COMMUNICATIONS

Microbiological Assay with Increased Sensitivity for Chlortetracycline in Eggs

A microbiological assay based upon the classical cylinder-plate assay procedure was developed for the determination of chlortetracycline in eggs. The procedure utilizes a pH adjustment with phosphoric acid, the surfactant polysorbate 20 to solubilize the lipid soluble fractions, and centrifuging to remove physical barriers to the diffusion of the antibiotic. Recoveries from eggs averaged 49.6% *vs.* 22.5% for presently used procedures. Epimerization of chlortetracycline accounted for only 10.9% of the added chlortetracycline. Limit of detectability for this procedure was 0.02 μg per g of egg as compared to 0.05 μg per g for presently used methods.

Chlortetracycline has been used in poultry feeds to increase feed conversion and egg production. As early as 1955, the efficacy of chlortetracycline in increasing egg production was reported (Carlson *et al.*, 1955). Although Durbin and coworkers (1954) reported measurable concentrations of chlortetracycline in eggs of birds fed 200 g per ton, no residues were detected at 100 g per ton. With the problems associated with antibiotic residues in foods, a review of the problems of measuring residue in edible products was undertaken.

Careful examination of the Food and Drug Administration-recommended (Kramer *et al.*, 1968) methods for antibiotic residues in milk, dairy products, and animal tissues revealed no specific suggestions for the determination of chlortetracycline in eggs. Abbey (1969) suggested a sample dilution of pH 4.5 phosphate buffer of 1 part egg + 2 parts buffer, or at most a dilution of 1 + 3, which was essentially in agreement with the FDA concept. Although the FDA-suggested procedures are not "official," a procedure used for residue studies should have at least the sensitivity and repeatability of those outlined. Studies using these suggested procedures and dilutions yielded recoveries considerably lower than anticipated. Although the problem of recoveries considerably less that quantitative is recognized and accounted for by corrections, low recoveries severely handicap the ability to detect trace residues.

It is the intent of this communication to report a sample preparation technique in conjunction with a cylinder-plate assay which achieves higher recoveries and hence increased sensitivity for detecting chlortetracycline residues in eggs. Similarly, since recoveries of the antibiotic are less than quantitative, the problem of the nature of the species not measured was also investigated to give insight into the problem of binding and/or epimerization.

REAGENTS

Phosphate Buffer pH 4.5 ± 0.1. Dissolve 13.6 monobasic potassium phosphate in distilled water and dilute to 1 l. with distilled water.

Agar Culture Medium. Antibiotic Medium 2 Difco or BBL. The pH of the medium should be adjusted so that after sterilization the pH is 5.95 ± 0.05.

Standard Solutions of Chlortetracycline. Dissolve chlortetracycline hydrochloride standard in 0.01*N* hydrochloric acid to give 1000 μg/ml. Dilute the stock solution with pH 4.5 buffer containing 0.5% polysorbate 20, Tween 20, to give a standard response curve of 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 μg/ml. The stock solution can be maintained in a refrigerator for 7 days. For recovery studies, prepare chlortetracycline standards containing 1.0, 2.0, 3.0, 5.0, and 10.0 μg/ml in 0.01*N* hydrochloric acid.

Test Organism. The test organism, *Bacillus cereus* var. mycoides, ATCC 11778, was prepared according to the AOAC procedure (1965). The stock solution so prepared is diluted 1:5 with sterile saline. This working diluted spore suspension can be maintained indefinitely if refrigerated and protected from evaporation and contamination. The actual amount of working spore suspension to be used for seeding the agar is determined by trial plates. A zone diameter approximately 11.0 mm can be obtained for 0.005 μg/ml chlortetracycline.

PROCEDURES

Recovery Studies. Several shelled eggs were blended in a Mason jar using a high speed blender. Fifty gram samples of blended egg, 50 ml of distilled water, and 1 to 10 μg of chlortetracycline were added to pint Mason jars and mixed well. The pH was adjusted to 4.5 with 3*N* phosphoric acid. One gram of Tween 20 (1 ml) was added and the total weight of the content of the Mason jar was brought to 200 g with pH 4.5 phosphate buffer. This mixture was blended for 30 sec. Portions of the blend were centrifuged in a clinical centrifuge at high speed for 5 min. The clarified centrifugate is saved for the cylinder-plate assay.

