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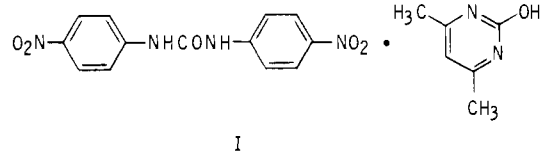
Modified Pulse Polarographic Determination of Nicarbazin in Chicken Tissue at the 0.1-Ppm Level

James S. Wood, Jr.,* and George V. Downing

The method described was devised to extend the assay of nicarbazin residues in chicken tissues by pulse polarography down to the 0.1-ppm level generally required by regulatory agencies. The 4,4′-dinitrocarbanilide portion of the complex is extracted with ethyl acetate. After removal of solvent, kidney and liver samples are cleaned up by a series of hexane washes of acetonitrile and acetonitrile/water solutions containing a small amount of dimethyl sulfoxide (Me_2SO), followed by extraction into methylene chloride. The methylene chloride is removed and pulse polarograms obtained on the residue dissolved in Me_2SO electrolyte after washing with hexane/toluene. For skin–fat and muscle, the acetonitrile/methylene chloride cleanup is unnecessary. The resulting polarograms were essentially clean for tissues from nonmedicated chickens, and recoveries of added drug at the 0.1–0.4-ppm level averaged 73% for liver (range 65–87), 76% for kidney (58–84), 85% for muscle (77–102), and 94% for skin–fat (79–106).

Nicarbazin (I) has been recognized as an effective coc-



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cidiostat for almost 25 years (Cuckler et al., 1956). The active component is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). Chickens dosed with nicarbazin have been shown to excrete the DNC portion of the complex more slowly than the HDP portion (Porter and Gilfillan, 1955) and consequently all residue analyses for nicarbazin are based on methods for the DNC moiety. The official FDA method for nicarbazin residues in chicken currently involves pulse polarography of DNC (Michielli and Downing, 1974).

The polarographic method involves reduction of the aromatic nitro groups. Dimethyl sulfoxide (Me_2SO) is used as the solvent largely due to solubility considerations, as nicarbazin and DNC are both extremely insoluble entities in most common solvents. In Me_2SO DNC yields two reduction peaks (potentials of ~−1.0 and −1.5 V vs. aqueous SCE). As protons are added to the solvent, the two waves merge until finally a single peak remains at about −0.98 V. The pulse polarographic system was designed to use an acid strength that generates a single wave.

The original polarographic method was estimated to have a lower limit of reliable measurement of about 2 ppm. In the United States a tolerance of 4 ppm has been established for nicarbazin and, hence, the method was satisfactory to determine compliance with FDA regulations. Recent experiments have demonstrated reliability at 1 ppm. At least for liver and kidney the method fails at levels much lower than that. For this study improvements

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in cleanup technique and other parameters have been made which extend the method to the 0.1-ppm level normally regarded as negligible for animal health drugs not known to be carcinogens.

EXPERIMENTAL SECTION

Apparatus. A PAR Model 170 electrochemistry system in the differential pulse mode and a mercury drop timer Model 172 (Princeton Applied Research) were used for the polarographic measurements. Instrumental settings were as follows: drop time, 2 s; pulse amplitude, 100 mV; current range, 5 μA; mercury drop rate, 1.31 mg/s. The cell, bridge, etc. were as described by Michielli.

Reagents. Solvents and reagents were as described by Michielli or distilled in glass (Burdick and Jackson Laboratories, Inc.). The Me_2SO electrolyte contained 2.30 g of tetraethylammonium perchlorate and 24 mg of benzoic acid per 100 mL presaturated with 400 mL of toluene/hexane (1:3 by volume).

