Improved Sample Preparation Procedure for Determining Chlortetracycline in Chicken Tissue and Related Organs

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The most commonly used procedures for determining antibiotic residues in edible tissues, organs such as the liver and kidneys resulting from the use of antibiotics in animal agriculture are based upon classical cylinder plate diffusion techniques (1). The basic cylinder-plate procedure as applied to these materials are summarized and described by the F.D.A. in a publication of methods and protocols (2). A laboratory study to determine the sensitivity of these procedures for chlortetracycline in tissues indicated that 0.025-0.080 ug/g could be determined consistently and levels of 0.02 ug/g on occasion.

The purpose of this manuscript is to report as improved sample preparation technique which coupled with a modified agar diffusion procedure that consistently detects levels of 0.015 ug/g chlortetracycline in edible tissue, liver, and kidneys.

Materials and Methods

Reagents:

1. Buffer pH 4.5 $\pm$ 0.1. Dissolve 13.6 g monobasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.
2. Agar Culture Medium. This medium is available in dehydrated form as Difto Antibiotic Medium 2 No. 10943. The pH after sterilization should be 5.95 $\pm$ 0.05.
3. Standard Solutions of Chlortetracycline. Dissolve chlortetracycline standard in 0.01 N hydrochloric acid to give 1000 ug/ml activity. Dilute stock solution with pH 4.5 buffer to give a standard response curve of 0.003, 0.005, 0.01, 0.02, 0.04, 0.08 and 0.16 ug/ml. The 0.04 ug/ml standard is the reference concentration. The acid

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stock solution of chlortetracycline is stable 7 days under refrigeration.
4. Test Organism. Bacillus cereus var. mycoides ATCC 11778 prepared according to the A.O.A.C. official procedures (3). This stock solution so prepared is diluted 1:5 with sterile saline and can be maintained indefinitely if refrigerated and protected from contamination. The actual amount of stock suspension is determined by trial plates. Zones for the 0.003 ug/ml and 0.005 ug/ml standards should approximate 9.0 and 11.0 mm respectively.

Apparatus:

1. High-speed blender capable of handling pint mason jars such as manufactured by the John Oster Company, Milwaukee, Wis.
2. Microbiological Assay Equipment. Cylinder dispenser, petri dish bottoms or equivalent plates, porcelain covers glazed on the outside, stainless steel cylinders, incubators described in the official A.O.A.C. procedures (3).

Procedures

Preparation of Standard Curves: For recovery studies and/or determinations themselves, a standard curve is prepared by diluting the stock solution with pH 4.5 buffer to obtain concentrations of 0.003 to 0.16 ug/ml. Reference concentration is 0.04 ug/ml.

To prepare a compensatory curve for tissue add 0.625 1.25, a.50, 5.00, 10.00 and 20.00 ug to 24g of tissue and 100g of pH 4.5 buffer in a mason jar. Blend at high speed for 2 minutes. Remove and centrifuge a portion at high speed in a clinical centrifuge for 10 minutes. Reference concentration is 0.04 ug/ml. Similar compensatory curves can be prepared for livers and kidneys.

Melt the agar and cool to 70°C before innoculating with the previously determined volume of organisms. Mix well. Add 6 ml of the seeded agar to the petri dishes, distributing the agar evenly. Use plates within 1 hr. of hardening. A complete description of the number of plates, placing and filling the cylinders, incubation time and temperature are found both in the FDA protocols and by the AOAC (2,3).

Determination of Chlortetracycline in Tissues and Related Materials: Place 25g of muscle tissue and 100 grams of pH 4.5 buffer in a mason jar and homogenize at high speed for 2 minutes. Remove and centrifuge a portion at high speed for 10 minutes as previously described. For liver, weigh either 25g or the whole liver into a tared mason

jar and add pH 4.5 buffer equivalent to 4 times the weight of liver. Homogenize and proceed as described. For kidney's weigh the organ into a tared mason jar and add pH 4.5 buffer equivalent to 9 times the weight of the kidneys. Blend and proceed as described.

Results and Discussion

The ability to measure active drugs with a macimum degree of recovery is the basic objective of analytical methods for residue analyses. Laboratory studies of the FDA recommended procedures for antibiotics in tissues and related products, basically the most versatile compendium of tissue residue procedures available, indicated that recoveries of chlortetracycline from chicken tissue and livers averaged slightly over 50% and from kidney tissue 40%. The lower limit of detectability of these procedures was 0.025 ug/g and occasionally 0.020 ug/g. Although recovery studies can eventually correct for these levels, the recoveries limit the sensitivity. Similarly, the assumption that the losses or inability to recover the antibiotic is simply a function of the media is not completely valid.

