Estimation of Decoquinate Residues in Chicken Tissues by Thin-Layer Chromatography and Measurement of Fluorescence on Plates

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A method for the estimation of decoquinate (ethyl 6 - decyloxy - 7 - ethoxy - 4 - hydroxyquinoline - 3-carboxylate) is described, based on thin-layer chromatography and direct measurement of its fluorescence on the plate. A sensitivity better than 0.05 ppm of drug in chicken tissues with a mean recovery of about 85% is obtained (89% for muscle, 87% for liver, 78% for kidney, and 80% for fat). Among the other currently used coccidiostats we have tried, only buquinolate interferes; nicarbazin, zoalene, and amprolium do not. Dimetridazole, which is used to control histomoniasis (blackhead) in turkeys, does not interfere either.

The use of decoquinate (ethyl 6-decyloxy-7-ethoxy-4-hydroxyquinoline-3-carboxylate) as a coccidiostat (Ball *et al.*, 1968; Yvore, 1968) necessitates a detailed knowledge of the magnitude and distribution of the residues likely to be present in the flesh and organs of treated chickens. For this reason, a method has been developed for estimating this coccidiostat in edible tissues (muscle, liver, kidney, fat).

Decoquinate in methanol solution at 1 μg per ml exhibits a relatively intense fluorescence. This fluorescence is increased by working in the presence of Ca^{2+} or Mg^{2+} ions in the form of a methanolic solution of calcium chloride at 10 g per l. or magnesium sulfate at 20 g per l.

Uncorrected values obtained with an Aminco-Bowman spectrofluorimeter are shown in Table I.

The fluorescence also decreases in the presence of water (decrease about 20% for 10% water, and more than 90% with 50% water). We have therefore measured the fluorescence of decoquinate directly on the tlc plate using magnesium to obtain an increase in sensitivity. The nature of the complex formed is not known exactly and is referred to as the magnesium complex of decoquinate. Our method therefore differs from those previously described for measurement of decoquinate in tissues (Button *et al.*, 1969), employing fluorescence of the corresponding acid in solution, and in feeds (Stone, 1968), using fluorescence measurement of the calcium complex in solution.

PRINCIPLE OF THE METHOD

Tissue samples were macerated and extracted with ethanolic hydrochloric acid (99/1 v/v). Maceration can be carried out on fresh, frozen, cooked, or lyophilized tissues. The extract was subjected to a double cleanup of an aliquot of this solution comprising firstly a liquid-liquid extraction followed by column-chromatography. The sample was then purified by thin-layer chromatography of the purified extract corresponding to 1 g of fresh tissue, and the fluorescence of the decoquinate measured directly on the chromatogram by comparison with the fluorescence of a quantity of pure decoquinate treated under the same conditions on the same chromatogram.

APPARATUS

Used were a uv lamp (254 nm), and a Chromoscan densitometer (Joyce and Loebl).

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MATERIALS AND STANDARD SOLUTIONS

A chromatographic column (200 nm x 12 mm) containing, to a height of about 150 mm, Sephadex LH 20 previously suspended in light petroleum (b.p. 40–65° C) was used. Standard solution S1 (100 ng/μl): Decoquinate—10 mg; Acetonitrile–hydrochloric acid (99/1 v/v) —97 ml; Triethylamine—to produce 100 ml. Standard solution S2 (10 ng/μl): Dilute the previous solution (1 to 10) with acetonitrile. Standard solution S3 (1 ng/μl): Dilute the previous solution (1 to 10) with acetonitrile.

PROCEDURE

Maceration and Extraction. Weigh, to about 50 mg, a 10 g test sample of chicken tissue. Coarsely chop the sample and place it in the macerator jar. Add 15 ml of ethanolic hydrochloric acid (99/1 v/v). Macerate until a fine suspension is obtained (30 sec to 5 min of maceration according to the type of macerator). Transfer the macerate to a 50 ml centrifuge pot. Wash the macerator and its pot with a total volume of 10–15 ml of ethanolic hydrochloric acid (99/1). Add to the preceding extract. Centrifuge for 5 min at about 5500 g (6000 rpm R = 135 mm for example). Transfer the supernatant liquid into another 50 ml centrifuge pot. Recover the solid residue from centrifugation and again macerate it for 5 min with 10 ml of ethanolic hydrochloric acid. Add the macerate to the supernatant of the first maceration. Rinse the macerator and its jar with 10 ml of ethanolic hydrochloric acid (99/1). Centrifuge the whole thing for 10 min at 5500 g. Transfer the supernatant to a 50 ml volumetric flask. Make up to the mark with ethanolic hydrochloric acid: Solution A.

Purification. Transfer to a 50 ml evaporation flask, 5 ml of the preceding solution (5 ml of the Solution A are equivalent to 1 g of tissue). Evaporate this solution under vacuum at 30° C until a volume of about 0.2 ml is obtained. Transfer to a 10 ml separation funnel, rinse the evaporation flask first with 3 ml of hexane and then with 3 ml of acetonitrile. Shake the mixture to achieve a liquid-liquid partition and allow to settle. Transfer the lower phase (acetonitrile) to the evaporation flask (Solution B). Wash the hexane layer again with 2 ml of acetonitrile and add the wash liquid to Solution B.