Assay Procedure. Fifteen grams of tissue (muscle, liver, kidney, or skin–fat) was homogenized with 0.1 mL of Me_2SO and 30 mL of ethyl acetate in a Sorvall Omni-Mixer homogenizer chamber. The ethyl acetate was allowed to settle briefly and decanted into a 500-mL, round-bottomed flask through a glass wool plug in a funnel. The procedure was repeated four additional times, each with 30 mL of ethyl acetate only. The resulting solution was concentrated to a volume of about 2 mL on a rotary evaporator and transferred quantitatively by disposable pipet to a 15-mL, stoppered, graduated centrifuge tube with about 4 × 2 mL of ethyl acetate. The contents of the tube was blown to dryness with nitrogen. Skin–fat was evaporated in each instance to as small a volume as possible (6–8 mL). The evaporations were accomplished at ca. 70 °C. Two milliliters of acetonitrile was added in the case of liver and kidney samples and 2 mL of hexane to muscle and skin–fat samples, the solids were resuspended with ultrasound, and all were reevaporated. For liver and kidney samples, 1 mL of acetonitrile was added and the tube shaken 10 min. Enough hexane was added to make 6 mL total volume and the tube was shaken gently and carefully for 1 min by hand and centrifuged for 5 min. The lower phase was moved to a second 15-mL tube and a second extraction performed on the hexane with 1 mL of acetonitrile. The combined acetonitrile phases were extracted three more times with 6 mL each of hexane and the hexane removed by aspiration after each wash. Distilled water was added to the 6-mL mark and the mixture washed with 3 mL of hexane as before. *No* interfacial material was removed in this wash. Six milliliters of methylene chloride was added, and the tube shaken for 10 min and centrifuged for 5 min. The lower phase was moved to a new tube by disposable pipet and the extraction repeated twice more with 6 mL each of methylene chloride. The combined methylene chloride layers were blown to dryness with nitrogen at 40 °C. For all tissues exactly 2 mL of Me_2SO electrolyte phase was added and the stoppered tube shaken for 10 min. Enough toluene/hexane phase was added to make 10 mL (13 for skin–fat), the tube shaken for 1 min and centrifuged for 5 min, and the upper phase removed by aspiration. The toluene/hexane wash was repeated and the lower phase transferred to the polarographic cell. The solution was deaerated for 5 min with high-purity nitrogen and a pulse polarogram recorded between −0.6 V and −1.2 V vs. SCE. The peak height was measured (Figure 1) and the amount of nicarbazin determined from a standard curve.

Standard Curve. A standard curve was obtained daily by diluting 0.1-, 0.2-, and 0.4-mL aliquots of a nicarbazin standard solution containing 1.5 μg/0.1 mL to 2 mL with the Me_2SO electrolyte bottom phase and recording the polarograms. These aliquots are equivalent to 0.1, 0.2, and 0.4 ppm nicarbazin. Response to a standard amount of nicarbazin varied somewhat from day to day, with a peak height of 0.61 ± 0.02 μamp (or 12.2 divisions) for each 0.75 μg/mL of standard (or each 0.1 ppm) being typical on an average day. As standard peaks were highly reproducible for a particular day, only single samplings for each point were generally used. For samples above 0.4 ppm, standard curves using higher current ranges were constructed. The standard curve was linear at least up to 60 μg/mL. If assays were desired in terms of DNC instead of nicarbazin, the results were multiplied by the molecular weight factor 0.71.

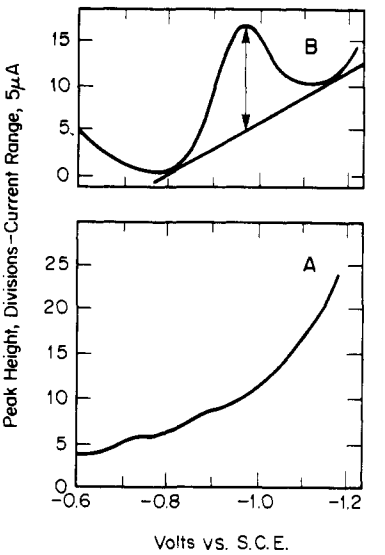

Figure 1. Differential pulse polarograms (no tissue): (A) reagent blank, (B) standard, 0.1 ppm.

Table I. Standard Series of Polarograms for Nicarbazin

ppm nicarbazin	peak height	
	divisions	μamp
0.108	13.2	0.66
0.216	26.4	1.32
0.432	52.0	2.60

Recovery Experiments. Tissues from nonmedicated chickens were fortified with nicarbazin at levels of 0.0–0.4 ppm. All 22 unfortified samples tested gave no peak indicative of nicarbazin. At 0.1–0.4-ppm fortification levels, the average recovery for muscle was 85% (nine samples), for liver 73% (nine samples), for kidney 76% (six samples), and for skin–fat 94% (nine samples).

