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A Comparison of Three Bioassay Techniques for the Detection of Chloramphenicol Residues in Animal Tissues

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In two experiments a total of six young steers weighing 135–196 kg were injected intramuscularly with 11 mg of chloramphenicol (CAP) in propylene glycol/kg body weight twice daily for 3 days. Another steer was injected with double that dose, and two steers were left untreated. Eighteen hours after the last CAP injection all steers were euthanized with a barbiturate overdose. Samples of kidney, liver, and muscle were collected from each steer. No microbial inhibition was obtained with any of the tissues with the swab test on premises (STOP). Only kidney from the steer given 22 mg of CAP/kg was positive by the microbial inhibition test. All muscle samples were positive for CAP by thin-layer chromatography/bioautography (TLCB). The CAP detection limit was 0.6 ppm for TLCB and 5 ppb for a gas chromatographic CAP method. Levels of CAP in muscle and liver decreased when stored at −20 °C.

Until recently, the antibiotic chloramphenicol (CAP) was used in Canada in food-producing animals because of its broad-spectrum antibacterial properties against pathogenic bacteria (Blood et al., 1983; Huber, 1982; Knight, 1981). CAP has been implicated in the occurrence of aplastic anemia in humans (Schmidt, 1983; Settepani, 1984), and as a consequence the use of CAP in food-producing animals has been prohibited in Canada, while the Food and Drug Administration of the United States has never approved the use of CAP in food-producing animals (Knight, 1981; Settepani, 1984). However, the illegal use of CAP in food-producing animals continues to be a concern.

Monitoring for antibiotic residues is done in Canadian abattoirs using the swab test on premises (STOP) (Johnston et al., 1981) and by the identification of suspect animals. The STOP procedure detects the presence of microbial inhibitors but does not identify them. A more specific screening procedure for antibiotics using thin-layer chromatography/bioautography (TLCB) is currently being tested in this laboratory as a confirmatory procedure (Neidert et al., 1987). This will replace, as a confirmatory laboratory test, a microbial inhibition test (MIT) previously used for this purpose. TLCB has been used for the detection and semiquantitation of CAP (Bogaerts et al., 1984). Gas–liquid chromatography (Nelson et al., 1983; USDA–FSIS, 1983) has also been used for CAP analysis. The current investigation was undertaken to compare the results obtained with the three bioassays (STOP, MIT, TLCB) for the detection of CAP residues in tissues from treated animals. For further comparison samples in the second half of the investigation were also analyzed by a quantitative gas chromatographic method for the determination of CAP residues in tissues (USDA–FSIS, 1983).

The effect of sample storage time prior to analysis on CAP levels in tissue was also investigated. Samples were stored at −20 °C and reanalyzed after 2, 5, and 12 or 13 weeks.

MATERIALS AND METHODS

1. Experimental Design and Animal Treatment. *Experiment 1.* Five young Holstein steers weighing 135–165 kg were purchased from a local dairy and maintained for 6 days. Three of the steers were then injected with 11 mg of CAP in propylene glycol/kg body weight twice daily for 3 days (Rogar-Mycine 500, Rogar/STB, London, Ontario), and the other two steers were untreated controls. On days 1 and 3 CAP was injected into the right gluteal muscle, and on day 2 it was injected into the right semitendinosis muscle. Eighteen hours after the last CAP injections, all five steers were euthanized with a barbiturate overdose. Samples (200 g) of kidney and liver, and muscle contralateral to the injection sites, were collected from each animal. Tissues for initial TLCB were immediately homogenized in methanol in the necropsy room. Tissues for STOP and MIT assays were stored 3 h with ice packs before the tests were conducted. The remaining tissues were immediately frozen with dry ice and then stored at −20 °C for 2, 5, and 13 weeks for TLCB analysis.

Experiment 2. Four young Holstein steers were purchased from a local dairy and maintained for 18 days. Three of the steers were then injected with 11 mg of CAP in propylene glycol/kg body weight, and the fourth steer was injected with 22 mg of CAP/kg body weight. At the time of the first injection the steers weighed 138–196 kg. The steers were injected twice daily for 3 days. Other procedures were similar to those described for the first experiment except that the longest time storage study was 12 weeks rather than 13 weeks. Tissues were also analyzed by a quantitative gas chromatographic procedure for CAP analysis. Serum was also collected and analyzed.

