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Note

Sensitive gas—liquid chromatographic method for chloramphenicol in animal tissues using electron-capture detection

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Chloramphenicol (CP) is considered to be an effective and almost essential antibiotic for the treatment of enteric and respiratory diseases of animals, where resistance has developed to the commonly used antibacterial drugs [1—3]. In the many countries where CP is used there is however, concern regarding the effects on consumers of CP residues in meat from treated animals. Varying degrees of bone marrow depression have occurred, relatively frequently, in humans undergoing therapy with this drug, and there is no data to indicate the minimal amount of drug or residue which can produce this situation [4, 5]. In view of these circumstances the F.A.O./W.H.O. Expert Committee on Antibiotics have recommended a zero tolerance for CP in meat products.

To accomplish this goal, the drug assay used to ascertain safe drug withdrawal periods and for monitoring residues in meat, should be capable of detecting chloramphenicol in the low ng/g range in tissue. A search of the relevant literature has failed to reveal a practical residue assay for animal tissue with this level of sensitivity.

The tissue residue studies reported to date involve the use of this drug in swine, cattle, and poultry [1, 6—10]; however, the assay procedures were not very sensitive (1 μg/g to 0.1 μg/g). In some studies the assay procedures were microbiological methods which lacked specificity. A gas—liquid chromatographic (GLC) method has been reported by Jacobson et al. [11] which appears to meet the required sensitivity level. It is too time consuming however, for routine residue assay procedures and has not been adopted by public health agencies.

Several methods of detection of CP in serum, urine and milk using GLC

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[12–16] or high-performance liquid chromatography (HPLC) [17, 18] were found. No practical sensitive assay procedures were reported for use in edible animal tissues.

The GLC methodology described in this paper was developed to provide a practicable tissue assay test with a lower limit of detection of $\leqslant$ 5 ng of CP per g of tissue. The test is not excessively time consuming, uses standard analytical techniques and is suitable for drug depletion studies and residue monitoring programs.

EXPERIMENTAL

Chemicals and reagents

Pharmaceutical grade (99.8%) chloramphenicol (CP), and chloramphenicol monoethanolamine succinate were donated by Pfizer Canada (Pointe Claire, Canada). Thiamphenicol (TP) was purchased from Sigma (St. Louis, MO, U.S.A.). For derivatization Trisil (standard mixture of hexamethyldisilazane (HMDS), trichloromethylsilane (TMS) and pyridine) was obtained from Pierce (Rockford, IL, U.S.A.). Solvents: ethyl acetate, methanol, light petroleum and benzene were distilled in glass (Caledon Laboratories, Georgetown, Canada).

Standard solutions

Standard solutions of CP and TP were prepared by dissolving 100 mg of either compound in 100 ml of methanol. These stock solutions were further diluted to produce an end concentration of 50 ng/ml. These standard solutions were then added to tissues for internal standardization (TP) and development of the calibration curve (CP).

Chromatographic conditions

A Varian Vista 401 gas–liquid chromatograph with dual electron-capture detectors (^{63}Ni) and dual automatic injectors was fitted with glass columns (1.83 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q, 100–120 mesh (Chromatographic Specialties, Brockville, Canada). Operating conditions were: injection port temperature, 230°C; column oven temperature, 220°C; ionization oven temperature 350°C; carrier gas high purity nitrogen, flow-rate 30 ml/min. Chromatograms were recorded on a 1-mV scale at detector range of 10, recorder attenuation 32, and the chart speed was 0.5 cm/min.

Procedure

A 5-g sample of ground frozen tissue (muscle, liver, kidney), was placed in a round bottom centrifuge tube (28 mm × 120 mm) fitted with a screw cap. A 2-ml aliquot of the standard solution (50 ng/ml) of TP in methanol, was added, stirred on a vortex mixer and allowed to stand for 15 min. Tissue was extracted twice by homogenizing, using a Tissumiser (Tek Mar, Cincinatti, OH, U.S.A.), in 10 ml ethyl acetate for 20 sec, then centrifuging at 700 *g* for 5 min. The ethyl acetate supernatants were pooled in a conical centrifuge tube (17 mm × 134 mm) and evaporated to dryness under a stream of dry nitrogen in an N-Evap (Organomation, South Berlin, MA, U.S.A.) at 60°C. The residue

was taken up in 0.2 ml methanol and 2.8 ml of 1 *N* hydrochloric acid were added. This solution was then washed three times with 1.5 ml of light petroleum. Sep-Pak C_{18} mini columns (Waters Assoc., Milford, MA, U.S.A.) were conditioned according to the manufacturer's directions by flushing with 2 ml of methanol, followed by 5 ml of double distilled water. Solution containing the residue was loaded onto the conditioned column and the initial fraction (3 ml) discarded. CP was eluted from the column using two 3-ml volumes of methanol—1 *N* hydrochloric acid (40:60). These fractions were collected in a screw-capped conical centrifuge tube (17 mm × 134 mm) and the methanol evaporated under a stream of dry nitrogen in a sandbath at 60°C. The drug residue was then extracted out of the aqueous phase with two 2-ml volumes of ethyl acetate.

