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Determination of lincomycin residues in salmon tissues by gas chromatography with nitrogen–phosphorus detection

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Abstract

A sensitive method for the determination of lincomycin residues in fish tissues is described. Lincomycin was extracted from fish tissues with phosphate buffer (pH 4.5). The extract was concentrated with a C₁₈ solid-phase extraction cartridge and further cleaned up by solvent extraction. Lincomycin was derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide to form a trimethylsilyl derivative before being analyzed by gas chromatography with nitrogen–phosphorus detection. Coumaphos was used as the internal standard. Assays showed good linearity in the range 25–250 ppb (ng/g) ($r=0.9994$). Recoveries of fortified lincomycin at 50, 100 and 200 ppb were >80% with relative standard deviations <6%. The limit of detection of the method was 1.7 ppb and the limit of quantitation was 3.8 ppb.

Keywords: Lincomycin

1. Introduction

Antibiotics have been used widely in animal agriculture for growth stimulation and disease prevention and treatment [1]. Lincomycin is one of the commonly used antibiotics. Improper use of antibiotics may result in undesirable residue levels in animal originated food products [1]. The improper and extensive use of antibiotics may also contribute to development of antibiotic resistant bacteria [2,3].

The tolerance levels of lincomycin residue set by

the US Food and Drug Administration are 150 ppb for milk and 100 ppb for the edible tissues of chicken and swine [4]. Lincomycin has not been approved for use on salmon and catfish, therefore no tolerance level has been established. To control and regulate the use of antibiotics and to detect the residual level of antibiotics, sensitive and reliable methods are needed.

Although the microbiological methods traditionally used for the assays of antibiotics are sensitive, they are not specific because of the co-existence of other antibacterial substances [5]. The difficulty associated with high-performance liquid chromatography (HPLC) analysis of lincomycin residues is the lack of fluorescence or strong UV absorbance. Several HPLC and gas chromatography (GC) methods

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have been developed for lincomycin analysis [6–11]. Some of these methods were for high concentrations and not suitable for the analysis of trace level residues in tissues. A GC–MS method reported by McMurray et al. [9] allowed determination of lincomycin at 100 ppb in animal feed, however an MS detector and overnight derivatization was required. An HPLC method for residue analysis developed by Moats [10] was able to detect 20 ppb of lincomycin in milk and muscle tissue, and 50 ppb in liver and kidney tissues. This method used a UV detector and required a long and extensive sample preparation procedure which included an HPLC clean-up step prior to HPLC quantitative analysis. A GC method for the analysis of lincomycin residue in porcine and bovine kidney tissues reported by Farrington et al. [11] was sensitive but suffered from low recoveries (40–50%).

This paper describes a GC method for the analysis of lincomycin residues in fish tissues at low ppb levels.

2. Experimental

2.1. Materials

Lincomycin (reference standard) was obtained from U.S. Pharmacopeial Convention (Rockville, MD, USA). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was supplied by Pierce (Rockford, IL, USA). 1-Pentanesulfonic acid, sodium salt (98% pure) was obtained from Aldrich (Milwaukee, WI, USA). All solvents were analytical reagent grade and supplied by J.T. Baker (Phillisburg, NJ, USA). All other chemicals were ACS reagent grade.

All fish samples were purchased from a local market and stored at -70°C until used.

2.2. Sample preparation

Salmon muscle or catfish muscle was blended with a food processor into a homogenous paste. Salmon skin was cut into small pieces and frozen with liquid nitrogen. The frozen salmon skin was then blended into a powder with a Waring blender.

2.2.1. Extraction and deproteinization

Fish samples (5.0 g) were weighed into 50-ml polyethylene centrifuge tubes. The fish samples were fortified with lincomycin standard solution ($5\ \mu\text{g}/\text{ml}$ in methanol) at the level of 50, 100 or 200 ppb. The samples were homogenized with 20 ml of 0.01 *M* sodium phosphate buffer (pH 4.5) using a tissue homogenizer (Model Ultra-turrax T25, Ika-Labortechnik, Janke and Hunkel, Staufen, Germany) for 2 min. The homogenized samples were then centrifuged (4000 *g*) for 10 min. The supernatants were filtered through plugs of glass wool and collected in 50-ml centrifuge tubes. The fish samples were then homogenized with another 20 ml phosphate buffer, centrifuged, filtered and combined with the original supernatants. A 1-ml portion of 10% sodium tungstate solution and a 1-ml portion of 0.34 *M* H_2SO_4 solution were added to the samples. These were then mixed and centrifuged (4000 *g*) for 10 min. The supernatants were filtered through plugs of glass wool and collected in 50-ml beakers or tubes. The final volume of each fish extract was approximately 40 ml.

