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## Short Communication

# Determination of thiamphenicol residues in chicken muscles by column liquid chromatography

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ABSTRACT

A column liquid chromatographic (CLC) method for the determination of thiamphenicol residues in chicken muscles was developed. The drug is extracted from minced muscles with ethyl acetate and the extract is evaporated to dryness. The residue is dissolved in 10% sodium chloride solution and partitioned with *n*-hexane. Thiamphenicol is extracted with ethyl acetate and, after evaporation of the solvent, the residue is cleaned up by alumina column chromatography. CLC analysis is carried out on a Nucleosil $C_{18}$ column with ultraviolet detection of thiamphenicol at 230 nm. The average recoveries of thiamphenicol added to muscles at 0.2 and 0.1 ppm were 92.8 and 90.0%, respectively. The detection limit was 5 ng for thiamphenicol standard, which corresponds to 0.05 ppm in muscles.

## INTRODUCTION

Thiamphenicol, like chloramphenicol, is a synthetic antibiotic with broad spectrum. In spite of their chemical analogy, the toxicity of thiamphenicol, unlike chloramphenicol, is low. Thiamphenicol was demonstrated to be valuable for the treatment of bacterial infections in animals [1]. The presence of drug residues in animal meats is undesirable from the standpoint of human safety. Therefore, it was decided to develop a sensitive method for the determination of thiamphenicol in animal tissues.

For the determination of thiamphenicol in biological fluids [2–6] or in swine muscles [7], a spectrophotometric method based on alkaline hydrolysis [2] and gas chromatographic methods [3–7] have been reported. The spectrophotometric method is time-consuming and lacks sensitivity. Gas chromatographic methods are complicated because they require the derivatization of thiamphenicol before analysis on a gas chromatograph equipped with an electron-capture [3,6], a flame photometric [7] or a flame ionization detector [4,5].

Column liquid chromatographic (CLC) methods for determining thiamphen-

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icol in biological fluids [8] and bovine plasma [9] have also been reported. These CLC methods are not suitable for the determination of thiamphenicol in chicken muscles, as the extract is not purified well using these methods. Many interfering peaks are observed in the chromatogram and thiamphenicol is not determined accurately.

In this study, an investigation was conducted to establish a convenient method for the determination of thiamphenicol residues in chicken muscles at levels as low as 0.05 ppm using CLC.

## EXPERIMENTAL

### *Materials and reagents*

Chemicals of analytical-reagent grade and deionized water were used unless specified otherwise. Ethyl acetate, acetonitrile, *n*-hexane and sodium chloride were purchased from Wako (Osaka, Japan).

A standard solution was prepared by dissolving 10 mg of thiamphenicol (Eizai, Tokyo, Japan) in 100 ml of acetonitrile (100 $\mu$g/ml). A working standard solution was prepared by diluting the standard solution with acetonitrile.

Alumina, Woelm B activity grady I (ICN Biochemicals, Eschwege, Germany), was packed into a chromatographic column (300 mm × 15 mm I.D.) with 3 g of alumina suspended in 30 ml of acetonitrile. The column was washed with 30 ml of acetonitrile followed by 30 ml of acetonitrile–water (95:5, v/v).

### *Chromatographic conditions*

A Shimadzu LC-3A chromatograph equipped with a Shimadzu SPD-2A UV spectrophotometer, set at 230 nm, and a Shimadzu CTO-2A column oven, set at 35°C, were used. Chromatographic separations were carried out with a 150 mm × 4.6 mm I.D. stainless-steel column containing 5-$\mu$m Nucleosil $C_{18}$ (Gasukuro Kogyo, Tokyo, Japan) and a stainless-steel guard column (50 mm × 4.0 mm I.D.) containing 10-$\mu$m Nucleosil $C_{18}$ (Gasukuro Kogyo). The mobile phase was acetonitrile–water (15:85, v/v) at a flow-rate of 0.7 ml/min.

### *Extraction and clean-up procedures*

Accurately weigh 10 g of minced muscle, homogenize it for 3 min at maximum speed with 50 ml of ethyl acetate, centrifuge for 10 min at 2300 *g* and transfer the supernatant into a round-bottomed flask. Homogenize the residue with 50 ml of ethyl acetate, centrifuge as above and combine the supernatants in the flask. Evaporate to dryness under vacuum on a rotary evaporator at 65°C.

