Determination of Ronidazole in Swine Tissues by Differential Pulse Polarography

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Thin-layer chromatography and differential pulse polarography have been combined to provide specificity and extreme sensitivity in analyzing swine tissues for trace levels of a new drug for treatment of dysentery, ronidazole. A sensitivity of 2 ppb (2 ng/g) and a limit of detection of 0.2 ppb were achieved with no interferences from tissue components. Individual results are reproducible within ±6%. Recoveries of spikes at levels of 2, 4, and 6 ppb average 68% for liver, kidney, and fat, and 89% for muscle.

With increasing public and government awareness of the problem of drug residues in edible animal tissues, the requirements for an acceptable tissue assay have become more stringent, and the analyst is therefore forced to push every technique for its maximum sensitivity and reproducibility. An acceptable assay must meet the following criteria: (1) desired sensitivity, (2) specificity, (3) a detector response from nonmedicated tissues sufficiently low to ensure no false positives, and (4) reproducible recoveries of ±10% from spiked tissues.

Ronidazole [1-methyl-5-nitroimidazol-2-yl methylcarbamate] (Figure 1) has been effective in experimental treatment of dysentery in swine. In order to study the tissue distribution of ronidazole in the pig, it was necessary to develop a sensitive, specific, and reliable chemical assay for the parent drug and any drug-related metabolites. Metabolism studies using radioactive tracer have shown that ronidazole undergoes rapid and complete biodegradation in the animal (Rosenblum et al., 1972). Virtually all of the radioactivity found in the animal tissues is accounted for as simple compounds normally present. The only nitroimidazoles found in any tissue are ronidazole and 1-methyl-2-hydroxymethyl-5-nitroimidazole (Figure 1), and this metabolite concentration is always less than 10% of the parent drug concentration. For this reason an assay was developed for the parent drug itself.

For a given compound the sensitivity required for a tissue assay is set by the toxicological studies. The term "sensitivity" (2 ppb or 2 ng/g in this case) as used in this work is not synonymous with "limit of detection". It is necessary to run reproducible recoveries at the "sensitivity" of the assay. The "limit of detection" (0.2–0.4 ppb) is 5–10 times lower than the "sensitivity". In residue samples with less than 2 ppb but more than 0.2–0.4 ppb, a residue could be detected but not adequately quantitated. Nonmedicated tissue samples show a response of less than 0.2 ppb, one-tenth the stated "sensitivity".

A good tissue assay must also be specific for the compounds of interest. Generally, this means that some type of chromatography—gas, liquid, or thin layer—must be used either as the method of detection or as the final separation method before a less specific detection system such as polarography or spectroscopy.

A number of methods for the determination of nitroimidazoles have been published. These include colorimetric methods based on reduction of the nitro group to the corresponding amine, followed by diazotization and coupling reactions to form colored products (Bratton et al., 1939; Stambaugh and Manthei, 1967); polarographic methods based on the electrolytic reduction of the nitro group (Cooper and Hoodless, 1967; Allen and McLoughlin, 1972; Laviron, 1963); an absorptiometric method (Lau et al., 1969); and gas–liquid chromatography (VandenHeuvel, 1971; MacDonald et al., 1971). Only thin-layer chromatography coupled with either gas chromatography using electron capture detection or differential pulse polarography has been successful in providing the necessary sensitivity and specificity for determination of ronidazole at parts per billion levels. The polarographic assay has the advantage of measuring ronidazole directly without the time consuming derivatization of the drug necessary for gas chromatography.

Table I. Thin-Layer and Electrochemical Properties of Ronidazole and Other Structurally Similar Nitroimidazoles

Compound	Structure	R_f[a]	E_{max} vs. SCE[a]
Ronidazole	R = $CH_2OC(=O)NH_2$	0.41	−0.51
Dimetridazole	R = CH_3	0.40	−0.57
Ipronidazole	R = $CH(CH_3)_2$	0.61	−0.55
1-Methyl-2-hydroxymethyl-5-nitroimidazole	R = CH_2OH	0.31	−0.52

[a] The TLC and polarographic systems were the same as described in the procedure section.

Differential pulse polarography (Parry and Osteryoung, 1965; Flato, 1972; Bond and Canterford, 1972) is a modern electroanalytical technique which has recently been used for the determination of pharmaceuticals in biological fluids (Brooks et al., 1973; deSilva and Hackman, 1972) and in tissues (Michielli and Downing, 1974). Recently, a pulse polarographic assay for dimetridazole was published (Craine et al., 1974). The procedure described here is four times more sensitive since only 25 g of tissue is required. It also has higher recoveries and lower blanks, and an internal standard is not necessary since there is no suppression of the peak response due to the presence of tissue components.