2.2.2. Solid-phase extraction

The 3-ml (500 mg) C_{18} solid-phase extraction cartridges (Waters, Milford, MA, USA) were mounted onto a vacuum operated solid-phase extraction manifold (Waters). The cartridges were conditioned with 15 ml methanol and washed with 5 ml water. After 1 ml of 3% sodium pentanesulfonate was added to each fish extract, they were loaded onto the C_{18} cartridges. The flow-rate was adjusted to about 1 ml/min. When all the fish extracts eluted through the cartridges, the cartridges were washed with 4 ml of 10% methanol (in water) followed by 2 ml of water. The lincomycin absorbed on the C_{18} cartridge was then eluted with 2 ml of 50% acetonitrile (in water) and the eluent was collected into a 16×150 mm screw-capped culture tube.

2.2.3. Solvent extraction

About 0.5 g sodium chloride and 0.2 ml of 1 *M* potassium hydroxide were added to the eluents in the culture tubes. The eluents were then extracted immediately with three 3-ml aliquots of ethyl acetate. The combined extracts were filtered through 3-g plugs of anhydrous sodium sulfate and collected in 50-ml

round bottom flasks. The ethyl acetate was evaporated to dryness with a vacuum rotary evaporator set at 35°C. The residues were dissolved and quantitatively transferred to 10×75 mm culture tubes using three 0.5-ml aliquots of methanol.

2.3. Silylation derivatization

The methanol was evaporated to complete dryness with a stream of dry nitrogen. Then 0.1 ml acetonitrile and 0.2 ml BSTFA were added to each culture tube. After vortexing for 30 s, the tubes were heated at 50°C for 30 min with a heating block. The tubes were then removed from the heating block and the acetonitrile and BSTFA were evaporated to complete dryness with a stream of dry nitrogen. The residues were each redissolved in 0.1 ml hexane containing 1 µg/ml coumaphos as the internal standard. The samples were then transferred to air tight screw-capped sample vials to await GC analyses.

2.4. Gas chromatographic analysis

Gas chromatographic analysis was carried out with a Tre-Metrics 9000 series gas chromatograph (Tre-metrics, Austin, TX, USA) equipped with a split-splitless injection port and a 702 nitrogen-phosphorus (NP)-specific detector. The splitless injection mode was used for the analyses. The column was a DB-1 30 m×0.25 mm with 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). The flow-rate of carrier gas (helium) was set at 0.78 ml/min. The flow-rates of hydrogen and make-up gas (air) for the NP detector were set at 2 ml/min and 125 ml/min, respectively. The temperatures of the injection port and the NP detector were set at 230°C and 300°C, respectively. The initial temperature of the column was 160°C. After injection, the column temperature was held at 160°C for 1 min, then heated at 40°C/min to 300°C and held for 10 min. A volume of 1.5 µl sample was injected using a hot needle injection technique. The chromatographic data was collected on a HP Vectra QS/16S ChemStation with HP 3365 series II ChemStation software version A. 03.21 (Hewlett-Packard, Memphis, TN, USA).

2.5. Calibration

The lincomycin calibration standards were prepared in the blank fish extract matrix. To prepare a six-point calibration curve, six blank fish samples each of 5 g were extracted by the procedure described above in Section 2.2. Before the silylation derivatization, 125, 250, 500, 750, 1000 and 1250 ng of lincomycin standard (5 µg/ml in methanol) were added to blank fish extracts, respectively. After the methanol was evaporated, these standards were derivatized by the procedure described in Section 2.3. The ratios of the peak areas of the lincomycin derivative and peak areas of the internal standard coumaphos were calculated. A calibration curve was constructed with the ratios of the peak areas vs. the amounts of the lincomycin standards.