Standard samples in the absence of tissue gave discernible peaks down to the 0.005-ppm level. Peaks were observed at the 0.01–0.02-ppm level for liver tissue samples fortified with nicarbazin and carried through the recovery scheme, but the data could not be quantified. The limit of detection of the method is 0.02 ppm since this is the lowest level that reproducibly gives a peak in the presence of tissue.

Discussion of the Method. Development of a satisfactory modification of the pulse polarographic method for nicarbazin, particularly for liver, at the 0.1-ppm level proved to be a more difficult task than originally envisaged. A variety of adjustments in sample size, Me_2SO volumes, and instrumental parameters easily gave the desired sensitivity on pure DNC. In all cases the same adjustments made to the assay of liver led to interfering peaks and a shift in the DNC wave to less negative voltages (Figure 3).

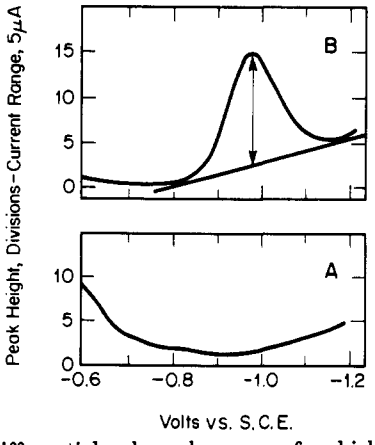

Figure 2. Differential pulse polarograms for chicken liver: (A) control liver, (B) control plus 0.1 ppm spike of nicarbazin.

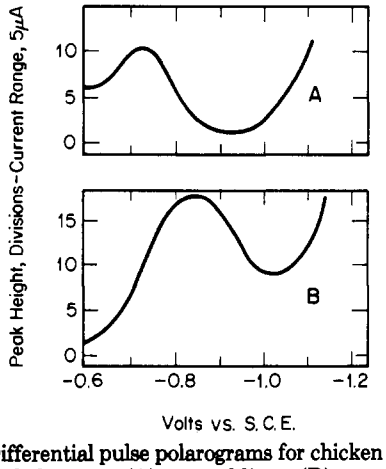

Figure 3. Differential pulse polarograms for chicken liver without the additional cleanup: (A) control liver, (B) control liver plus 0.1 ppm spike of nicarbazin.

Obviously additional cleanup was called for. The poor solubility characteristics of DNC severely limited the choice of solvent–solvent systems, and the presence of tissue changed markedly the distributions actually observed. The latter effect presumably results from the tendency of DNC to complex a wide variety of substances. The method that was finally achieved relies on complexation with Me_2SO and acetonitrile to swamp out tissue complexation until the greater part of the tissue components are removed and the problem alleviated.

Five ethyl acetate extractions were shown to be necessary for complete drug removal from the tissue compared to the three previously reported. The additional extractions may be necessary at the lower drug levels reported or they may be needed because of differences in the geometry of the homogenization vessels.

The recoveries reported are adequate for a sensitive tissue residue assay. Nevertheless, additional experimental data that were available indicated that the actual average recovery was higher than the observed polarographic values. Distributions measured by ultraviolet absorption in the absence of tissue indicated that a nearly quantitative result should have been accomplished. Several such UV measurements gave recoveries ranging from 95 to 99%. Radioactive ^{14}C-labeled DNC containing nicarbazin fortified into liver at the 0.1-ppm level gave >90% recovery by radioactive measurement. The losses observed, then, are apparently largely not in the extractions nor are they mechanical. Most of the loss must be due to matrix effects on the polarography. One such effect involves suppression of the polarographic signal by adsorption of the tissue components on the electrode (Jacobsen and Lindseth, 1976). Another involves simply the increased curvature of tissue polarographic curves compared to curves without tissue (see Figures 1 and 2). Several schemes were applied in an attempt to correct for this curvature. Such corrections generally yielded higher recoveries, but the corrected answers were so erratic that the corrections were not justified.

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Supplementary Material Available: Recovery data for nicarbazin in chicken tissues (1 page). Ordering information is given on any current masthead page.

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