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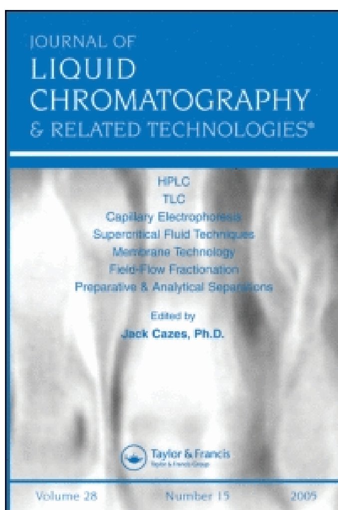

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HPLC DETERMINATION OF CHLORAMPHENICOL AND THIAMPHENICOL RESIDUES IN GAMEBIRD MEATS

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ABSTRACT

Liquid chromatographic methods for the determination of chloramphenicol (CAP) and thiamphenicol (TAP) residues in gamebird (pheasant, mallard and quail) meats were developed. The drugs were extracted from the homogenised tissues (muscle and liver) by water and the extracts were purified by subsequent partitions with Chem Elut CE 1020 cartridge - ethyl acetate and water - toluene. The recoveries were 67% for CAP and 72% for TAP. Chromatographic separations were performed on a Hypersil C-18 column and the peak identification and quantitation was made with a photodiode array detector. Using the first derivative of the chromatographic peak the selectivity of the analysis was improved. The elimination kinetics of CAP and TAP from the quail tissues were also evaluated.

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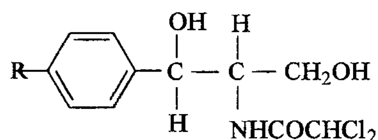
INTRODUCTION

Chloramphenicol (CAP) and Thiamphenicol (TAP) are synthetic broad-spectrum antibiotics which are used by veterinary practice in the prevention and treatment of many bacterial infections (1, 2). The drugs are structurally correlated, the nitrogroup (R= $-NO_2$) in CAP being replaced with a methylsulphonyl group (R= $-SO_2CH_3$) in TAP.



CAP may display toxic side-effects to humans such as irreversible aplastic anaemia (3); these toxic effects have not been observed for TAP. For its toxicity CAP is banned for food producing animals in the United States; in the European Community (EC) the CAP use is prohibited for poultry and it is restricted for other animals. Consequently, appropriate control programs based on a proposed maximum residue level (MRL) of 10 μg CAP/Kg are recommended (4).

Among the various techniques, several liquid chromatographic (HPLC) methods have been proposed for the selective and sensitive determination of CAP residues in meat and fish (4-13, 15, 17, 18,) milk (4, 16, 17) and eggs (14, 15).

For the determination of TAP residues in edible tissues (8, 19, 21) and milk (23) few HPLC methods have been described.

The present study was undertaken to provide HPLC procedures suitable for the determination of CAP residues in gamebird meats whose importation in Italy has turned prominent in the last years. The animal "type" traded (gamebirds

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shooted in game reserve but almost always obtained by intensive breeding) and the provenience from countries where the drug use is differently regulated, led us to investigate about a possible presence of CAP residues in the pheasant and mallard meat. The work also included the Japanese quail, a gamebird which is intensively reared in large quantity in hour country. To this end, a reversed-phase liquid-chromatographic (HPLC) method was developed as useful screening method for monitoring CAP and TAP residues in commercial gamebird meats.

Diode array UV-VIS detection was used for the peak purity evaluation and the drug identification. The HPLC method was also applied to achieve informations on the CAP and TAP elimination kinetics from the muscle tissues after the drug administration at therapeutic doses.

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EXPERIMENTAL

Chemicals

Chloramphenicol (CAP) was obtained from Fluka (Switzerland); thiamphenicol (TAP) was supplied from Zambon S.p.A. (Italy). Solvents for chromatography were of HPLC grade from Mallickrodt (MO, USA); triethylamine (TEA) and all the other chemicals were from Farmitalia-C. Erba (Italy). Chem Elut CE 1020 cartridges from Analytichem International (USA) were used for liquid-liquid extraction.

Apparatus

Omni-mixer (Sorval Inc., Connecticut, USA), Sonorex super AK 102H (Bandelin, Germany) and Centrifuge 4225 ALC (Italy) were used.

The chromatographic system consisted of a 5020 Varian liquid chromatograph equipped with a Rheodyne 7125 model injector (100 μL sample

loop) and connected to a HP 1040A photodiode array detector (Hewlett-Packard, USA).

