Residue Analysis of Ipronidazole and Its Metabolite at the 2 ppb Level in Turkey Tissue

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A method for the analysis of ipronidazole (1-methyl-2-isopropyl-5-nitroimidazole) and its major metabolite from turkey tissue at the 2 part per billion level is described. The initial tissue extraction with benzene is followed by silica gel column cleanup and concentration. The column eluate is divided for individual extraction procedures followed by a gas chromatographic assay using electron capture detection specific for each compound. The following factors affecting the assay are discussed: form of the initial tissue sample, vapor pressure, extraction characteristics, chemical stability, and light stability of the two compounds. The assay methodology was evaluated using turkey muscle, skin and fat, kidney, liver, and blood with average recoveries of 75% for ipronidazole and its metabolite. Tissue clearance data show that turkeys fed a 0.00625% ipronidazole ration for 12 weeks are free of the drug and its metabolite 4 days after cessation of treatment.

Ipronidazole (1-methyl-2-isopropyl-5-nitroimidazole) is a safe (Marusich *et al.*, 1970) and effective (Mitrovic and Schildknecht, 1970) histomonastat for turkeys that is metabolized to 1-α,α-trimethyl-5-nitroimidazole-2-methanol (Fellig *et al.*, 1969). It is the active compound in Ipropran medicated premix for use in feeds for control of histomoniasis in turkeys. The chemical structures are shown in Figure 1.

Methodology for determining ipronidazole in feed has been reported using gas chromatography, spectrophotometry (Osadca *et al.*, 1970), and automated analysis utilizing alkaline hydrolysis and the Bratton–Marshall reaction (MacDonald *et al.*, 1970). Ipronidazole has been determined in poultry tissue at the 0.05-ppm level using pulse polarography and gas chromatography (MacDonald *et al.*, 1969).

This paper describes a procedure for the recovery and analysis of ipronidazole and its metabolite 1-α,α-trimethyl-5-nitroimidazole-2-methanol from turkey tissue with a lower limit of 2 ppb using a 100-g sample.

DEVELOPMENT OF METHOD

The earlier work on assay methodology for ipronidazole at the 0.05-ppm level in tissue utilized both pulse polarography and gas chromatography (MacDonald *et al.*, 1969). In the development of methodology at the 2-ppb level, gas chromatography was used exclusively because of its specificity and its suitability for the small final extract volume. The following factors directly influence the utility of the method: form of the initial tissue sample; sensitivity of the electron-capture detector to both compounds; vapor pressure of both compounds; extraction characteristics of both compounds; stability of both compounds to hydrolysis; and effect of light on both compounds.

a. Tissue Sample. The assay for both ipronidazole and its metabolite should be done on the same physical sample with the initial extraction step being the same to provide positive correlation between the two compound levels. Because of the extreme sensitivity of the method, the tissue used for method validation (*i.e.*, control tissue) must be collected in an ipronidazole-free environment from birds raised in an ipronidazole-free environment. Sample preparation (tissue grinding) in the laboratory must also be carefully monitored to prevent any introduction of ipronidazole or its metabolite into the sample.

b. Electron-Capture Detector Sensitivity. The sensitivity of the electron-capture detector (Ni^{63} Microtek) was established for ipronidazole as 1.0 ng and its metabolite as 0.5 ng for a 15–20% of scale response. This response for a 10 μl injection would mean a minimum concentration of 0.5×10^{-7} g/ml for the metabolite final extract and 1×10^{-7} g/ml for the ipronidazole final extract. These values dictate a minimum of 50 g of tissue for each compound or a total of 100 g of tissue for both if the criteria noted for tissue, the assay level of 2 ppb, and a final extract volume of 2 ml (metabolite) and 1 ml (ipronidazole) for each are to be met.

c. Extraction Characteristics. The extraction of both compounds into benzene is most efficient from aqueous solutions with pH's greater than 8, but due to the base hydrolysis noted, the pH of all aqueous solutions to be extracted was maintained at 8. At this pH an extraction efficiency greater than 98% could be maintained if the following maximum volume ratios (aqueous to benzene) were not exceeded: ipronidazole (5 to 1) and metabolite (1 to 1).

The extraction of both compounds from benzene into aqueous is greater than 98% with 3 *N* HCl if the following maximum volume ratios (benzene to aqueous) are not exceeded: ipronidazole (4 to 1) and metabolite (1 to 0.7).

The above information indicates that both compounds can be extracted and the extraction used as part of a cleanup, but only with ipronidazole can a concentration be effected with the extraction step.

d. Vapor Pressures. The evaporation of benzene from a benzene solution of either compound results in loss of compound due to their high vapor pressures. The vapor pressure of ipronidazole is high enough to result in losses of 25 to 100% of compound when a benzene solution is evaporated to dryness. Losses from evaporation of ethyl ether solutions are less, but still can reach 50% if allowed to go to dryness. The vapor pressure of the metabolite is much lower, with losses from 5 to 25% on evaporation of a benzene solution to dryness. Loss of metabolite on evaporation of an ethyl ether solution to dryness is from 0 to 10%.

