Determination of Residues of Nitromide in Poultry Tissues to Concentrations of Less than 0.010 Part per Million

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A procedure for the determination of nitromide in poultry tissue has been developed. The nitromide is extracted from the sample with acetone and chloroform and prepared for chromatography by removing the aqueous phase and evaporating the solvents. The extract residue is then chromatographed on alumina to remove lipid components and residues of other drugs. The benzamide eluate is passed through a column of Dowex-50 resin to remove arylamines (e.g., 3-amino-5-nitrobenzamide), the nitromide fraction is reduced with $TiCl_3$ to an amine, and the reduced fraction is rechromatographed on another Dowex-50 column. After removal of the interfering substances with washings, the arylamine residue is eluted with 4*N* HCl. Colorimetric measurement is made in a 100-mm. cell at 530 mμ. after reacting the residue with Bratton-Marshall reagents. Recoveries were in a range from 66.2% for skin to 72.6% for liver and kidney. The interference was less than 0.006 p.p.m. in all tissues and the sensitivity is less than 0.010 p.p.m.

Nitromide, or 3,5-dinitrobenzamide (3,5-DNBA), is a drug which is effective in preventing cecal and intestinal coccidiosis in chickens (Morehouse and McGuire, 1959). The drug is one of the active ingredients in Unistat-3 available from Salsbury Laboratories, which is used in poultry feed at 0.1% and provides 0.025% 3,5-dinitrobenzamide, 0.030% acetyl-(*p*-nitrophenyl)-sulfanilamide and 0.005% 3-nitro-4-hydroxyphenylarsonic acid. An analytical procedure with a sensitivity of less than 0.010 p.p.m. has been developed to determine the amount of 3,5-DNBA in various tissues of chickens on medication and after withdrawal of medication.

EXPERIMENTAL

Solvents and Reagents. Alumina, Fisher 80- to 200-mesh absorption, A-540, Fisher Scientific Co. Other sources of alumina gave erratic results.

Ammonium sulfamate, 1.25% solution in water prepared fresh weekly. Refrigerate when not in use.

Special denatured alcohol Formula 3A (3A alcohol). 100 parts 95% ethanol plus 5 parts absolute methanol.

3,5-Dinitrobenzamide, recrystallized and purified for standard use, m.p. 185–6° C. (Salsbury Laboratories, Charles City, Iowa).

N-(1-Naphthyl)-ethylenediamine dihydrochloride, 0.25% solution in water prepared fresh weekly. Refrigerate when not in use.

Sodium nitrite, reagent grade, 0.25% solution in water prepared fresh weekly. Refrigerate when not in use.

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Cation exchange resin Dowex 50W-X8, 200–400 mesh, prepared as described by Thiegs *et al.* (1961).

Eluting reagent, 3A alcohol: absolute ethyl alcohol (1 to 1, v./v.).

Apparatus. Absorption cells, Beckman Instrument No. 75195 matched set of two cylindrical silica cells with 100-mm. optical path.

Chromatography tubes, Corning No. 38460, 20 mm. × 400 mm. with a ⅋ 29/40 joint with a coarse fritted disc.

Ion-exchange column, as described by Thiegs *et al.* (1961).

Spectrophotometer, Beckman DK-2 or equivalent.

Sample Preparation and Extraction. For liver, kidney or blood, homogenize 350 grams in a Waring Blendor. For skin with adhering fat and muscle, pass 350 grams through a meat grinder and divide and quarter to obtain a homogeneous sample. The tissues must be obtained from either freshly killed or quickly frozen birds; the latter should be analyzed as soon as thawed. Weigh 100 ● 0.5 grams of the homogenized or gound tissue in triplicate and transfer each sample with 250 ml. of acetone into separate one-quart blender jars containing a 100-ml. beaker full of Hyflo-Super-Cel (Fisher Scientific). The sample is blended for five minutes at medium speed and then filtered through wetted Whatman No. 5 paper in a Buchner funnel (Coors No. 4) into a one-liter suction flask. The pulp and paper are transferred back into the blender jar, re-extracted with 250 ml. of chloroform and refiltered into the same suction flask containing the acetone extract. The extracted tissue is then discarded.

The combined filtrates are transferred with three 25-ml. rinses of chloroform into a one-liter separatory funnel, mixed, and allowed to stand until phase separation occurs. The aqueous phase (30–50 ml.) is not always emulsion free. Losses from emulsions have not been significant. If an upper phase does not appear, add an additional 100 ml. of chloroform and 10 ml. of H_2O and repeat the mixing step.

The lower acetone-chloroform phase is withdrawn into a one-liter round bottom evaporation flask and the upper aqueous phase is discarded. The organic phase is evaporated in a rotary flash evaporator at 45–50° C. until an oily residue remains. *Caution: Do not overheat the sample or allow it to go to dryness.* The acetone and chloroform evaporated from the sample are discarded. The residue (<30 ml.) should be free of traces of water. If the oil is turbid indicating the presence of water, add 100 ml. each of acetone and chloroform and repeat the evaporation step to remove the water, since water may interfere with the subsequent chromatographic separation.