Dissolve the residue in 5 ml of 10% sodium chloride solution in an ultrasonic bath and transfer the mixture into a separating funnel with two 10-ml portions of the same solvent. Add 25 ml of *n*-hexane to the separating funnel and shake gently, then allow it to stand until the two layers separate and discard the upper layer. Add a further 25 ml of *n*-hexane to the separating funnel, shake vigorously

for 5 min, allow it to stand until the layers separate and transfer the lower phase into another separating funnel. Add 40 ml of ethyl acetate and shake for 5 min. Transfer the upper phase into a flask and repeat the extraction with another 40 ml of ethyl acetate.

Collect the ethyl acetate layers in the flask and evaporate to dryness under vacuum on a rotary evaporator at 65°C. Dissolve the residue in 5 ml of acetonitrile–water (95:5, v/v) in an ultrasonic bath and apply to an alumina column. Drain the solvent to *ca.* 0.5 cm above the alumina layer. Rinse the flask with two 5-ml portions of the same solvent, add the rinsings to column and drain as above. Elute thiamphenicol with 15 ml of the same solvent at a flow-rate of 5–7 ml/min, collect all the eluates and evaporate to dryness under vacuum on a rotary evaporator at 65°C. Dissolve the residue in 1 ml of CLC mobile phase, filter the solution through a membrane filter of 0.45-$\mu$m porosity and apply to the CLC instrument.

## RESULTS AND DISCUSSION

To extract thiamphenicol from samples, ethyl acetate [3,4,6,7,9] and methanol [8] have hitherto been used. In this study, ethyl acetate was used to obtain cleaner extracts. In order to remove lipids by partitioning with *n*-hexane, the extract was concentrated to dryness and the residue was dissolved in sodium chloride solution. When the solution was shaken with *n*-hexane, the two layers did not separate clearly, and therefore the residue was dissolved in 10% sodium chloride solution. The solution was shaken gently at first, then, after adding more *n*-hexane, the separating funnel was shaken vigorously.

To extract thiamphenicol, the aqueous solution was partitioned with ethyl acetate. Ethyl acetate was found to extract thiampenicol more effectively than other solvents such as diethyl ether, chloroform or dichloromethane.

This ethyl acetate extract showed considerable polar material in the chromatogram. To remove interferents, purification on an alumina column was attempted. A small amount of alumina (3 g) in a column was used for extract clean-up. When acetonitrile was used as the mobile phase, thiamphenicol was not eluted from alumina. It was observed that the inclusion of a certain amount of water in the mobile phase was necessary. Comparative studies showed that 5% of water in acetonitrile was the minimum acceptable to elute thiamphenicol and effectively retain interfering substances. An increased polarity of the eluent did not improve the recovery of thiamphenicol but increased the number of extraneous peaks in the solvent front in the chromatogram of muscle extracts.

Methanol–water has previously been used as the mobile phase to determine thiamphenicol [8,9]; in this study, appropriate conditions of the mobile phase and wavelength for chromatographic detection were investigated. When methanol–water was used, the sample solution gave an interfering peak in the chromatogram. Therefore, acetonitrile–water was selected as the mobile phase.

Two absorbance peaks of thiamphenicol in this mobile phase were observed at 224 and 270 nm. At 270 nm, the absorbance was too low to determine thiamphenicol residues sensitively. Even though there was another peak at 224 nm, 230 nm was selected for detection, because at this wavelength the baseline was more stable and there were considerably less interfering peaks from muscles than at 224 nm. Typical chromatograms of a muscle extract are shown in Fig. 1.

Other drugs such as sulphonamides and chloramphenicol did not interfere in the determination of thiamphenicol. The former were not eluted from the alumina column under the conditions used and the latter was not eluted from the CLC column with the mobile phase adopted. The equation for the calibration graph for thiamphenicol isolated from fortified chicken muscles was $y = 0.7938x - 0.1015$, where $y$ = peak height (cm) for thiamphenicol and $x$ = concentration (ppm) of thiamphenicol in the sample solution, over the range of 5–100 ng with a correlation coefficient of 0.9964. The detection limit, defined as three times the baseline noise, was 5 ng, which corresponds to 0.05 ppm of thiamphenicol in chicken muscles.

To test the deviation of results on different days, recoveries were determined for one set of fortified chicken muscles on day 1 and for a second set on day 2. Recovery studies were performed by adding 1.0 or 2.0 ml of the working standard solution to 10 g of minced chicken muscles. As shown in Table I, there was no significant difference statistically between days 1 and 2 at the 95% confidence level. When ten results at 0.1 and 0.2 ppm were considered, the average recoveries were 90.0 and 92.8%, with standard deviations of 0.035 and 0.060% and relative standard deviations (R.S.D.) of 3.88 and 3.23%, respectively. The peak heights of thiamphenicol in sample solutions were 81.0% of those of the added working standard solutions which were applied directly to CLC.