The chromatographic separations were performed on a 5 μm RP-18 Hypersil column (150x4.6 mm I.D.) using a mobile phase consisting of 0.05M TEA phosphate buffer (pH 3) - acetonitrile 79:21 (v/v) for CAP and 86:14 (v/v) for TAP at a flow rate of 1 mL /min.

UV detection at 278 and 224 nm was used for the routine analysis of CAP and TAP, respectively.

Sample preparation

Fresh or thawed meat was homogenised with Omni-mixer and an accurately weighed sample (about 10g of muscle and 5g of liver) was subjected to ultrasonication with 40 mL of water at ambient temperature for 8 min. The mixture was then centrifuged for 10 min and 20 mL of the supernatant were applied to a Chem Elut CE 1020 cartridge allowing to equilibrate for 15 min. The drug was then eluted with 50 mL of ethyl acetate and the eluate was evaporated to dryness with a rotavapor. The residue was mixed with 0.5 mL of water and extracted (2x1mL) with toluene (gentle vortex mixing and centrifugation). After careful removal of toluene, the aqueous phase was filtered through a 0.45 μm filter and injected (100 μL) into the LC system.

This general procedure was applied to the analysis of all the samples (Blank tissues, samples fortified with known quantities of CAP and TAP, and real samples).

Assay

Sample solutions and working standard solutions were sequentially subjected to the HPLC analysis according to an alternate order. The content of CAP and

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TAP in each sample was calculated by comparison of the analyte peak height of the sample with that of standard solutions equivalent to initial concentrations of 20 μg/Kg for CAP and 80 μg/Kg for TAP.

Calibration graph

Chloramphenicol- Homogenised samples (quail muscle) were fortified with known quantities of CAP to give sample containing 10-75 μg/Kg of the drug. The described sample handling was followed and the peak heights were plotted against the corresponding added drug concentrations to obtain the calibration graphs.

Alternatively, when the first derivative of the chromatographic peak was used, the amplitude of the first satellite peak to the zero line was measured and plotted against the added drug concentration to obtain the calibration graph.

Thiamphenicol - The same procedures described for CAP were applied to homogenised samples (quail muscle) fortified with TAP at the level of 10 -100 μg/Kg.

Each sample solutions for the calibration graphs of CAP and TAP were also compared with the corresponding standard solution in order to evaluate the drug recovery at different concentration levels.

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Elimination of CAP and TAP residues

Chloramphenicol- 18 quails were orally dosed with a 0.8 g/L CAP aqueous solution for three days for a mean total dose of 85 mg/Kg. From the fourth day, three quails were daily slaughtered and the breast tissues were subjected to the described extraction and HPLC methods for quantifying the CAP residues. Tissues from control quails and tissues fortified with CAP at 40 μg/Kg level were concurrently analysed.

Thiamphenicol- 14 quails were orally dosed with TAP for three day for a mean total dose of 108 mg/Kg. Then, two quails were slaughtered at different time intervals up to 16th day. The muscle tissues from treated and control quails were then concurrently analysed with tissues fortified with TAP at 80 μg/Kg level.

RESULTS AND DISCUSSION

Sample preparation

For the extraction of CAP or TAP from edible tissues ethyl acetate (7, 8, 19, 21-24), ethyl ether (11), water (4-6, 10, 27), diphasic dialysis (16) and matrix solid-phase dispersion (26) have been used. In this study, to obtain a rapid and practical sample clean-up suitable for a screening method, an aqueous extraction (4-6) was chosen with minor modifications. Briefly, the homogenised tissues was subjected to ultrasonication with water; an aliquot of the aqueous extract was applied to a Chem Elut CE 1020 cartridge and the drug elution was carried out with ethyl acetate. After the solvent evaporation the residue was subjected to water-toluene partition to remove interfering co-extractants and to give a suitable aqueous sample solution. Ethyl acetate was used instead of dichloromethane (4-6) for the cartridge eluition and this resulted in improved overall recoveries (67%; RSD% = 5.1-8.2) for CAP and acceptable overall recoveries (72%; RSD% = 5.4-9.1) for TAP in the 10 - 75 μg/Kg range. The repeatability, as indicated by RSD%, was decreasing with the drug concentration. When aqueous standard solutions of CAP and TAP were applied to the Chem Elut column, the ethyl acetate elution gave mean recoveries of about 85%; this suggest that considerable analyte losses take place during the cartridge elution.