Neutralize Solution B (~5 ml) with 0.5 ml of triethylamine. Evaporate under vacuum at 30° C until a volume of about 0.5 ml is obtained. Dilute with 1.5 ml of chloroform-methanol (50/50 v/v). Pour this solution onto the column; for quantitative transfer, rinse the flask with 5 ml of light petroleum and pour it similarly onto the column. Develop the column with

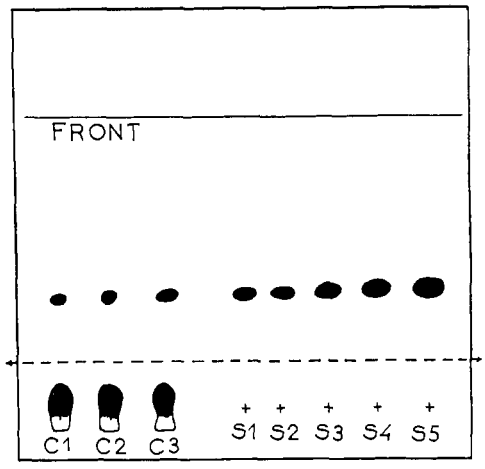

Figure 1. Thin-layer chromatogram after the first migration. C1, C2, C3: tissue extracts; S1, S2, S3, S4, S5: standard solutions. Cut the plate using a diamond, as shown by the dotted line

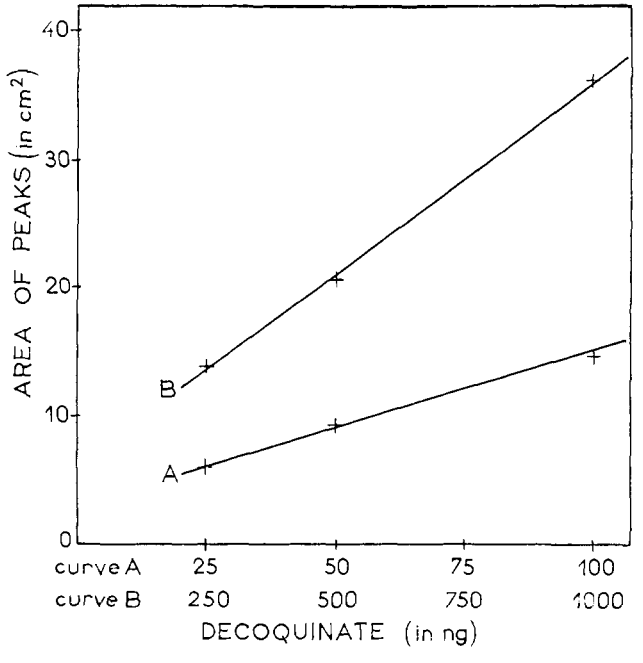

Figure 2. Calibration curves. λ exc = 290 nm; λ Fl = 440 nm. Curve A: 25 to 100 ng–Optical Wedge 2 D—Cam D. Curve B: 250 to 1000 ng—Optical Wedge 2 D—Cam B

petroleum ether and collect the first 100 ml of eluate. Evaporate this eluate in a 250 ml flask under vacuum at 30° C to obtain a volume of about 5 ml of solution. Pour into a 50 ml evaporation flask. Wash the 250 ml flask with 10 ml of solvent and evaporate the whole in the 50 ml evaporation flask until a volume of about 0.1 ml remains, giving Solution C.

Thin-Layer Chromatography. Prepare a plate of Merck silica F 254 by activating it at 130° C for 1 hr and quantitatively apply to this plate the following products: Solution C [quantitatively collecting this solution by rinsing the flask successively with 200 μl of light petroleum and 200 μl of chloroform-ethanol (50/50 v/v)]; Solutions C1, C2, etc. (solutions originating from other tissues at the same time since it is possible to apply up to three samples to the plate).

Standard solutions of decoquinate

With solution S3 apply 50 ng (50 μl)

With solution S2 apply 100 ng (10 μl) and 250 ng (25 μl)

With solution S1 apply 500 ng (5 μl) and 1000 ng (10 μl).

Table I. Fluorescence of Decoquinate in Methanol Solution at 1 μg per ml

	Excitation (nm)	Emission (nm)	Relative intensity
Decoquinate alone	340	380	0.32
Decoquinate + Ca^{2+}	265–270	390	1.30
	330	390	2.50
Decoquinate + Mg^{2+}	265–270	390	2.50
	330	390	2.50

Table II. Results Obtained on Tissues Fortified with Decoquinate

Nature of the tissue examined	Concentration of decoquinate (in ppm) Added	Recovered	% Recovery
	0.05	0.052	104
	0.10	0.095	95
	0.25	0.172	69
Muscle	0.25	0.25	100
	0.50	0.44	88
	0.75	0.65	88
	1.00	0.72	72
Muscle	0.05	0.046	92
(after cooking)	0.05	0.047	95
	0.05	0.040	80
	0.10	0.088	88
Liver	0.35	0.34	97
	0.75	0.55	74
	1.00	0.95	95
	0.05	0.039	78
	0.10	0.068	68
Kidney	0.50	0.395	79
	1.00	0.88	88
	0.05	0.04	80
Fat	0.50	0.40	80
	1.00	0.81	81

It is essential to make all the applications in a very reproducible manner in the form of an oval 0.3 cm wide and 0.6 cm long.

