rpm. (5600g). Aliquots of the filtrate (20µL) were injected into the HPLC at intervals of 8 min. for the determination of AMP.

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Calibration Curves And Recovery Studies

The calibration curves for AMP and ETB were obtained by spiking muscle tissue samples with standard solutions, to yield 5, 10, 15, 20, 30, 50, 100, 200, 300 and 500 ng/g and 1, 2, 5, 10, 20, 30 and 50 ng/g of AMP and ETB, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked muscle samples with those of standard solution. The linearity of the standard curves for AMP and ETB in muscle were tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of extract of blank samples, and spiked samples from chicken meat are shown in Fig. 1 and 2. The standard curves were linear in the investigated areas; 5 - 500 and 1 - 50 ng/g for AMP and ETB in meat, respectively. The linearity of the standard curves was 0.9998 for AMP and

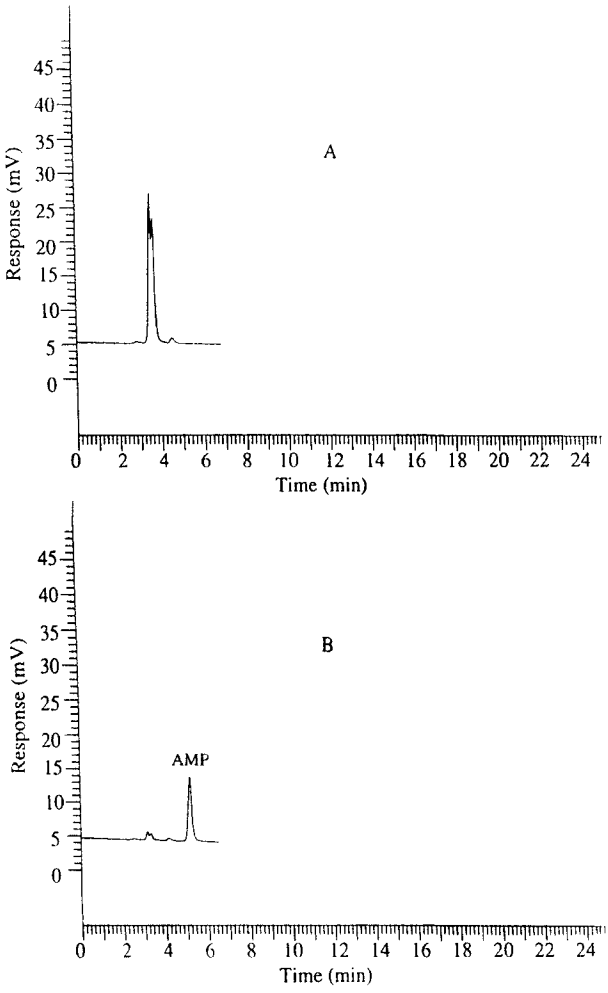

Figure 1. Chromatograms of extracts from chicken meat. **A:** drug-free meat, **B:** meat spiked with amprolium (200 ng/g).

0.999 for ETB in meat, when using the external standard method of calculation. The precision and recovery for AMP and ETB from meat were also calculated and are shown in Table 1.

The extraction procedures were validated, and showed good recovery of AMP and ETB. The recovery was 99% for AMP and varied from 98 to 99% for ETB in meat. The precision of these recovery studies varied from 0.8 to