Plates were prepared by adding 6 ml of seeded agar to Petri dishes or Petri dish bottom, and distributing the agar evenly, letting the agar harden before placing the cylinders on the plates. Plates should be used within 1 hr of hardening. The placing of the cylinders, the use of porcelain covers, and the filling of cylinders are completely described in the AOAC procedures (1965) for antibiotics in animal feeds.

The standard curve was prepared by diluting the stock solution of chlortetracycline with pH 4.5 buffer containing 0.5% Tween 20 to obtain concentration of 0.005 to 0.16 μg/ml. Reference concentration was 0.04 μg/ml.

DETERMINATION OF RESIDUES IN EGGS

A standard response curve in eggs is prepared by blending 1, 2, 8, 16 and 32 μg chlortetracycline to 50 g of shelled, blended egg, adding 50 ml of distilled water, adjusting the pH to 4.5 with 3*N* phosphoric acid, buffering and adjusting the weight to 200 g, adding 1 g of Tween 20, blending for 30 sec,

centrifuging at high speed for 5 min, and filling the cylinders with the centrifugate. The final concentrations of chlortetracycline are 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 μg/g. The advantage of preparing a response curve in this fashion is that it allows for a simplified mode of determining the concentration of antibiotic in egg.

To determine the concentration of chlortetracycline in eggs of laying hens fed this antibiotic, place the shelled egg in a preweighed Mason jar. After determining the weight of the egg, add 50 ml of distilled water and blend for 30 sec. Adjust the pH to 4.5 with 3*N* phosphoric acid and add sufficient pH 4.5 buffer and surfactant so that the final weight is four times the weight of the egg and the Tween 20 concentration equals 0.5%. This mixture is reblended for 30 sec at high speed, followed by centrifuging. The clear centrifugate is then assayed as previously described.

RESULTS AND DISCUSSION

If common laboratory detergents are used to wash equipment employed in antibiotic assays, they must be completely removed. The presence of detergent residues on equipment usually manifests itself as a nonspecific inhibition of the test organism. It is mandatory that any antibiotic activity found be derived from the sample and not from any external laboratory sources. The use of Tween 20, a nonionic detergent, in the analysis of antibiotic residues appears to be a contradiction. However, its use did not result in any measurable inhibition or in a change in the zone diameters of response curves when compared to response curves using pH 4.5 phosphate buffer as the diluent. The addition of surfactants to increase the release of antibiotics from petrolatum based formulations was reported by Sharma and Gupta (1967). These investigators found that Tween 20, while not showing antimicrobial activity, increased the release of tetracycline antibiotics from a petroleum base.

Comparison of the results obtained using the procedures in agreement with the FDA concept (Kramer *et al.*, 1968); Abbey, 1969) and the procedure using Tween 20 as an emulsifying agent showed that considerably higher recoveries were obtained using the surfactant procedure (Table I). The presence of 2 μg in 50 g of egg (0.04 μg per g) is not detected by techniques based upon the FDA-suggested methods for tissue assays. Although a recovery factor will correct for the low recoveries, the overall sensitivity of any procedure is dependent upon the ability to recover the active agent. The surfactant procedure allows for the detection and semi-quantitative measurement of levels as low as 1 μg per 50 g of egg (0.02 μg per g). The just detectable designation in Table I indicates that the levels detected are below the limit of the standard curve, but still show measurable inhibition. Extrapolation of the calibration curves to cylinder diameter gives estimated recoveries of 0.58, 0.50, and 0.62 μg, and correspond to 58, 50, and 62% recovery. The data presented in Table I were developed on individual days and indicate that reasonable repeatability is possible and detection of the 0.02 μg per g of egg level is not the result of a laboratory fluctuation on a given day.

Increasing the dilution factor of the FDA-suggested method to 1 part egg + 3 parts buffer neither raised recoveries nor increased sensitivities. The 2 μg per 50 g of egg level could not be detected. Recoveries at the 3 μg per 50 g of egg level were 33.3%, at the 5 μg per 50 g of egg were 26.4% and 26.0% at the 10 μg per 50 g of egg level. Similarly increasing the dilutions of the surfactant procedure to 1 part egg + 4 parts buffered diluent did not increase recoveries; instead it eliminated the ability to detect 1 μg per 50 g of egg level (0.02 μg/g).