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Chromatography

The described sample clean-up allowed adequate chromatographic separations to be performed under isocratic conditions.

Routine analyses were carried out on 5 μm Hypersil C-18 column using binary mixtures of TEA phosphate solution (pH 3.0) - acetonitrile 79:21 (v/v) for CAP and 86:14 (v/v) for TAP as the mobile phase.

Typical chromatograms obtained from the HPLC analysis of quail, pheasant and mallard tissues fortified at 75 μg/Kg level of CAP are reported in Figs 1, 2 and 3, respectively. In each case, by comparing the sample chromatogram with that from the corresponding control tissues the absence of matrix interferences can be ascertained. UV Diode Array Detection (DAD) was used to check the peak purity and to confirm the analyte peak identity (5, 7). In order to avoid false positive results, three dimensional plots (spectrochromatograms) were obtained, giving direct informations about the peak identity (retention and UV spectrum) and the background interferences.

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A 3D plot for TAP is shown in Fig. 4; similar 3D plots for CAP were obtained according to previous reports (11). The described HPLC method with DAD detection enabled a reliable identification of CAP at ≥8 μg/kg level, with a detection limit (S/N = 3) of about 2 μg/Kg. This, should prevent false positives at the proposed tolerance level of 10 μg/Kg. On the other hand, positive samples should be confirmed by approved confirmatory methods, such as GC-Mass spectrometry (4).

Analysis of CAP and TAP residues

For the quantitation of the CAP and TAP residues in the muscle tissues a calibration graph for each drug was constructed. Quail muscle homogenates were

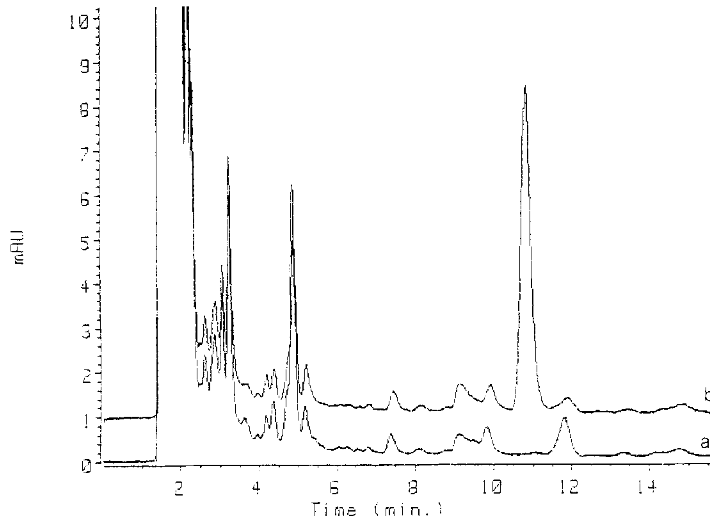

Fig. 1 - HPLC chromatograms obtained from quail muscle tissues: a) control tissue, b) tissue fortified with CAP at 75 µg/Kg level. Chromatographic conditions: 5 µm Hypersil C-18 column (150x4.6 mm I.D.) using a mobile phase of 0.05M TEA phosphate (pH 3.0) - acetonitrile 79:21 (v/v) at a flow - rate of 1 mL/min.

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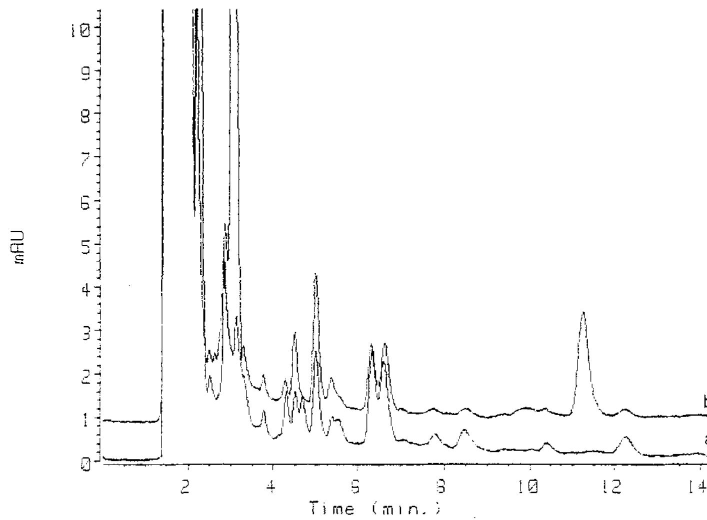

Fig. 2 - HPLC chromatograms obtained from pheasant muscle tissues: a) control tissue, b) tissue fortified with CAP at the level of 75 µg/Kg. Chromatographic conditions as in Fig. 1.