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2. Swab Test on Premises (STOP). STOP procedures were conducted by the method described by Johnston et al. (1981) with kidney, liver, and muscle. Plates were incubated with *Bacillus subtilis* at 32 °C for 16–18 h.

3. Microbial Inhibition Test (MIT). Tests were conducted with kidney, liver, and muscle.

Reagents. (*a*) *Tryptose Agar Slants* (*TAS*). A 12.3-g sample of tryptose agar (Difco; Canlab, Edmonton, Alberta) was dissolved in 300 mL of boiling distilled water, and then 7-mL portions of this agar were dispensed into 15 × 150 mm screw-cap tubes. These tubes were autoclaved for 15 min at 121 °C, slanted, and then stored at 4 °C.

(*b*) *Trypticase Soy Broth* (*TSB*). TSB (30 g) (BBL; Canlab) was dissolved in 1 L of distilled water and the resultant solution mixed thoroughly and warmed gently. Of the mixture 7 mL was dispensed into 15 × 150 mL tubes and then autoclaved for 15 min at 121 °C, final pH 7.3.

(*c*) *Micrococcus luteus ATCC 9341 and Bacillus cereus ATCC 11778.* These organisms were maintained on TAS, with subcultures made every 2 weeks, and used to inoculate TSB as necessary.

(*d*) *Phosphate-Buffered Saline* (*PBS*). Into distilled water was dissolved 8.0 g of NaCl (Difco), 0.2 g of KCl (Difco), 0.2 g of KH_2PO_4 (Difco), 1.15 g of Na_2HPO_4 (Difco), and 0.2 g of dextrose (Difco) and the resultant mixture diluted to 1 L and autoclaved at 121 °C for 15 min, final pH 7.4.

(*e*) *Plate Count Agar* (*PCA*). A 23.5-g sample of PCA (Difco) was dissolved in 1 L of boiling distilled water and then autoclaved at 121 °C for 15 min, final pH 7.0.

Apparatus (equivalent apparatus may be substituted): (a) Petri dishes, 100 × 15 mm (Canlab) disposable; (b) Polytron homogenizer, Model PT 10-35 with probe generator, Model PT 20 ST (Brinkman Instruments, Rexdale, Ontario); (c) blank specimen disk, 7-mm diameter (Difco); (d) incubator, 37 °C, Fisher Isotemp Oven 300 series (Fisher Scientific Co., Ltd., Edmonton, Alberta).

Sample Preparation. A 10-g sample of diced, partially thawed tissue was weighed into a disposable 50-mL centrifuge tube, and to this was added 10 mL of PBS. Tissue was homogenized for 30 s and the homogenate centrifuged for 15 min at 1000*g*.

Preparation of Assay Plates. Molten PCA (12–15 mL) was poured into Petri plates and incubated overnight to dry the agar and detect any contaminant organisms. The surface of the plate was seeded with 18-h TSB culture of the test organisms (one plate per organism for each sample) by dipping a sterile swab into the inoculum and shaking it to express excess fluid. The entire agar surface of the plate was streaked three times, turning the plate 60° between streakings. Fresh plates were poured weekly and stored at 4 °C until use.

Microbial Inhibition. For each sample, two disks saturated with tissue supernatant (20 μL) and one control CAP disk (5 μg; BBL, Canlab) were placed on one plate for each organism. The plates were incubated in an inverted position at 37 °C for 18–24 h. Zones of inhibition that extend more than 1 mm from the edge of the disk on one or both plates were considered positive for the presence of microbial inhibitors.