Table I. Comparison Between Recoveries of Chlortetracycline from Eggs Obtained Using FDA Suggested Protocols and the Surfactant Procedure Chlortetracycline

Egg Sample g	Chlortetracycline Added μg	Found μg	Recovered[a] %	Found μg	Recovered[b] %
		FDA 1 + 2[c]		Surfactant	
50	none	none	...	none	...
50	none	none	...	none	...
50	none	none	...	none	...
50	1	none	...	J.D.[d]	...
50	1	none	...	J.D.[d]	...
50	1	none	...	J.D.[d]	...
50	2	none	...	1.04	52.0
50	2	none	...	1.04	52.0
50	2	none	...	0.98	49.0
50	3	0.75	25.0	1.52	50.7
50	3	1.00	32.0	1.34	44.7
50	3	0.69	23.0	1.65	55.0
50	5	1.14	22.8	2.40	48.0
50	5	0.99	19.8	2.60	52.0
50	5	1.11	22.2	2.20	44.0
50	7	1.50	21.4	3.15	45.0
50	7	1.62	23.1	3.60	51.5
50	7	1.82	26.0	3.40	48.6
50	10	2.25	22.5	5.20	52.0
50	10	2.60	26.0	5.00	50.0
50	10	2.50	25.0	5.00	50.0

[a] Average 22.5%. [b] Average 49.6%. [c] 1 part egg, 2 parts 4.5 buffer. [d] J.D. = just detectable zone inhibition size 9.5 to 10.0.

Recoveries of chlortetracycline increased with higher surfactant concentrations up to the 0.5% level. Above this concentration but less than 1% surfactant increased recoveries were not apparent. Levels of 1% surfactant showed inhibition of the test organism. Blending times greater than 30 sec did not increase recoveries; instead there was a trend toward lower recoveries. The increased temperatures resulting from extended blending in all probability increased the epimer content. Not only has the epimer limited microbiological activity (Eisner and Wulf, 1963), but also the conversion to the epimer form is related to temperature. Measurement of the epichlortetracycline content (Katz and Fassbender, 1967) in eggs showed the conversion to be 10.9%.

Centrifuging to remove physical barriers to diffusion showed interesting phenomena. Egg samples adjusted to pH 4.5 with phosphoric acid and centrifuged yielded recoveries lower than those found with the same samples not centrifuged. Egg samples diluted with pH 4.5 buffer (1 + 2) and centrifuged showed a similar tendency. The addition of the surfactant, blending and centrifuging, yielded results higher than those which were only blended with the surfactant. Those eggs blended with the surfactant yielded recoveries greater than those not containing the Tween 20. Evidently there are two factors which play a role in the recoveries of chlortetracycline from eggs: one is a physical barrier of diffusion, and the other is the solubility or binding to or with egg material. Centrifugation and the surfactant tend to lower or minimize the effects of both factors.

The overall changes in procedure also included adjustment of pH to 4.5 and dilution with buffer. This step was added to standardize the procedure. All response curves, whether made up in the egg media or not, eventually are compared to a response curve made up with pH 4.5 phosphate buffer.

The pH of the eggs diluted with buffer ranged between 5.0 and 5.2, and not 4.5.

Recoveries of added chlortetracycline even with the increase in sensitivity remained below anticipated levels. Total active chlortetracycline recovered was 49.6%, with an additional 10.9% in the epimer form; this made a total recovery of 60.5%. There remains an additional 39.5% unrecovered, the activity and nature of which are unknown. Categorizing this data, some 25% of the chlortetracycline activity is easily extractable. An additional 25% is extractable using a surfactant to solubilize that fraction dissolved in the lipid portion. Ten percent can be found in the epimeric form. The remaining unknown quantity can be defined as "bound" and not measurable.

Attempts to recover this fraction using various enzymatic digestions did not prove overly successful. Although there was a measurable increase of some 10% in recovery from eggs, incubations of chlortetracycline with the enzymatic preparations showed a 20% loss of activity. Indirectly it can be inferred that the slight increase in recovery coupled with the loss of activity in the enzymes' presence indicates that a good portion of the bound chlortetracycline activity is potentially available through normal digestive processes. This avenue of discussion is based upon inference and not upon any direct evidence, but is offered for consideration concerning the significance of the "bound" species. Whether the "bound" material has any biological ramifications remains to be shown through research.

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Stanley E. Katz*
Carol A. Fassbender

Department of Agricultural Chemistry
Rutgers–The State University of New Jersey
New Brunswick, N.J. 08903

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** To whom correspondence should be addressed.*