The procedure described here is for the recovery and polarographic analysis of ronidazole from swine muscle, liver, kidney, and fat with a sensitivity of 2 ppb and a limit of detection of 0.2–0.4 ppb. This method is one of the most sensitive of its type ever developed. Recoveries ranged from 89 ± 5% for muscle to 68 ± 6% for kidney, liver, and fat. The procedure is specific for ronidazole because of (a) the choice of the initial extracting solvent, (b) the TLC step, and (c) the reduction potential (E_{max}) which is not the same for nitroimidazoles with different substituents. The thin-layer and electrochemical characteristics of

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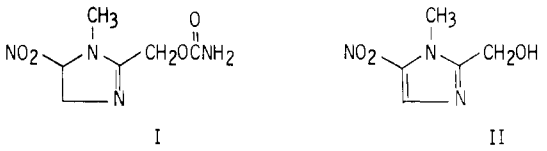

I II

Figure 1. Structure of ronidazole (I) and the only drug related metabolite (II).

ronidazole and other similar nitroimidazoles are presented in Table I. The metabolite, 1-methyl-2-hydroxymethyl-5-nitroimidazole, is poorly extracted in the initial step and is completely separated from ronidazole in the TLC step so that it does not interfere with the assay for ronidazole.

EXPERIMENTAL SECTION

Reagents. All reagents except ethyl acetate were of analytical grade purity and were used without further purification. The ethyl acetate used was doubly glass distilled from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. All aqueous solutions except the pH 11 buffer were prepared with water which had been doubly distilled. The doubly distilled water was redistilled from an acidified permanganate solution in an all glass apparatus to prepare the water used in the pH 11 buffer.

A standard solution of ronidazole was prepared by dissolving 5 mg of a reference sample of ronidazole in 100 ml of 0.1 M hydrochloric acid. One milliliter of this solution was diluted to 10 ml with doubly distilled water to give a solution containing 5 ng/μl. Suitable aliquots of this solution were added to pH 11 buffer with a microsyringe, and polarograms were recorded to obtain a standard curve of ronidazole concentration vs. polarographic response.

A ronidazole marker solution was prepared by dissolving 50.0 mg of a reference sample in 10 ml of methanol to give a stock solution containing 5 μg/μl. One microliter of this solution was used as a marker spot on TLC plates. The solution was further diluted with methanol to prepare solutions for recovery experiments.

Thin-Layer Chromatography Plates. Particular attention was paid to the preparation of the TLC plates since their condition is the principal determinant of the ultimate sensitivity of the method. The plates (Analtech, silica gel GF 5 × 20 cm, 250 μm thickness) as received always contain polarographically reducible impurities which must be removed by washing (developing) once with acetonitrile–water (1:1) and twice with acetonitrile in solvent-saturated containers. The plates were dried thoroughly between washings. After washing, the plates were stored in a convection oven (40–45 °C) to prevent the water content of the silica from varying with relative humidity changes in the laboratory. The plates were washed on the same day that they were to be used in assays.

Apparatus. A Princeton Applied Research Model 170 Electrochemistry System equipped with a P.A.R. Model 172 Drop Timer was used for analysis. A special small volume polarographic cell was required to permit the use of small sample volumes, generally 0.6–0.9 ml. A standard dropping mercury electrode (DME) was used as the indicating electrode. A saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode were employed. The cell and electrodes were constructed as shown in Figure 2. The instrumental parameters were: pulse amplitude, 100 mV; current sensitivity, 2 μA; scan rate, 1 mV/s; drop time, 5 s.

Technique. The sensitivity of this assay requires that careful and consistent techniques be applied to all its phases. Sources of contamination such as dirty glassware, benchtops, or apparatus were avoided. All glassware was washed by hand with soap and water, rinsed with water, distilled water, and methanol, and then was oven dried. It was even necessary to rinse the disposable pipets with distilled water and methanol and to dry them in an oven before use. The homogenizing mill and all pipets were rinsed once with the solvent or solution for which they were intended before actual use. Consistency, without which trouble-shooting was virtually impossible, was the only way to track down and identify as the source of poor assay results, a bad batch of TLC plates, a contaminated buffer solution or other reagent, or a simple human error in procedure.