3. Results and discussion

This method was developed following the FDA's general guidelines described in [12]. Lincomycin was fortified at three levels with $n \geq 5$ samples within each level. The recoveries, within-day variations and day-to-day variations were evaluated.

3.1. Sample extraction and clean-up

A phosphate buffer (pH 4.5) was used to extract lincomycin from the fish tissues. The pK_a value of lincomycin is 7.6. Therefore, lincomycin is positively charged and very soluble in any buffer with a pH lower than 5.6. The buffer's pH value of 4.5 was close to the isoelectric point (pH) of fish proteins. The fish tissues were homogenized with buffer. After centrifugation, the fish proteins precipitated and were well separated from the aqueous extract with no emulsion being formed. The fish samples were extracted twice with 20 ml phosphate buffer for a complete extraction of lincomycin.

A C_{18} cartridge was then used for the solid-phase extraction of lincomycin from the fish extract. Sodium pentanesulfonate, added to the fish extract as an ion-pairing reagent, increased the retention of

lincomycin on the C_{18} cartridge. The flow-rate through the cartridge was 1 ml/min. Higher flow-rates (>1.5 ml/min) decreased the recovery of lincomycin.

After the lincomycin was eluted from the C_{18} cartridge, the pH of the eluent was adjusted to greater than pH 9.6. Thus the lincomycin was not charged at that pH and was extracted into the ethyl acetate.

3.2. Silylation derivatization of lincomycin

BSTFA was used for the trimethylsilyl (TMS) derivatization of lincomycin. Its effectiveness as a TMS donor is approximately the same as its unfluorinated analog BSA, *N,O*-bis(trimethylsilyl)acetamide. The advantage of using BSTFA over many other silylation reagents is the high volatility of its reaction by-products. After the silylation reaction was complete, the unreacted BSTFA, the reaction by-products and acetonitrile were easily removed by a stream of nitrogen. All these compounds contained nitrogen and if present in the extract, would have given an oversize solvent peak when injected into the GC with a highly sensitive NP-specific detector. Since BSTFA reacts with methanol, the methanol in the samples should be removed completely before proceeding with the TMS derivatization reaction. TMS derivatives are sensitive to moisture, therefore the TMS derivative of lincomycin should be analyzed within 24 h of its preparation.

3.3. GC analysis

The splitless injection mode was used for the analysis because of its higher sensitivity. A hot-syringe needle technique was used for the manual injection of the samples in order to obtain good reproducibility. The relatively low column initial temperature (160°C) was designed for the cold focusing of the analytes. The cold focusing was necessary due to the long mass transfer time from the injector to the column in the splitless injection mode. Coumaphos was used in the method as an internal injection standard. The internal standard was important for good precision in the analysis of lincomycin. The retention times for the internal stan-

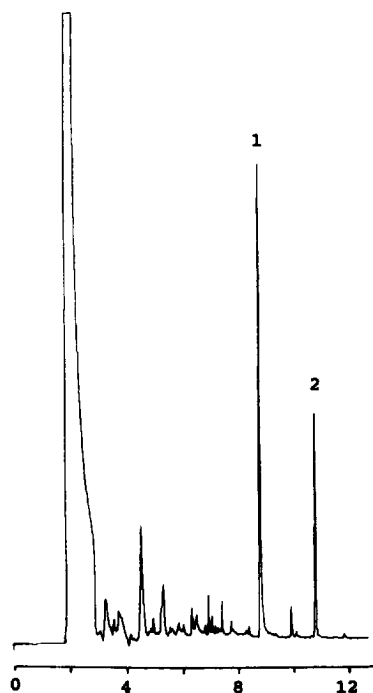


Fig. 1. Chromatogram of salmon muscle sample fortified with lincomycin at 100 ppb. Peak 1 is the internal standard coumaphos and peak 2 is the lincomycin TMS derivative.

dard and the lincomycin derivative are shown in Fig. 1.

3.4. Matrix effect and calibration

The calibration standards were prepared in the fish extract due to a GC matrix effect. It was found early in this research that when the calibration standards were prepared in pure solvent, the day-to-day variations of the lincomycin recoveries were very large. This was due to the large variation of the peak area ratios of the internal standard and the lincomycin derivative. It was also discovered that the peak sizes of both the internal standard and lincomycin derivative were much smaller when the standards were prepared in pure solvents as opposed to preparation in the fish extract matrices. An experiment was designed to investigate this matrix effect. Duplicate standards were prepared in pure hexane, a catfish extract matrix and a salmon muscle extract matrix.