The extracts were pooled in a clean conical centrifuge tube (17 mm × 134 mm) and evaporated to dryness under a stream of nitrogen as described above. The residue was redissolved by washing the tube with 1 ml of methanol. In a nitrogen atmosphere, methanol was evaporated and 400 μl of Trisil added. The tube was then stoppered, vortexed for a few seconds and reacted in a sandbath at 35°C for 30 min. After the Trisil was evaporated to dryness under a stream of dry nitrogen the residue was redissolved in 1 ml of benzene for injection into the gas chromatograph.

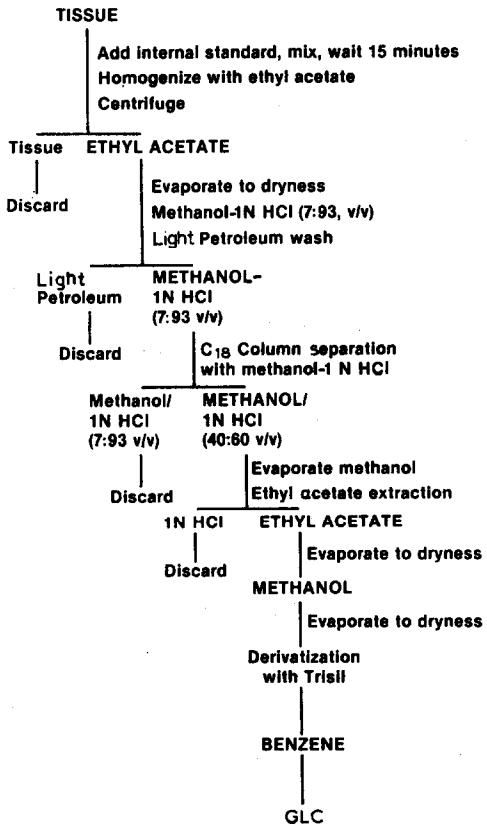

Fig. 1. Extraction and cleanup procedure for chloramphenicol (CP) from edible animal tissues.

Calibration graph

The calibration graph was developed by spiking tissue with standard solutions of CP and TP to yield concentrations of 5, 10, 20, 40, 60, 80 ng/g. The tissues were extracted using the above procedure and each level was assayed in triplicate. GLC analyses of spiked tissue were compared to those of derivatized standard solutions to calculate recovery rates. Peak areas were plotted against drug concentrations for the six levels of drug and a regression analysis carried out.

A summary of the extraction and cleanup procedure is shown in Fig. 1.

RESULTS AND DISCUSSION

Typical chromatograms of broiler chicken muscle and liver, and muscle and liver spiked with 10 ng/g of CP are shown in Fig. 2. The retention times for CP and TP varied from 5.9 to 7.4 and 11.9 to 15.0 min, respectively, with the retention time increasing over a run of fourteen samples. The small peak, which was eluted just after the CP, was present in all samples including the

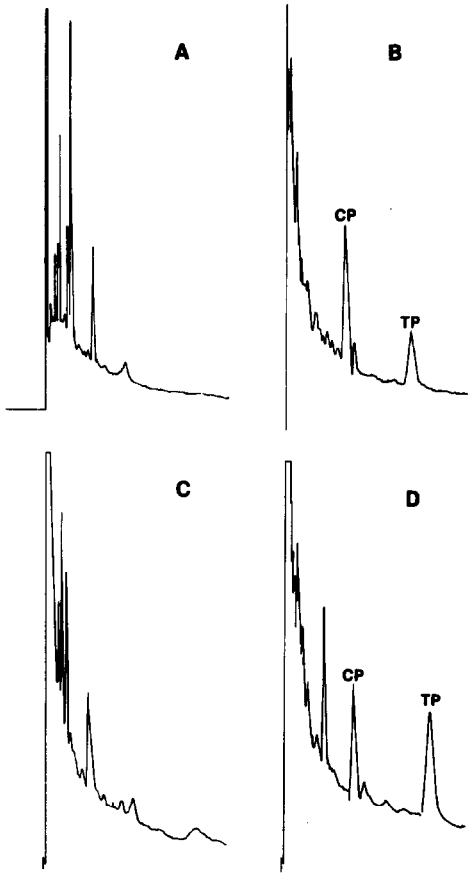

Fig. 2. Typical chromatograms of (A) broiler chicken muscle; (B) muscle with 10 ng/g chloramphenicol (CP) and 10 ng/g thiamphenicol (TP) added; (C) broiler chicken liver; (D) liver with 10 ng/g CP and 20 ng/g TP added.

derivatized blank. In liver a small peak occurred about 0.2 min before the CP. A similar but much larger peak was reported by Wal et al. [16] in milk when derivatized with heptafluorobutyrate. This peak represents about 9% of the area of chloramphenicol at 5 ng/g. A peak also occurs in liver, in the area of the TP but it could be differentiated from the TP. Chromatograms of chicken kidney were similar to muscle in that no interfering peaks were observed.