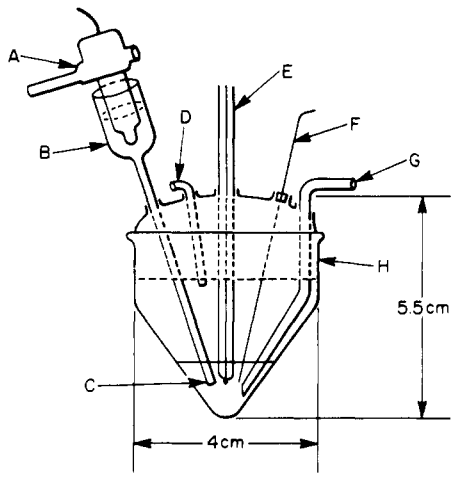

Figure 2. Small volume polarographic cell: (A) Beckman fiber junction aqueous saturated calomel electrode; (B) salt bridge containing pH 11 buffer; (C) fiber junction; (D) glass tube for nitrogen blanket over solution; (E) dropping mercury electrode; (F) platinum auxiliary electrode; (G) fine tipped glass tube for deaeration with nitrogen; (H) ground glass joint.

Analytical Procedure. The detailed procedure devised for isolation of the drug in a form suitable for polarographic analysis is presented below. The main steps are summarized as follows: (1) extraction of the tissue with ethyl acetate; (2) back extraction into acid; (3) benzene and petroleum ether washes of the acid phase; (4) adjustment to pH 10 and extraction into ethyl acetate; (5) evaporation of the extract almost to dryness; (6) spotting of the extract onto a TLC plate and development in acetonitrile; (7) extraction of the drug from the silica gel TLC plate into pH 11 buffer; (8) back extraction into ethyl acetate and evaporation to dryness; (9) dissolution of the residues in pH 11 buffer; (10) polarographic analysis.

Twenty-five grams of tissue was homogenized with 30 ml of ethyl acetate in a Virtis homogenizing flask. The ethyl acetate was decanted into a 250-ml round-bottomed flask, and the procedure was repeated two additional times. The combined extracts were evaporated almost to dryness on a rotary evaporator using a water bath of ca. 50 °C around the flask. The sample was quantitatively transferred to a 15-ml centrifuge tube with a disposable pipet and evaporated to less than 0.5 ml under a stream of nitrogen in a warm water bath. Five milliliters of 1 M hydrochloric acid was added to the sample, and the acid phase was then washed twice with 5 ml of petroleum ether, twice with 5 ml of benzene, and twice more with 5 ml of petroleum ether. Each time the organic phase was discarded by aspiration. The sample was transferred to a 125-ml separatory funnel and made basic by the addition of 5 ml of 1 M sodium hydroxide and buffered by the addition of 2 ml of 2 M pH 10 carbonate buffer. The drug

was then back extracted with 2 × 15 ml of ethyl acetate which was subsequently evaporated almost to dryness in a 250-ml round-bottomed flask on a rotary evaporator. The sample was quantitatively transferred to a 15-ml centrifuge tube and evaporated to less than 0.1 ml under a stream of nitrogen in a warm water bath.

The extract was streaked on a 5 × 20 cm prewashed silica gel GF TLC plate, and the plate was developed in an acetonitrile-saturated developing tank. Since the R_f of ronidazole is affected by the moisture content within the developing tank and by differences between individual TLC plates, it was necessary to mark each plate with a spot of ronidazole which can be seen under ultraviolet light (254 nm). The marker spot was placed at the origin close to one edge of the TLC plate and away from the sample streak before the plate was developed. After development, an area on the sample side of the TLC plate adjacent to the marker spot was scraped from the plate and slurried with 1 ml of pH 11 sodium borate–sodium carbonate buffer. The sample was centrifuged, the supernatant transferred to a 15-ml centrifuge tube, and the volume of the supernatant was noted. The drug was extracted from the buffer into 3 × 1 ml of ethyl acetate and the ethyl acetate was evaporated to dryness under a stream of nitrogen in a warm water bath. The residue was dissolved in the same volume of pH 11 buffer that was obtained from the sample after centrifugation in the previous step. The sample was transferred to the polarographic cell and deoxygenated for 5 min with nitrogen, and a polarogram was obtained by scanning from –0.300 V (vs. SCE) to –0.700 V (vs. SCE) using the differential pulse mode of operation.