4. Thin-Layer Chromatography/Bioautography (TLCB). The method used was a modification of the method described by Neidert et al. (1987). A centrifuge tube and centrifugation were used rather than a separatory funnel to separate extracts 1 and 2. The bioautography medium used was developed by C. E. Rigby (unpublished) and was prepared as follows. Into distilled water were dissolved 1.5 g of beef extract (BBL, Canlab), 3.0 g of yeast extract (Difco), 4.0 g of tryptone (Difco), 6.0 g of peptone (Difco), and 1.0 g of dextrose (Difco), 5 mL of triple salt solution was added (0.5 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, and 0.5 g of $MnSO_4 \cdot H_2O$ in 200 mL of distilled water), and the resultant mixture was diluted to 1000 mL. Aliquots of 100 mL were dispensed into 10 250-mL Erlenmeyer flasks each containing 1 g of noble agar and autoclaved at 121 °C for 20 min. After autoclaving, the pH should be 6.7.

Recovery determinations were conducted with muscle tissues from the control steers. Portions (10 g) of minced tissue were spiked with 33, 16, 8, 7, 5.5, or 4.0 μg of CAP. The tissue was in contact with the CAP for 1 h before being extracted with methanol.

5. Quantitative Gas Chromatographic Method for CAP. Samples were analyzed by the USDA–FSIS method (USDA–FSIS, 1983).

Table I. Effect of Tissue Storage on Chloramphenicol Concentrations in Muscle from Steers Injected Intramuscularly with Chloramphenicol[a] (Experiment 1, TLCB Analysis)

test steer	CAP after storage at −20 °C, ppm			
	0 weeks	2 weeks	5 weeks	13 weeks
A	0.8	0.6	<0.6	<0.6
B	2.1	1.5	<0.6	<0.6
C	3.8	3.0	1.8	<0.6

[a] Dose: 11 mg of CAP/kg, twice daily for 3 days. The steers were killed 18 h after the last injection with a barbiturate overdose.

Table II. Comparison of Initial Assay Results in Muscle (Experiment 2)

CAP dose,[a] mg/kg	test steer	CAP levels, ppm	
		GC	TLCB
11	D	2.94	2.2
	E	1.31	1.1
	F	2.76	1.0
22	G	3.05	4.1

[a] CAP injected intramuscularly, twice daily for 3 days. The steers were killed with a barbiturate overdose 18 h after the last CAP injection.

RESULTS AND DISCUSSION

In the initial experiment, CAP residues were detected in muscle but not kidney or liver samples on the date of sampling by TLCB (Table I). Residues of CAP could no longer be detected in muscle samples from two of the three treated animals after 5 weeks of storage at −20 °C and could not be detected from the third treated animal after 13 weeks of storage. All tissues tested negative by the STOP and MIT procedures. It was decided to repeat the experiment, with use of the more sensitive gas chromatographic assay as a reference method to confirm these observations.

Results of the second experiment were consistent with those observed for the first. A comparison of results obtained for subsamples from the same muscle tissues from this experiment (Table II) reveals that the TLCB procedure, used for semiquantitative estimation, generally provided a good level of agreement with the gas chromatographic assay results.

On the basis of results obtained for fortified muscle in the range 0.83–3.33 ppm, average recoveries for the TLCB procedure as applied to CAP were in the range 71–83%

Table III. Recovery Data for Beef Muscle Samples Fortified with Chloramphenicol (TLCB)

CAP fortifcn level,[a] ppm	no. of determin	av zone diam, mm	CAP found,[b] ppm	rec, %
0.83	16	4.9 ± 1.2	0.64	77
1.66	16	8.3 ± 1.9	1.38	83
3.33	16	10.7 ± 1.7	2.36	71

[a] CAP was added to 10 g of minced muscle, left in contact for 1 h, and then taken through the extraction procedure. Of the final extract, 30 μL was spotted on the thin-layer chromatography plates. [b] Based on average zones of 6.4, 10.0, and 15.1 mm, respectively, when 0.25, 0.50, and 1.0 μg of CAP standard was spotted directly on the thin-layer plates.

(Table III). Coefficients of variation calculated for the method ranged from 16% at 3.33 ppm to 24% at 0.83 ppm, indicating the method can be applied semiquantitatively at these levels. The normal analytical range for the gas chromatographic procedure used in these experiments is recommended at 10–100 ppb, although it can be used to estimate higher levels with a suitable standard curve or by employing appropriate dilutions (USDA–FSIS, 1983).