The vapor pressure of ipronidazole dictates that evaporation of benzene or ethyl ether from its solution would not be a

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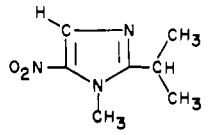
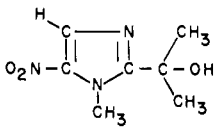


IPRONIDAZOLE

1-methyl-2-isopropyl-5-nitroimidazole



1-α,α-trimethyl-5-nitroimidiazole-2-methanol

Figure 1. Ipronidazole and metabolite structures

satisfactory method of concentration for ipronidazole extracts.

Evaporation of ethyl ether from solutions of the metabolite would be a satisfactory method of concentration provided the solution did not go to dryness. The addition of a small volume of benzene to the ethyl ether solution, followed by evaporation of the ethyl ether until only the added benzene remains, is a statisfactory method of concentration of the metabolite with negligible losses.

e. Stability to Hydrolysis. The two compounds are stable in acid but unstable in base. Base hydrolysis with evolution of nitrite has been reported for ipronidazole (Lau *et al.*, 1969) and is also a valid reaction for the metabolite. The effect of this hydrolysis during the extraction with benzene is minimized by keeping the pH of the aqueous solutions at 8 and by limiting the contact time. The compounds should not be allowed to stand in the pH 8 solution or in contact with the pH 8 aqueous layer for prolonged periods of time.

f. Light Stability. The effect of light on solutions of both of these compounds is very noticeable at the levels involved for part per billion assays. This effect is illustrated in Figure 2 where 2×10^{-7} g/ml of benzene solutions in 100 ml of clear pyrex volumetric flasks were allowed to stand in bright sunlight and the solutions were analyzed *via* gas chromatography on an hourly basis for a 7–8 hr period. The plot shows 80% or greater decomposition of both compounds after 7 hr of bright sunlight. The same solutions were stored in amber flasks under identical conditions with no change in the original starting concentration.

The effect of light on the low concentrations in solution of both compounds involved dictates that amber glassware is necessary throughout the procedure and the exposure to light of any solutions of either compound must be avoided.

The 100-g tissue sample used for assay of both compounds can be satisfactorily extracted for both compounds (Figure 3) with 300 ml of glass-distilled benzene after addition of borax and salt to the tissue. The volume of benzene extract that is used for the assay of all tissues is 250 ml.

The concentration of this 250 ml of benzene extract into a small volume was accomplished by means of a silica gel column. The 250-ml benzene extract was passed through a short (3–4 cm) column of activated silica gel which will retain both ipronidazole and its metabolite. The column is then stripped with 30 ml of water-saturated ethyl ether which will

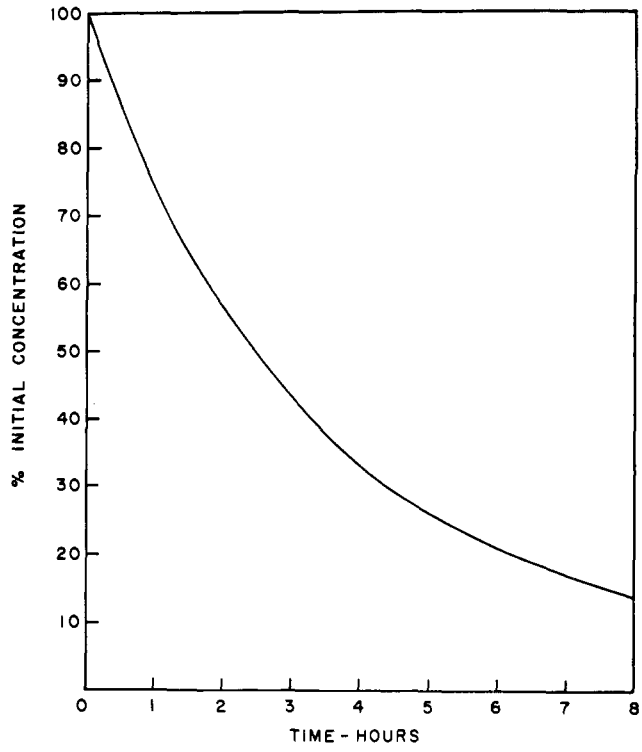


Figure 2. Photodecomposition of ipronidazole and 1-α,α-trimethyl-5-nitroimidazole-2-methanol in bright sunlight (initial concentration = 2×10^{-7} g/ml in benzene)

remove both compounds. The two compounds can be separated on a silica gel or florisil column but longer columns and larger volumes of an elution solvent series are necessary, thereby defeating the desired concentration step.

The ethyl ether stripping eluent is then divided into equal portions for separate ipronidazole and metabolite assays. The portion used for ipronidazole utilizes this compound's desirable extraction characteristics and is extracted with 3 ml of acid. The acid portion is washed with 2×2.0 ml of benzene and the pH of the aqueous layer is adjusted to 8 and extracted with 1 ml of benzene. The benzene layer is used for the gas chromatographic analysis of ipronidazole.