Table 1
The effects of matrices on the GC detector signal response

Chemical compound	Peak-area counts		
	Pure solvent	Catfish	Salmon
Coumaphos (I.S.)	1400	3100	4100
Lincomycin TMSderivative	900	1200	1500
Ratio of I.S./lincomycin	1.56	2.58	2.73

The peak areas of the internal standard and lincomycin derivative in the three matrices are summarized in Table 1. These data indicated the differences of the peak areas as well as the differences of the peak area ratios of the lincomycin derivative and the internal standard among the three different matrices.

A similar matrix effect was reported by Erney et al. [13]. They observed that chromatographic responses for organophosphorus pesticides in extracts from milk and butterfat were matrix dependent. They suggested that the matrix protected the organophosphorus compounds from adsorption and/or decomposition in the hot injection port and as a result the amounts of the compounds transferred from the injection port to the column were more than that of the standards prepared in the matrix-free solvents.

Since the responses of both the internal standard and lincomycin derivative were dependent on the matrix of the sample extract, it was necessary to prepare the calibration standards in the same matrix extract as that of the sample. Lincomycin standards added into the extracts of blank salmon muscle, skin and catfish muscle before the derivatization were used for the determination of lincomycin in the respective tissues as described in Section 2.5. If no blank samples were available, the method of standard additions could be applied to a sample extract for the preparation of calibration standards. In this research, a calibration curve was prepared with blank salmon muscle tissue extracts. The linear regression equation was: $y = a_1x + a_0$, where y is the concentration (ppb) of lincomycin and x is the ratio of the peak area of lincomycin derivative to the peak area of the internal standard. The coefficients a_1 and a_0 are 224.84 ± 3.38 and 1.55 ± 2.10 , respectively. The linear regression coefficient was $r = 0.9994$. The intercept of the regression equation was negligible compared to the value of $224.84x$. Therefore, for

practical reasons a one-point calibration standard could be used for routine analyses.

3.5. Specificity and sensitivity

More than twenty blank samples of salmon muscle and skin and catfish muscle tissues have been analyzed. No interfering peaks were found at the retention time of the internal standard or the lincomycin derivative. An amplified typical baseline of a blank salmon muscle sample is shown in Fig. 2A. No interfering peak at the retention of the lincomycin TMS derivative was observed.

In order to determine the sensitivity of the method, duplicate salmon muscle samples fortified with 5 ppb lincomycin were analyzed. The recovery was $73.5 \pm 2.2\%$. The lincomycin derivative peak indicated in Fig. 2B was equivalent to 3.6 ppb of lincomycin in salmon muscle.

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated according to the guidelines of the American Chemical Society [14]. The LOD was defined as mean blank response plus three times the standard deviation of replicate analysis of blank samples. LOQ was defined as mean blank response plus ten times the standard deviation of replicate analysis of blank samples. Therefore based on the blank salmon muscle samples analyzed, the LOD and LOQ of the method were calculated to be

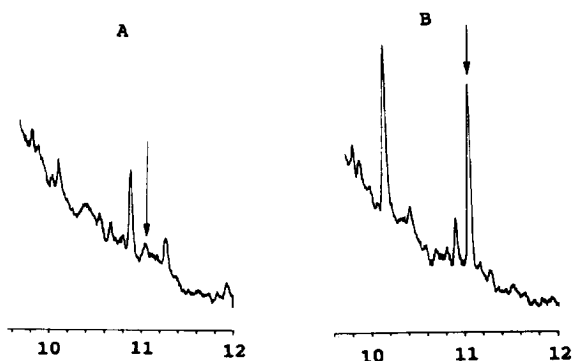


Fig. 2. Chromatogram A illustrates a magnified baseline of a typical blank salmon muscle sample at the retention time of lincomycin TMS derivative (pointed by the arrow). Chromatogram B shows a peak of lincomycin TMS derivative equivalent to 3.6 ppb lincomycin in salmon muscle.