Alumina Chromatography. Fill a 20- × 400-mm. column with alumina to a height of 60 mm. Tap the column to settle the alumina and add anhydrous sodium sulfate for an additional height of 25 mm. to absorb any water left in the chloroform extract. Prewash the column with 50 ml. of chloroform, transfer the oily residue from the evaporation step onto the column with several 15-ml. rinses of chloroform (approximately 75 mm. total) and wash the column with 100 ml. of chloroform. Discard these fractions. Elute the drug with 65 ml. of eluting reagent (see solvents and reagents section).

Ion Exchange Chromatography, No. 1. Arylamines, such as 3-amino-5-nitrobenzamide, a metabolite of 3,5-DNBA, are separated from 3,5-DNBA by cation exchange chromatography. Prepare the ion exchange column with regenerated Dowex 50W-X8 resin to give a bed depth of 4–5 cm. after settling. Prewash the resin bed with 10 ml. of 3A alcohol. Transfer the eluate from the alumina column to the ion exchange column, and wash the column with 50 ml. of 3A alcohol. Collect and combine both effluents in a 250-ml. beaker. (Slight air pressure can be used to increase the flow rate.) The arylamines, if present, are retained on the column and are discarded.

Reduction. The effluent containing the 3,5-DNBA is evaporated to 5–10 ml. in a 90° C. water bath under a stream of air. *Caution: Do not overheat the sample by allowing the sample to go to dryness.* Transfer the sample to a 50-ml. centrifuge tube with 3A alcohol and re-evaporate to about 1 ml. Add 5 ml. of water and reduce to an arylamine with 2 drops of 20% $TiCl_3$ and 4 drops of 10*N* NaOH while being mixed on a Vortex mixer. The sample is mixed until the grayish color turns to a chalky white. Dilute to 50 ml. with 3A alcohol to precipitate the titanium salts and centrifuge for 5 minutes at 2000 r.p.m.

Ion Exchange Chromatography, No. 2. Decant the alcohol phase of the centrifuged sample, which contains the reduced 3,5-DNBA, from the titanium salts onto a second ion exchange column prepared as described in "Ion Exchange Chromatography No. 1." The titanium salts are discarded. After the alcohol phase has passed through the column, wash the column with 50 ml. of 3A alcohol and then 50 ml. of water. The eluates are discarded. Elute the reduced 3,5-DNBA with 42 ml. of 4*N* hydrochloric acid into a low actinic 50-ml. volumetric flask. Avoid direct sunlight since the reduced 3,5-DNBA has been found to be photosensitive.

Colorimetry. Cool the eluted sample to 0–5° C. in a freezer or ice bath and then add 1 ml. of 0.25% sodium nitrite, 1 ml. of 1.25% ammonium sulfamate, and 1 ml. of 0.25% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5-minute intervals while the sample is kept in subdued light. Dilute the sample to volume and allow the color to develop for 10 minutes. Measure the absorbance in 100-mm. matched cells at 530 mμ in a spectrophotometer. Obtain the observed level of 3,5-DNBA from a standard curve of parts per million 3,5-DNBA *vs.* absorbance.

Preparation of Standard Curve. Dissolve 100 mg. of purified 3,5-DNBA in acetone and dilute to 1000 ml. (100 μg. per ml.). Dilute 1 ml. of this solution to 100 ml. with acetone (1 μg. per ml.). From this solution add aliquots containing from 1 to 10 μg. of 3,5-DNBA to centrifuge tubes containing 5 ml. of water. Evaporate the acetone from the tubes in a hot water bath. While mixing on a Vortex mixer, reduce the 3,5-DNBA by adding 2 drops of 20% $TiCl_3$ and four drops of 10*N* NaOH. Continue mixing until the sample is chalky white in appearance. Add 2 ml. of concentrated HCl, mix, and allow to stand for five minutes. Transfer the sample to 50-ml. low actinic volumetric flasks and dilute with 4*N* HCl to 40–45 ml. Follow the colorimetric procedure described above for tissue samples. Construct a standard curve by plotting the mean absorbance at 530 mμ for each concentration of 3,5-DNBA from several runs *vs.* the equivalent concentration in parts per million in tissues.

RESULTS AND DISCUSSION

Evaluation. An evaluation of this method was performed in a randomized block of 20 experimental runs, four each on muscle, skin with adhering fat, kidney, liver, and blood. Each tissue was fortified with 3,5-dinitrobenzamide standard at either 0, 10, 20, 50, or 100 ng. drug per gram of tissue. The order of fortification was determined from a table of random numbers. Each day's run consisted of five samples, in triplicate, of one of the five tissues listed. The daily sequence of tissues to be analyzed was prearranged so that one set of all tissues was analyzed before a second set was started.