Using the extraction method described and spotting 60 μL of the final extract on the thin-layer chromatographic plate, we have established 0.6 ppm as a reliable limit of detection for CAP in muscle tissue using TLCB. Nonspecific inhibition appeared on the bioautographs for kidney and liver extracts. These zones of inhibition had a retardation factor (R_f) similar to that for CAP (0.67) but could be distinguished from CAP zones by shape. Nonspecific inhibition zones were rectangular and restricted to the thin-layer chromatography channel on which the extract was spotted whereas the CAP zones were round and, if large, diffused beyond the limits of the channel. Gas chromatographic analysis of these tissues for the second treatment group indicated the presence of CAP levels above the TLCB detection limit in kidney from three of the four steers, but levels in liver were well below the TLCB detection limit.

The published literature is somewhat contradictory on the stability of CAP residues in stored tissue samples. O'Brien et al. (1981), using a microbiological assay, reported a decrease of CAP levels in muscle tissue stored at −20 °C for 12 and 24 weeks. In their study, samples were taken from cattle weighing 190–300 kg that had been dosed on three successive days with 4.5 mg of CAP/kg and slaughtered 2 h after the third treatment. They aged the carcasses for 5–7 days at 4 °C before muscle samples were stored frozen at −20 °C. Singer (1984; Singer and Katz, 1985) developed a microbiological assay for CAP in tissues of various species and reported no loss of CAP when bovine muscle was fortified with CAP and stored frozen at −20 °C for 27 and 57 days.

In our experiments, levels of CAP in muscle and liver decreased with time when the tissues were stored at −20 °C (Tables I and IV). Considerable variation was noted in replicate tissue analysis for the same animal, as well as the expected variation between animals. Further testing of the gas chromatographic procedure is planned to provide a clearer evaluation of these observations. On the basis of our findings, we recommend minimum storage of incurred tissue prior to analysis.

The choice of target tissue for analysis is related to the time between treatment and slaughter. Sisodia et al. (1973) injected 135-kg calves intravenously with 20 mg of CAP/kg and found higher CAP levels in skeletal muscle than in liver or kidney at 2 and 4 h after injection, while O'Brien et al. (1981) were only able to detect CAP in muscle samples 2 h after intramuscular injection of CAP. Mercer et al. (1978) injected swine intravenously with 22 mg of CAP/kg, collected samples at periods ranging from 5 min to 24 h postinjection, and found that the highest CAP levels appeared in the liver and kidney shortly after treatment but that the half-life of CAP was longer in muscle. In our experiments, where a period of 18 h was allowed to elapse between treatment and slaughter to approximate a shipping period, highest levels were observed in muscle, with approximately one-fourth the muscle levels appearing in kidney and much lower levels in liver. Except in cases of slaughter almost immediately posttreatment, muscle tissue would therefore appear to be the preferred target tissue for analysis, based on these results. To further assess this issue, a long-term withdrawal study with calves injected with CAP within 3 weeks of birth is now in progress.

Table IV. Effect of Sample Storage at −20 °C on CAP Levels (Experiment 2, Gas Chromatographic Analysis[a])

	CAP levels after storage, ppb			
tissue[b]	0 weeks	2 weeks	5 weeks	12 weeks
serum	390^{de}	450^{cd}	520^{c}	330^{c}
kidney	680^{cd}	1000^{c}	370^{cd}	120^{d}
muscle	2340^{c}	1700^{d}	1090^{e}	1350^{de}
liver	23^{c}	20^{cd}	11^{de}	8^{e}

[a] Detection limit equals 15 ppb for serum and 5 ppb for kidney, muscle, and liver. [b] Means for three steers injected intramuscularly twice daily for 3 days with 11 mg of CAP/kg. The steers were killed with a barbiturate overdose 18 h after the last CAP injection. Horizontal means with different superscripts are different ($P < 0.05$) based on an analysis of variance and a least significant difference test (Steel and Torrie, 1960). Least significant difference ($P = 0.05$) equals 94, 878, 534, and 10 ppb, respectively, for serum, kidney, muscle, and liver.