1.7 and 3.8 ppb of lincomycin in salmon muscle samples. The sensitivity of this GC method was superior to an HPLC–ECD method previously developed in our laboratory [15]. The HPLC method's LOD and LOQ were 7 and 17 ppb, respectively.

Fig. 3 illustrates the retention times of the TMS derivatives of two analogs of lincomycin, lincomycin B and clindamycin. They were well separated from the lincomycin TMS derivative. McMurray et al. [9] reported that it was not possible to separate the TMS derivatives of lincomycin from clindamycin with a short OV-1 (100% poly dimethylsiloxane) column or on an SE-54 column. However, Fig. 3B shows the lincomycin derivative completely separated from clindamycin derivative on a 30 m DB-1 (100% poly dimethylsiloxane) column.

3.6. Recoveries and variations

Samples of salmon muscle tissue were fortified with lincomycin at 50, 100 or 200 ppb ($n=5$ for each level). These were analyzed in one day to evaluate the within-day recoveries and variations. Also three samples of salmon muscle, each fortified with lincomycin at one of the three levels (50, 100 and 200 ppb) were analyzed each day for six days to evaluate the day-to-day recoveries and variations. These data are summarized in Table 2 and Table 3. The average recoveries of all three levels both within-day and day-to-day were about 89%. The relative standard

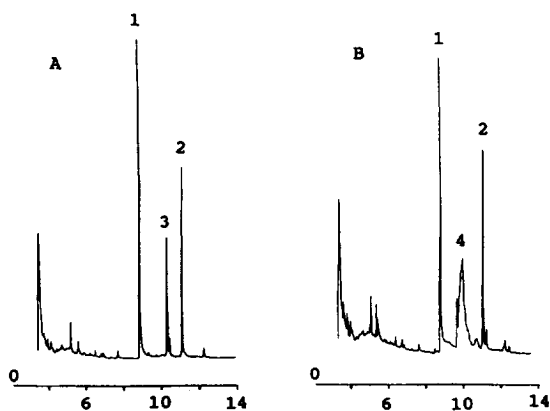


Fig. 3. Chromatograms indicate the relative retention times of the (1) internal standard coumaphos, (2) lincomycin TMS derivative, (3) lincomycin B TMS derivative and (4) clindamycin TMS derivative.

Table 2

Recoveries of lincomycin from fortified salmon muscle tissue samples (within-day)

Fortification level of lincomycin (ppb)	Recovery (%)		n	C.V. (%)
	Range	Mean		
50	85.5–94.0	89.0	5	3.7
100	85.1–93.4	89.4	5	4.4
200	83.0–94.8	88.4	5	5.8

Table 3

Recoveries of lincomycin from fortified salmon muscle tissue samples (day-to-day)

Fortification level of lincomycin (ppb)	Recovery (%)		n	C.V. (%)
	Range	Mean		
50	81.2–93.0	89.1	6	5.5
100	81.4–96.2	89.8	6	5.7
200	83.8–94.3	87.7	6	4.7

deviations for both within-day and day-to-day were less than 6%. These results met the requirements described in the US FDA's general guidelines [12].

The method was also applied to the analyses of lincomycin in salmon skin and catfish muscle tissues. Five samples of salmon skin or catfish muscle were fortified with 100 ppb of lincomycin and analyzed in one day. The average recoveries were greater than 85% and the relative standard deviations were less than 6% for both salmon skin and catfish tissues (Table 4).

3.7. Conclusions

A highly sensitive method for the GC determination of lincomycin residues in fish tissues has been developed. The sample extraction and clean-up procedure gave high recoveries consistently and no

Table 4

Recoveries of lincomycin from fortified salmon skin tissue and catfish muscle tissue samples (within-day)

Fish tissue	Recovery (%)		n	C.V. (%)
	Range	Mean		
Salmon skin	83.3–94.7	89.1	5	4.8
Catfish muscle	80.2–91.0	86.0	5	5.0

Lincomycin fortification level was 100 ppb for both fish tissues.

significant interferences. Preparation of calibration standards in blank fish extract matrices was necessary for the precise and accurate measurement of lincomycin residues in fish tissues. The method described offers the sensitivity, accuracy and precision for monitoring lincomycin residues in fish at low ppb levels.

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