A sufficient amount of each tissue was homogenized and 100 ± 0.5-gram aliquots were transferred to 15 one-quart Waring Blendor jars. The tissue samples were then fortified with 3,5-DNBA and extracted according to the described method. Since 3,5-DNBA is rapidly metabolized *in vitro* by tissue homogenates, the acetone was added to the tissue to denature the enzymes before the tissue was fortified, thus eliminating metabolic conversion of 3,5-DNBA. Observed levels of 3,5-DNBA were determined from the standard curve. Calculations to correct for losses in recovery and tissue interference were made on each tissue by determining a straight-line function of ng. per gram 3,5-DNBA added *vs.* p.p.m. 3,5-DNBA observed using the method of least squares. In all tests, the means of triplicate values were used to calculate the least squares fit. The slope of the resulting line times 100 was taken as an estimate of recovery and the y-intercept was taken as an estimate of the interference in the method.

Standard curves were run 10 times to determine the amount of data to obtain a reliable standard curve. After each run, all the data were combined and a new standard curve was calculated by the method of least squares. Although only five runs were found to be needed to obtain a curve that did not change, the standard curve data from all 10 runs were used for calculating the equation for the curve for the evaluation of the tissue method.

The results of the recovery of 3,5-DNBA added to the five tissues are summarized in Table I which gives the means and standard deviations for each drug level assayed. These data were used to calculate the least squares fit to the equation $y = a + bx$ for each tissue curve. The precision (s_b, s_a) of the calculated recovery and interference was estimated as described by Snedecor (1956) from the data used to construct the least squares lines. Percent recoveries were determined by multiplying the slope of the best fitting line by 100; the tissue

Table I. Recovery of 3,5-DNBA from Various Tissues

P.P.M. Added	P.P.M. Observed ± Standard Deviation				
	Muscle	Skin	Blood	Liver	Kidney
0.000	0.0008 ± 0.0006[a]	0.0058 ± 0.0052	0.0016 ± 0.0021	0.0028 ± 0.0038	0.0022 ± 0.0022
0.010	0.0088 ± 0.0010	0.0121 ± 0.0038	0.0097 ± 0.0030	0.0090 ± 0.0008	0.0114 ± 0.0014
0.020	0.0159 ± 0.0029	0.0189 ± 0.0033	0.0188 ± 0.0062	0.0166 ± 0.0028	0.0174 ± 0.0018
0.050	0.0357 ± 0.0054	0.0404 ± 0.0027	0.0392 ± 0.0027	0.0392 ± 0.0026	0.0371 ± 0.0006
0.100	0.0722 ± 0.0041	0.0715 ± 0.0089	0.0746 ± 0.0050	0.0738 ± 0.0039	0.0762 ± 0.0015

[a] Mean of 12 samples (triplicate samples of 4 different days).

Table II. Precision of Calculated Recovery of 3,5-DNBA and Its Interference of Various Tissues[a]

Tissue	Recovery ± s_b, %	Interference ± s_a (as 3,5-DNBA), P.P.M.
Muscle	71.2 ± 1.5	0.0015 ± 0.0008
Skin	66.2 ± 3.0	0.0059 ± 0.0015
Blood	72.3 ± 2.4	0.0028 ± 0.0012
Liver	72.6 ± 1.7	0.0023 ± 0.0009
Kidney	72.6 ± 1.2	0.0028 ± 0.0006

[a] Calculated from 20 determinations on each tissue.

Table III. Depletion of 3,5-Dinitrobenzamide[a] from Broilers

Tissue	Tissue Concentration (P.P.M.) Days after Withdrawal of Medication						
	0	1	2	3	4	5	6
Muscle	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Skin	0.0204	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Liver	0.0221	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Kidney	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Blood	0.0737	0.0383	0.0321	0.0150	<0.0116	<0.010	<0.010

[a] Supplied from 0.1% Unistat-3 in feed for 8 weeks.

interference, as 3,5-DNBA, is the y-intercept of the best fitting line (Table II).

The performance of the method was remarkably consistent between samples of liver, kidney, and blood where recoveries of 72.3–72.6% and an interference of 0.0023–0.0028 p.p.m. were observed. Both recovery and interference from muscle tissue were lower than the other tissues. This was expected since a lesser amount of substance is extracted from this fibrous tissue. The results for skin were not as good since recovery was 4 to 6% lower (66.2%) and the interference was about 0.003 p.p.m. higher than most other samples. The precision was also lower for skin than for the other samples. The higher interference, lower recovery, and lower precision on skin is probably related to high and varying fat content and the problem of preparing samples of uniform composition. However, the performance of the method is satisfactory for measuring all samples to levels of less than 0.010 p.p.m.

Residues. Broilers were treated with 0.1% Unistat-3 in the feed which provided 250 p.p.m. of 3,5-DNBA for eight weeks and then were placed on a nonmedicated ration. Groups of 50 birds were killed on the last day of medication and on daily intervals of a six-day withdrawal period to prepare pooled tissue samples for analysis. The results obtained from the analyses of these tissues are given in Table III. The highest level of 3,5-DNBA (0.0733 p.p.m.) was found in blood of birds on medication. The drug decreased to <0.010 p.p.m. in five days. The 3,5-DNBA in all other tissues ranged from <0.010 p.p.m. to 0.0221 p.p.m. on medication and decreased to <0.010 p.p.m. in less than 24 hours.

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