Because of the nature of fat samples, they had to be handled differently in the initial extractions and clean-up procedure. Twenty-five grams of fat was homogenized with 30 ml of petroleum benzin in a 250-ml homogenizing flask. The petroleum benzin was decanted into a 50-ml centrifuge tube and centrifuged. The supernatant was discarded, and any solid material returned to the homogenizing flask. No ronidazole was lost in this pre-extraction step, but it removed a good portion of the fat which was difficult to carry through the assay procedure. The fat sample was then homogenized with ethyl acetate as described in the general procedure. The ethyl acetate from a fat sample could only be evaporated down to 10 to 15 ml rather than 0.5 ml as for other tissues. The fat extract was transferred to a 50-ml centrifuge tube and extracted three times with 3 ml of 1 M hydrochloric acid. The acid extracts were combined in a 15-ml centrifuge tube, washed as described in the assay procedure, made basic with 9 ml of 1 M sodium hydroxide and 2 ml of pH 10 carbonate buffer, and then carried through the rest of the assay as described.

Calculations. The quantitation of ronidazole is based on the direct proportionality of the diffusion current produced vs. the concentration of the drug in solution. A standard curve was obtained by recording polarograms of standard solutions containing 50 ng/ml (2 ppb based on a 25-g sample size), 100 ng/ml (4 ppb), and 150 ng/ml (6 ppb). The standard curve was plotted as peak height in millimeters vs. concentration in parts per billion. Fifty nanograms of ronidazole (2 ppb) gave a peak height of 20 mm (0.16 μA) under the conditions given in the Experimental Section.

The concentration of ronidazole in a sample solution was determined by measuring the peak height at a potential of –0.50 V (vs. SCE) as shown in Figure 3. The concentration was then read directly from the standard curve.

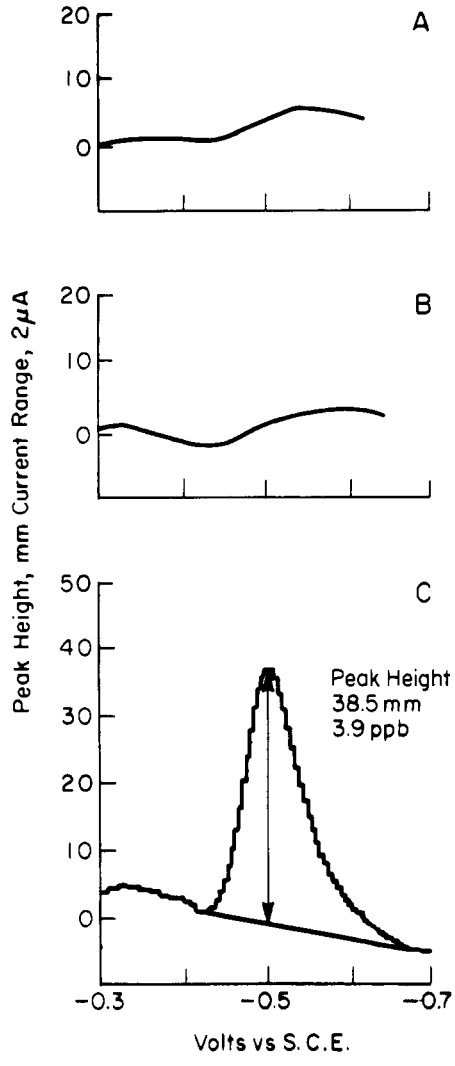

Figure 3. Differential pulse polarograms of nonmedicated and ronidazole medicated swine tissues: (A) reagent blank; (B) nonmedicated muscle; (C) on-drug kidney.

When the concentration of ronidazole in a sample was greater than the highest point on the curve, the sample was diluted with pH 11 buffer so that it would fall in the curve and the polarogram was rerecorded. If no peak was seen at the correct peak potential for ronidazole, the sample contained less than 10 ng (0.4 ppb) and had no detectable residue.

RESULTS AND DISCUSSION

Nonmedicated swine muscle, fat, liver, and kidney were spiked with 0.1–0.2-ml aliquots of methanolic ronidazole solutions for recovery studies at 2-, 4-, and 6-ppb levels. Each tissue was assayed six times at each level. Average fat, liver, and kidney recoveries were 68% with a standard deviation of 6%. Muscle recoveries averaged 89% with a standard deviation of 5%. Recovery data are presented in Table II. Typical polarograms for reagent blank, medicated, and nonmedicated tissues are shown in Figure 3. Tissue blanks were indistinguishable from reagent blanks and had no detectable residue since there was no peak at the peak potential of ronidazole.