The detection limit of 0.6 ppm CAP in muscle by TLCB, together with the nonspecific interferences observed in liver and kidney, limits the applicability of TLCB for the detection of CAP residues in bovine tissues to injection sites and muscle samples for recently treated animals. Our experience with survey samples suggests that CAP levels in tissues from animals sampled at the time of slaughter are usually below 0.6 ppm. We have, therefore, adopted a policy of routinely analyzing all submissions for antibiotic testing from federally inspected Canadian abattoirs for CAP by the gas chromatographic method when an injection site is present.

Despite this apparent limitation, the TLCB procedure has been demonstrated in this work to be more sensitive as a test for CAP residues in muscle than the in-plant STOP procedure or the confirmatory laboratory MIT previously used to analyze STOP-positive tissues by our division. No microbial inhibition was observed for STOP tests on any tissues from treated animals in these experiments, while only the kidney from the steer treated at 22 mg of CAP/kg body weight (twice the recommended maximum therapeutic dose) gave a positive response to the laboratory MIT. Analysis of kidney tissue from this animal by gas chromatography indicated a CAP level of 2.4 ppm.

The TLCB screening procedure used in our laboratory for the identification of antibiotic residues can therefore be reliably used for the detection and identification of CAP residues in muscle and injection site samples at levels above 0.6 ppm. However, based on our TLCB and gas chromatographic results, a negative STOP result is not necessarily indicative of an absence of CAP residues in a suspect animal. Prudence dictates submission of samples from such animals for laboratory gas chromatographic analysis.

ABBREVIATIONS USED

Chloramphenicol, CAP; swab test on premises, STOP; thin-layer chromatography/bioautography, TLCB; microbial inhibition test, MIT; tryptose agar slants, TAS; trypticase soy broth, TSB; phosphate-buffered saline, PBS; plate count agar, PCA; retardation factor, R_f.

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Evaluation of Molecular Interactions in Myosin, Fibrinogen, and Myosin–Fibrinogen Gels

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Myosin, fibrinogen, and myosin–fibrinogen gels formed by heating at a rate of 12 °C/h were disrupted with guanidine hydrochloride, urea, and 2-mercaptoethanol. The degree of disruption was quantitated by turbidity, and complexes that remained soluble were analyzed by gel filtration in 6 M guanidine hydrochloride and SDS–polyacrylamide gel electrophoresis. Myosin and fibrinogen together or individually formed gels at 70 °C that were more difficult to solubilize than gels formed at 50 °C. Myosin and fibrinogen gels formed at 70 °C were stabilized by both noncovalent and disulfide bonds. Noncovalent and disulfide bonds are formed in myosin–fibrinogen gels at 50 and 70 °C.

Thermally induced gelation of muscle proteins is important to muscle foods because it affects texture and water-holding properties of comminuted meat products (Acton et al., 1983). Thus, the gelling properties of proteins added to muscle foods, such as the blood protein fibrinogen, should be relevant to their functionality in a meat system.

Thermally formed myosin, fibrinogen, and myosin–fibrinogen gels were investigated in two previous studies (Foegeding et al., 1986a,b). Gel strength was shown to be dependent upon (1) the specific protein or proteins used to form a gel matrix, (2) protein concentration, and (3) the heating method used to form the gel. The characteristics of the protein gel matrices that caused the variations in gel strength were not determined.

Strength of myosin gels is a function of the gel matrix geometry (microstructure) and also may be affected by the chemical bonding within the matrix. The formation of a fine-stranded gel microstructure, as opposed to a coarse structure, makes a more rigid myosin gel (Ishioroshi et al., 1979; Hermansson et al., 1986). The microstructure is affected by variations in pH and ionic strength; however, the heating temperature from 55 to 65 °C has no effect (Hermansson et al., 1986). The rigidity of gels heated from 55 to 65 °C was not determined so the study by Hermansson et al. (1986) did not establish a relationship among heating temperature, microstructure, and rigidity.

Van Kleef (1986) investigated the chemical bonding within gel matrices by determining the ability of urea or urea plus 2-mercaptoethanol to dissolve ovalbumin and soybean protein gels. The combination of urea and 2-mercaptoethanol dissolved all gels, whereas urea alone dissolved soy protein but not ovalbumin gels. Thus, disruption of gel matrices with denaturants provides information on the bonding within the matrices.

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