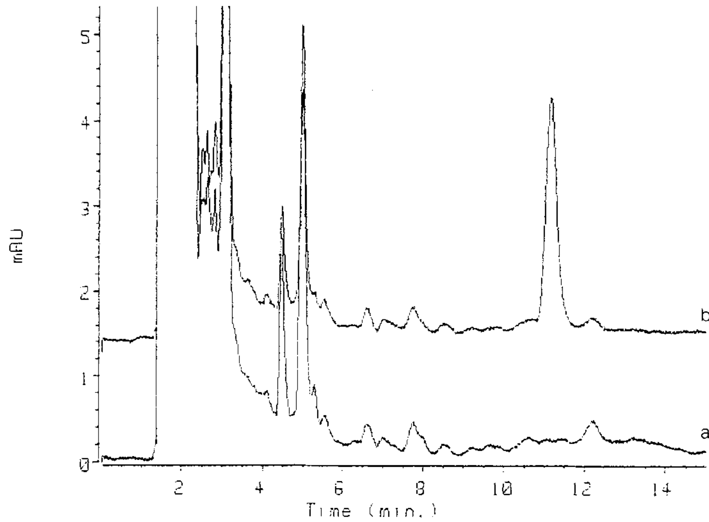

Fig. 3. - HPLC chromatograms obtained from mallard muscle tissues: a) control tissue, b) tissue fortified with CAP at the level of 75 μg/Kg.
Chromatographic conditions as in Fig. 1.

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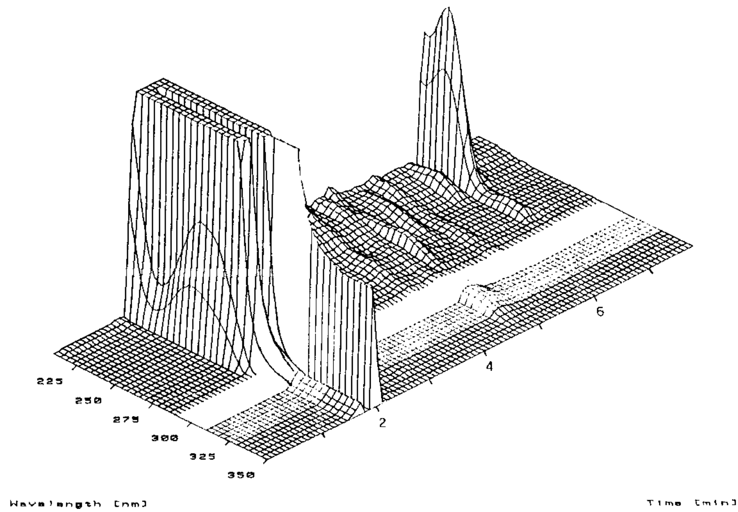

Fig. 4 - Three dimensional plot (spectrochromatogram) of a homogenised muscle tissue from a quail orally treated with TAP. Retention time of TAP: 6.62 min.

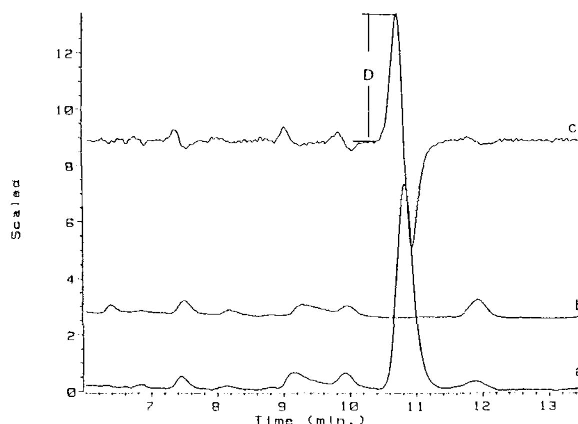

Fig. 5 - HPLC chromatogram obtained from quail muscle tissues: a) zero-order chromatogram of tissue fortified with CAP at 40 µg/Kg level, b) zero-order chromatogram from a control tissue, and c) first-order derivative of the chromatogram (a).
The amplitude D was used for the CAP quantitation.