TP as suggested by Least et al. [13] and Nakagawa et al. [14] proved to be a suitable internal standard to improve the accuracy of the test by correcting for variation in the extraction and injection procedure. This drug was selected also because it is commercially available in Canada and its extraction and chromatographic characteristics are similar to CP.

Calibration curves of CP in chicken muscle, liver and kidney were linear throughout the range of 5—80 ng/g with correlation coefficients of 0.974 for muscle, 0.939 for liver and 0.924 for kidney. This, plus the observation that the recovery rates of CP calculated at the points of the calibration curve were not significantly different, suggests that the level of drug did not affect recovery rate within the 5—80 ng/g range. Levels of less than 5 ng/g were readily detected.

Initially the recovery rate of TP in muscle, at every point on the calibration curve, was determined. Since there was no effect of drug concentration on the recovery rate, 20 ng/g was chosen as the level for internal standardization and for development of the calibration curves in liver and kidney.

Recovery rates (Table I) for CP and TP are similar to those reported by Least et al. [13] from blood serum. Liver values tended to be more variable than muscle or kidney. Kidney results were based on a smaller sample due to lack of tissue availability.

TABLE I

RECOVERY (PERCENT OF DERIVATIZED DRUG) OF CHLORAMPHENICOL (CP) AND THIAMPHENICOL (TP) FROM MUSCLE, LIVER AND KIDNEY OF BROILER CHICKENS

Tissue	No. of samples	Recovery of CP		Recovery of TP	
		Mean (%)	C.V. (%)*	Mean (%)	C.V. (%)*
Muscle	15	56.2	10.9	45.5	16.0
Liver	15	51.9	24.7	40.5**	12.8
Kidney	8	68.0	17.5	45**	14.6

*Coefficient of variation.
**Based on levels of 20 ng/g in tissue.

The method was applied to an experiment to determine CP residues in liver and muscle from chickens given a single oral dose of chloramphenicol monoethanolamine succinate (Fig. 3). The method showed good sensitivity in these tissues at 5 ng/g. This level of detection is 20 times lower than the presently used methods. After GLC analysis derivatized samples in benzene were sent to an independent analytical laboratory for analysis using GC—chemical ioniza-

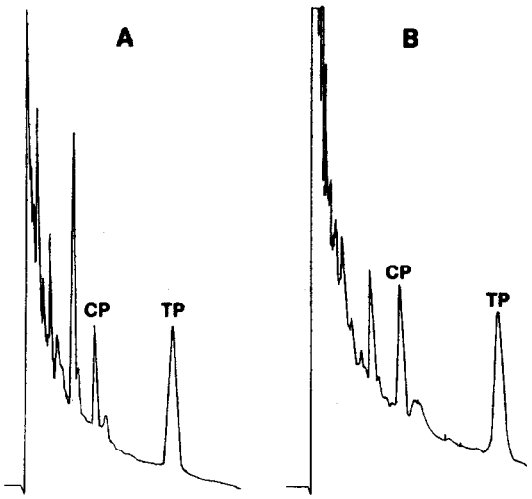

Fig. 3. Chromatograms of tissue extracts from broiler chickens treated with a single oral dose of chloramphenicol monoethanolamine succinate showing chloramphenicol (CP) at the level of 5.2 ng/g in muscle (A) and 6.4 ng/g in liver (B). Thiamphenicol (TP) was added as the internal standard at 20 ng/g.

tion mass spectroscopy (GC—CIMS). GC—CIMS analysis confirmed the presence of di-trimethylsilyl derivatives of CP and TP at concentrations as low as 1.4 ng/g. Since regulatory procedures require confirmatory tests for legal actions, the ability to use samples prepared for GLC without further extracting for GC—CIMS assays is a distinct advantage over existing tissue residue methodology.

CONCLUSIONS

The method described above provides a system which meets the generally accepted requirements for a tissue residue monitoring system. Inclusion of the cleanup steps, particularly the C_{18} mini columns, resulted in removal of many interfering tissue components and thus an improvement in the sensitivity of the test. The procedure is extremely sensitive (5 ng/g or less) and is capable of being carried out using standard analytical equipment and commercially available reagents. Further studies indicate that with minor modifications a lower limit of 1 ng/g may be possible.

Experience gained in residue studies with broiler chickens and swine tissues indicate that two technicians can process 70 samples in a week. Results of the above studies will be published elsewhere.

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