The centrifugation of samples, which is the basic change from the FDA suggested procedure removes the physical barrier to diffusion of the antibiotic and hence allows for greater recoveries. The use of 1 part tissue plus 4 parts pH 4.5 buffer remains the same. Although the final pH of the homogenate is not in the range of 5.5-5.7, a study of recoveries in the range of 4.0 to 6.0 indicated pH adjustment did not improve recoveries. For the sake of simplicity no adjustment of pH was used.

The use of a single layer of seeded agar spread at a relatively elevated temperature allows for the spreading of an extremely uniform plate. A study of the effect of the temperature at which the agar is innoculated and poured indicated no change in the response of the organism to chlortetracycline from 48° to 75°C. The simplicity of the single layer change is a time saving feature when considerable numbers of samples must be run.

Recoveries of chlortetracycline from "spiked" tissue were compared using both the FDA suggested procedure and the centrifuge modification. Table I shows the recoveries of chlortetracycline from edible tissue of chickens.

Table 1

Recoveries of Chlortetracycline From "Spiked" Chicken Tissue

Supplementation per 25g Tissue		Procedure Recoveries				
		FDA Suggested		Centrifuged		Ratio
ugs	ugs/g	ugs/g	%	ugs/g	%	Centrifuged / FDA
0.5	0.02	N.D.[1]	-	0.018	90.0	-
1.0	0.04	0.024	60.0	0.033	82.5	1.37
2.0	0.08	0.048	60.0	0.061	76.2	1.26
3.0	0.12	0.052	43.3	0.080	75.0	1.73
5.0	0.20	0.108	54.0	0.172	86.0	1.59

1 Not detected.

The FDA suggested procedure averaged 54.3% with the centrifuged procedure averaging 81.9%. The ratio of recoveries averaged 1.48 indicating that approximately 50% greater recoveries possible using the centrifuge technique of sample preparation.

Examination of the epimerization reaction in the meat matrix showed 14 to 18% of added chlortetracycline could epimerize in the analytical procedure. There was no apparent binding of the muscle tissue resulting from the "spiking" since the combination of recoveries of active chlortetracycline and epimer are 90% or greater.

The same pattern exists in measuring residues resulting from the feeding of the antibiotic to poultry. Table 2 shows a comparison of tissue residues resulting from the feeding of broilers over a 12-week interval at 4 different levels. Each value shown is an average of results from 3 birds on the same ration and the levels are uncorrected for recoveries.

Table 2

Comparison of Residue Values Using the FDA Suggested Procedure and the Centrifuge Modification

Centrifuge Modification

Chlortetracycline Level g/ton	3-week ug/g	6-week ug/g	9-week ug/g	12-week ug/g
50	0.016	0.030	0.029	0.030
100	0.024	0.137	0.056	0.037
150	0.037	0.111	0.061	0.052
200	0.059	0.108	0.070	0.144

Table 2-Continued

Chlortetracycline Level g/ton	3-week ug/g	6-week ug/g	9-week ug/g	12-week ug/g
	FDA Suggested Procedure			
50	0.015[1]	0.018[1]	0.016[1]	0.022
100	0.022	0.100	0.041	0.029
150	0.030	0.081	0.043	0.035
200	0.041	0.079	0.048	0.090

1 Calculated from extrapolations of calibration curve.

Comparison of the ratios of the residues found indicate the ratios are essentially the same as found with "spiked" samples.

The same trend is noted with assays in liver tissue. The ratio's are very similar with the centrifuge procedure having recoveries approximately 35% greater. Table 3 shows a comparison of recoveries from "spiked" liver samples.

Table 3

Recoveries of Chlortetracycline from "Spiked" Liver Samples

Supplement per 25 g ugs	Tissue ugs/g	Procedure Recoveries FDA Suggested ugs/g	%	Centrifuged ugs/g	%	Ratio Centrifuged / FDA
0.5	0.02	N.D.[1]	-	0.015	75.0	-
1.0	0.04	0.024	60.0	0.031	77.5	1.29
2.0	0.08	0.044	55.0	0.060	75.0	1.36
3.0	0.12	0.063	52.5	0.087	72.5	1.38
5.0	0.20	0.126	63.0	0.166	83.0	1.33

1 Not detected.

Again as was noted with the muscle tissue, the FDA suggested procedure could not detect the 0.02 ug/g level. The epimerization reaction accounts for 15% of the added chlortetracycline. Binding does not appear to be significant.

Comparison of the results obtained with kidney analyses cannot be applied directly. The FDA protocols indicate for tissue 1 part tissue plus 4 parts 4.5 buffer. Because the concentration of chlortetracycline in the kidney is some 10 to 15 times that of the tissue, a

dilution of 1+9 seemed more reasonable. Recoveries by the centrifuge procedure using the 1+9 dilution averaged 85-90%. Obviously with a 1+9 dilution, the limit of detectability is not 0.015 ug/g: but since this is not an edible tissue and is used only as measure of the excretion of the drug, the sensitivity previously noted is not necessary.

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