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fortified with known quantities of the drug over the 10-75 µg/Kg range; after the sample clean-up process and the HPLC analysis a linear relationship (y=0.606x + 2.86; r=0.9987; n=5) was obtained by plotting the CAP peak height (y) against the CAP concentration (x; µg/Kg). The obtained linear curve exhibits a significant y-intercept; in effect, the UV spectra (upslope, apex, downslope) showed the peak not to be homogeneous, owing to some interference at higher times. Thus, the first-order derivative spectrum of the chromatographic peak was recorder and the amplitude (D) of the first positive peak was measured (Fig. 5). By plotting this amplitude (y) against the drug concentration (x) an improved linear relationship (y=0.310x + 0.205; r=0.9993; n=5) with reduced y- intercept was obtained. Similar effect was observed when the analysis of TAP was carried out. The best linear relationship was observed using the first

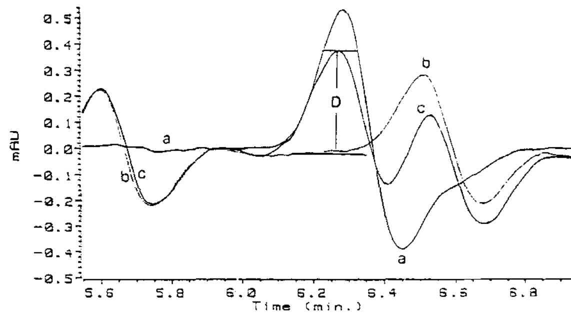

Fig. 6 - First-order derivative chromatograms obtained from: a) standard solution of TAP (100 ppb), b) control quail tissue, and c) tissue from quail orally treated with TAP. The amplitude D was measured for quantitative applications.

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derivative of the TAP chromatogram (Fig. 6) and measuring the amplitude (D) of the first positive peak (y=0.0056x - 0.0031; r=0.9996; n=5). Thus, the application of the derivative approach to the analyte chromatographic peak allowed a more specific "analytical window" to be used in order to reduce the matrix background interferences.

The described HPLC method was applied to the determination of CAP residues in quails (52 muscle tissues), pheasants (33 muscle and 10 liver tissues) and mallard (30 muscle and 7 liver tissues) obtained from the market. No positive results were found; therefore, according to the method sensitivity, the presence of CAP at > 8 µg/Kg level was excluded for all the analysed samples. On account of this result, it was considered of interest to achieve informations on the elimination kinetic of CAP from the tissues after the drug assumption at therapeutical level. Thus quails were dosed with CAP and the resulting drug residues in the muscle tissues were monitored over a 6 days period. Analogous investigations were then performed on the elimination of TAP

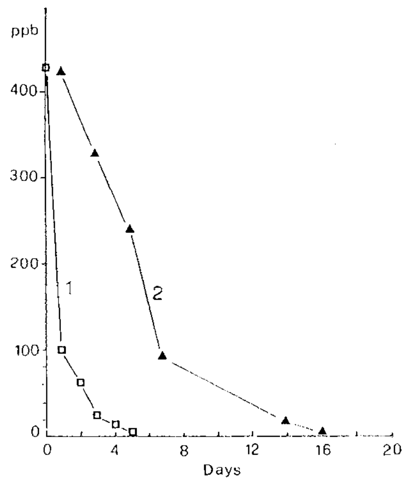

Fig. 7 - Curves concentration - time obtained for quail muscle tissues after CAP (1) and TAP (2) oral administration at therapeutical doses.

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residues. The resulting curve concentration - time observed for each drug are reported in Fig. 7.

As shown, after five days the CAP residues were lower of the maximum residue level (10 μg/Kg). The observed rapid elimination of CAP from quail tissues was comparable to previous results obtained for chicken tissues (27).

Differently, TAP residues were found to be longer present in the quail tissues; after 14 days TAP residues at the level of 20 μg/Kg were still present. According to this slow elimination, the italian low states a withdrawn period of 21 days before the slaughter of food animals medicated with TAP. These data are consistent with the known different pharmacokinetics of CAP and TAP (2).

In summary, convenient HPLC screening methods have been developed for the determination of CAP and TAP residues in edible tissues of a variety of gamebird (pheasants, mallards and quails). The methods offer adequate

selectivity and sensitivity; using photodiode array detection the peak identity can be confirmed and the interferences from co-extractants in the quantitative assay can be eliminated by a derivative approach to the chromatographic peak evaluation.

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