Develop the chromatogram by the ascending technique with dichlorethane-methanol (90/10 v/v) as mobile phase.

After the solvent front has traveled not less than 12 cm, remove the plate, dry it in a current of air, and examine it under ultraviolet light (λ = 254 nm). Locate the decoquinate on the chromatogram and then cut the plate 2 cm beneath the zone of migration of the decoquinate, as in Figure 1 (this allows removal of polar compounds likely to interfere with subsequent development).

Next develop the chromatogram to a height of 15 cm from the original base by the ascending technique with diethyl ether-tetrahydrofuran (90/10 v/v) as mobile phase. This development, which does not cause the decoquinate to migrate, is intended to eliminate certain less polar substances. It may be repeated several times if necessary, each time checking progress under the ultraviolet lamp. If the decoquinate spots are too diffuse during these successive migrations it is possible to concentrate them by a shorter development (2–3 cm) with dichlorethane-methanol (90/10) as mobile phase. Spray the dry chromatogram evenly with a 10% w/v aqueous solution of magnesium sulfate ($MgSO_4$, 7 H_2O) and dry the chromatogram at 110° C for 2 min.

Measurement. Measure the fluorescence of the range of decoquinate and that of the spots of decoquinate finally

present in the extracts originating from the tissues taken from the treated animals by means of the Chromoscan, using an excitation of 290 ± 20 nm and emission at 440 ± 10 nm. The reading may be taken in either of two directions—perpendicular to the direction of migration (in the region of R_f of decoquinate) and measuring for each spot the integrated values given by the apparatus, or in the direction of migration and measuring the area of the peaks (approximating them to triangles).

In each case a curve is plotted, with the values of the weight of decoquinate as abscissae and the integrated values or areas of peaks as ordinates. Integrated values or areas of peaks obtained with the samples are read from the curve enabling the weight of decoquinate present in 1 g of tissue examined to be found.

VALIDITY OF THE METHOD

Several estimations carried out with pure decoquinate have established the following points. The minimum quantity of decoquinate detectable on a plate with the Chromoscan densitometer by the fluorescence of the magnesium complex of decoquinate is 10 ng. The minimum quantity of decoquinate detectable on a plate by visual examination of the plates at 254 nm is 100–200 ng (0.1–0.2 μg), depending on the shape of the spots. This simple visual examination is important, since it allows the concentration of decoquinate to be assessed and the conditions of reading with the Chromoscan to be determined. The curve—amount of decoquinate *vs.* area or integrated values—is a straight line.

This sensitivity depends on the conditions of reading of the Chromoscan; *i.e.*, the settings of the density wedge, the cam, the reference lamp slit and the sample slit. To illustrate this point, we give as examples, in Figure 2, two plots obtained with ranges of decoquinate with different conditions of reading of the Chromoscan apparatus.

In the presence of tissue extracts, the limit of detection is 50 ng in 1 g (*i.e.*, 0.05 ppm). This limit could be lowered to 10 ng but, in this case, the range of reading is very restricted (10–50 ng) and is inconvenient for a series of measurements.

The method is applicable to liver, kidney, to fresh or cooked muscle, and also to fat. In none of the tissues have we found substances interfering with decoquinate at the usual R_f of this compound. Assays on control chicken did not give significant values (less than 0.01 ppm).

Percentage Recovery. We have added 0.05–1 ppm to 10 g test samples of various tissues. The results of these determinations are summarized in Table II. The mean percentage recovery is 85%, the extreme values being 68 and 104%. The mean percentage recovery for each tissue is as follows: 89% for muscle; 87% for liver; 78% for kidney; and 80% for fat.

Interferences. Under the conditions described, buquinolate interferes. On the other hand, amprolium, dimetridazole, nicarbazin, and zoalene do not interfere, either because they are separated in the course of purification or during final chromatography, or because they do not give fluorescence similar to that characteristic of decoquinate.

LITERATURE CITED

Ball, S. J., Davis, M., Hodgson, J. N., Lucas, J. M. S., Parnell, E. W., Sharp, B. W., Warburton, D., *Chem. Ind.* **56,** 2 (1968).
Button, R. G., Muggleton, D. F., Parnell, E. W., *J. Sci. Food Agr.* **20,** 70 (1969).
Stone, L. R., *J. Offic. Anal. Chem.* **15,** 1279 (1968).
Yvore, P., *Rec. Méd. Vét.* **144,** 1 (1968).

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