Tissue Residue Study. A residue experiment was carried out with pigs medicated with ronidazole through the drinking water. This study consisted of five groups of pigs (two gilts and one barrow per pen). One group served to furnish nonmedicated control tissue, and the remaining four groups received 0.012% ronidazole in the

Table II. Recovery Data and Nonmedicated Swine Tissue Assays

Samples	Recovery	
	ppb	%
Muscle		
Nonmedicated control	ndr,[a] ndr, ndr	
50 ng of spike (2 ppb)	1.9, 1.7, 1.7, 1.8, 1.6, 1.8	95, 86, 86, 91, 82, 91
100 ng of spike (4 ppb)	3.4, 3.8, 3.6, 3.3, 3.3, 4.0	86, 95, 91, 82, 82, 100
150 ng of spike (6 ppb)	5.5, 5.1, 5.0, 5.2, 5.3, 5.7	91, 85, 83, 87, 89, 95
Fat		
Nonmedicated control	ndr, ndr, ndr	
50 ng of spike (2 ppb)	1.3, 1.3, 1.3, 1.5, 1.3, 1.3	68, 68, 64, 77, 68, 64
100 ng of spike (4 ppb)	2.8, 2.8, 2.4, 2.6, 2.6, 2.4	71, 71, 62, 66, 66, 62
150 ng of spike (6 ppb)	3.7, 4.4, 4.2, 3.7, 3.8, 4.1	62, 74, 70, 62, 64, 68
Liver		
Nonmedicated control	ndr, ndr, ndr	
50 ng of spike (2 ppb)	1.4, 1.3, 1.4, 1.3, 1.4, 1.6	73, 68, 73, 68, 73, 82
100 ng of spike (4 ppb)	2.4, 2.9, 2.4, 3.1, 2.8, 3.1	62, 73, 62, 77, 71, 77
150 ng of spike (6 ppb)	3.7, 4.1, 4.4, 3.7, 4.3, 4.0	62, 68, 73, 62, 71, 67
Kidney		
Nonmedicated control	ndr, ndr, ndr	
50 ng of spike (2 ppb)	1.3, 1.3, 1.3, 1.6, 1.4, 1.5	68, 68, 68, 82, 73, 77
100 ng of spike (4 ppb)	2.4, 3.1, 3.2, 2.4, 2.8, 2.6	62, 77, 82, 62, 71, 66
150 ng of spike (6 ppb)	4.9, 4.2, 3.7, 3.7, 3.7, 4.3	82, 70, 62, 62, 62, 71

[a] ndr, no detectable residue, i.e., no peak was observed at the peak potential for ronidazole.

Table III. Polarographic Assay Results from Nonmedicated Swine and Swine Fed 0.012% Ronidazole in the Water

Pig no.	ppb			
	Muscle	Liver	Kidney	Fat
Nonmedicated				
12-2M	ndr[a]	ndr	ndr	ndr
24-8F	ndr	ndr	ndr	ndr
36-4F	ndr	ndr	ndr	ndr
On drug				
12-3M	4930	ndr	16	51
33-8F	2400	ndr	21	72
30-12F	1700	ndr	4	50
1 day off				
25-4M	58	ndr	ndr	ndr
30-10F	138	ndr	ndr	ndr
31-9F	44	ndr	ndr	ndr
3 days off				
33-12F	ndr	ndr	ndr	ndr
32-2M	ndr	ndr	ndr	ndr
30-11F	ndr	ndr	ndr	ndr
5 days off				
35-4M	ndr	ndr	ndr	ndr
34-14F	ndr	ndr	ndr	ndr
33-7F	ndr	ndr	ndr	ndr

[a] ndr, no detectable residue, i.e., no peak was observed at the peak potential for ronidazole.

drinking water for 7 days. The average amount of drug taken was 15 mg/kg of body weight per day. The nonmedicated control group was sacrificed immediately prior to the start of the medication period. One group of medicated pigs was sacrificed at each of 0, 1, 3, and 5 days off drug, and their tissues were assayed. The results are summarized in Table III.

Residues were found in on-drug muscle (1.7–4.9 ppm), kidney (4–21 ppb), and fat (50–72 ppb). At one day off drug only muscle had a residue (44–138 ppb). No drug was found in any tissue that had been withdrawn for 3 or 5 days. Control, 3 days off, and 5 days off tissues were indistinguishable from a reagent blank.

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