The accuracy of detection was tested by preparing eleven samples of chicken muscles, fortified with ten levels of thiamphenicol, in duplicate by one analyst.

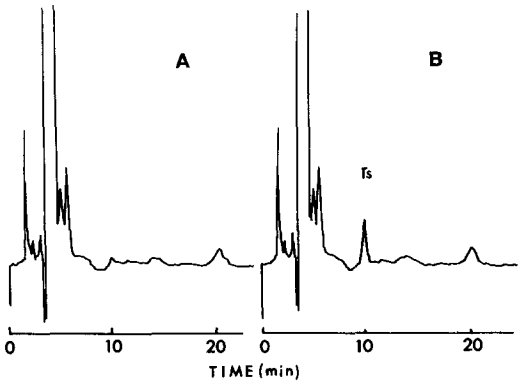


Fig. 1. Chromatograms of commercial chicken muscle extracts. (A) Chicken muscle blank; (B) chicken muscle fortified with 2 $\mu$g of thiamphenicol (Ts).

TABLE I

RECOVERY OF THIAMPHENICOL ADDED TO 10-g PORTIONS OF CHICKEN MUSCLES

| Experiment No. | Day 1 | | | | Day 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Recovery of 2 μg added | | Recovery of 1 μg added | | Recovery of 2 μg added | | Recovery of 1 μg added | |
| | μg | % | μg | % | μg | % | μg | % |
| 1 | 1.878 | 93.9 | 0.867 | 86.7 | 1.887 | 94.4 | 0.910 | 91.0 |
| 2 | 1.815 | 90.8 | 0.896 | 89.6 | 1.888 | 94.4 | 0.886 | 88.6 |
| 3 | 1.853 | 92.6 | 0.950 | 95.0 | 1.863 | 93.2 | 0.857 | 85.7 |
| 4 | 1.715 | 85.8 | 0.885 | 88.5 | 1.939 | 97.0 | 0.966 | 96.6 |
| 5 | 1.885 | 94.2 | 0.905 | 90.5 | 1.847 | 92.4 | 0.878 | 87.8 |
| Mean | 1.829 | 91.4 | 0.901 | 90.1 | 1.884 | 94.2 | 0.899 | 89.9 |
| S.D. (μg) | 0.069 | | 0.031 | | 0.034 | | 0.041 | |
| R.S.D. (%) | 3.77 | | 3.44 | | 1.80 | | 4.56 | |

TABLE II

RECOVERY OF THIAMPHENICOL FROM TEST SAMPLES FORTIFIED WITH THIAMPHENICOL AT TEN LEVELS IN A SINGLE BLIND EXPERIMENT

A standard solution of thiamphenicol was added to a 10-g portion of minced chicken muscle by one analyst and these samples were submitted blind to another analyst.

| Added (ppm) | Found (ppm) | | Recovery (%) | |
|---|---|---|---|---|
| | No. 1 | No. 2 | No. 1 | No. 2 |
| 0[a] | 0.0153 | 0.0127 | – | – |
| 0.03[a] | 0.0398 | 0.0359 | 132.7 | 119.7 |
| 0.05 | 0.0505 | 0.0427 | 101.0 | 85.4 |
| 0.07 | 0.0639 | 0.0597 | 91.3 | 85.3 |
| 0.1 | 0.0930 | 0.0920 | 93.0 | 92.0 |
| 0.15 | 0.1597 | 0.1480 | 106.5 | 98.7 |
| 0.3 | 0.3147 | 0.3042 | 104.9 | 101.4 |
| 0.5 | 0.4855 | 0.4965 | 97.1 | 99.3 |
| 0.6 | 0.5664 | 0.6270 | 94.4 | 104.5 |
| 1.0 | 1.032 | 0.895 | 103.2 | 89.5 |
| 1.5 | 1.434 | 1.467 | 95.6 | 97.8 |

[a] The concentrations of thiamphenicol at these two levels were out of linear range of the calibration graph.

The samples were then submitted blind to another analyst to determine the concentration. As shown in Table II, the recoveries ranged from 85.3 to 106.6% for all the fortified levels, which were within the linear range of the calibration graph. To test the suitability of the method, sixteen commercial chicken muscles were examined. Thiamphenicol was not detected in any of these samples.

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