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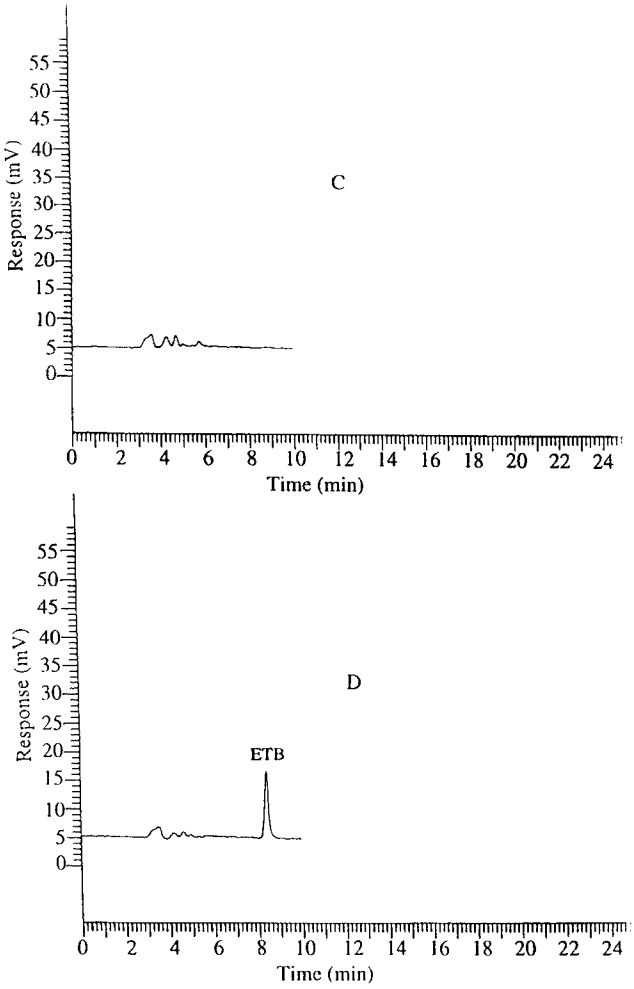

Figure 2. Chromatograms of extracts from chicken meat. **C:** drug-free meat, **D:** meat spiked with ethopabate (20ng/g).

1.0% and from 0.2 to 0.8% for AMP and ETB in meat, respectively. The limit of quantification was 5 ng/g and 1ng/g for AMP and ETB in meat, respectively.

The method was tested under practical conditions by analysing about 50 samples from different chickens, with no interfering peaks being observed. No interference was seen during analysis, when calibrating the curves, nor when performing recovery studies.

Table 1

Precision and Recovery of AMP and ETB

Tissue 3g	No. of Samples	Amount in Spiked Samples (μg/g)	Recovery% AMP Mean	AMP SD	ETB Mean	ETB SD
Meat	8	0.05	99	0.8		
3g	8	0.30	99	1.0		
Meat	8	0.005			98	0.8
3g	8	0.030			99	0.2

S.D. = standard deviation

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AMP was separated from interfering substances, using a reverse phase ion-pair system, and was post-column oxidized to amprochrome with ferricyanide in alkaline solution, giving a fluorophore that can be determined with greater sensitivity than by U.V. detection.[2,8] The LC operating conditions, i. e., mobile phase composition, flow rate, column temperature, concentrations of NaOH and ferricyanide, and detection wavelength were investigated, each of these parameters being optimized to provide maximum drug response.

Conditions were as reported by T. Nagata and M. Saeki[8] apart from column temperature which do not have any particular effect between 20 - 40°C, and the mobile phase composition which was adjusted to a 15 cm ABZ analytical column. The reaction coil length used gave a satisfactory result.

Because of the high salt concentration in the mobile phase (0.2M KH_2PO_4 - 0.005M hexane sulfonic acid Na salt) the HPLC system, including the analytical column (15 cm) for AMP, was flushed with water-acetonitrile (90 : 10) for 15 min. (1 mL/min.) prior to and after running a serie of samples.

It is also important to follow the recommendations of the producer to store Supelcosil LC-ABZ column in 100% acetonitrile, to avoid serious retention loss.

Regarding the ETB analytical column (25 cm) it is not necessary to take special precautions since the mobile phase is water - acetonitrile. However, the producers recommendations concerning the storage of Supelcosil LC-ABZ+Plus in 100% methanol must be followed.

This study has shown that residues of the coccidiostatic compounds AMP and ETB in chicken meat can be determined using minimal sample manipulation. The cost of chemicals is reduced and the manual work-up procedures are less laborious, compared to previously published methods.

An experienced technician can carry out sample clean-up of about 18 samples per day. The pretreatment of tissues by liquid-liquid extractions combined with centrifugation filters, is preferable to solid-phase extraction columns when performing the pretreatment manually. The assay shows good precision when using the external standard method. The method is robust, sensitive and is efficient for quantification of residues of AMP and ETB. The quantification is linear over a wide concentration range.

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