The portion of the ethyl ether eluent used for the metabolite utilizes the favorable evaporation of ethyl ether under a stream of nitrogen after the addition of 2 ml of benzene. The 2 ml of benzene solution left after the evaporation of the ethyl ether is extracted with 1.4 ml of acid and the aqueous layer is washed with 2×2.0 ml of benzene. The pH of the aqueous layer is adjusted to 8 and extracted with 2 ml of benzene. The benzene layer is used for the gas chromatographic analysis of the metabolite.

The gas chromatographic conditions and columns were tailored for the individual final extracts to be run on separate instruments.

APPARATUS

Chromatograph. Micro-Tek MT 220 equipped with Nickel-63 electron-capture ionization detector and 10-in. strip chart recorder (Micro-Tek, Austin, Texas).

Tissue Grinders and Homogenizer. Hobart Kitchen Aid Model 5A, Intedge Model C-2 and Virtis Homogenizer Model 45 with 500-ml amber flasks.

Chromatographic Column. Fischer & Porter amber 9 mm i.d. × 150-mm long Teflon stopcock, Teflon seal, and sintered glass disc with a 500-ml amber reservoir.

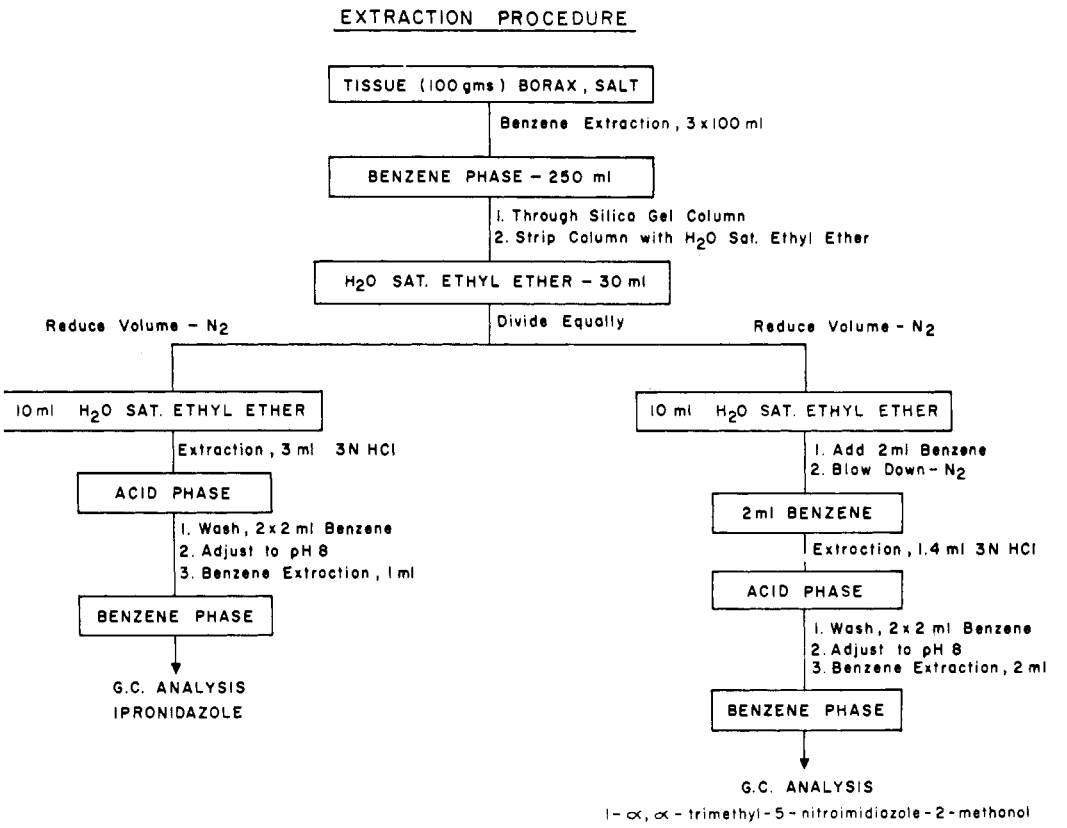


Figure 3. Extraction procedure

Glassware. All glassware used must be amber and cleaned as follows. Wash glassware with detergent (Alkonox) and rinse with water, distilled water, and acetone. Prior to use, rinse with ether followed by benzene and drain thoroughly.

REAGENTS

All chemicals used are reagent grade unless otherwise noted. The anhydrous ethyl ether (Mallinckrodt, AR, 1-lb can) should be opened fresh on a daily basis and the benzene should be nanograde glass-distilled quality (Burdick and Jackson). The 3 *N* HCl and 6 *N* NaOH solutions should be washed three times with an approximately equal volume of benzene. Check purity by injecting 10 μl of the third benzene wash onto glc column for 1-α,α-trimethyl-5-nitroimidazole-2-methanol. If necessary, repeat benzene wash until interfering peaks are no longer detected.

PREPARATION OF STANDARDS

The standards for both ipronidazole and 1-α,α-trimethyl-5-nitroimidazole-2-methanol are prepared so as to be equivalent to 2 and 4 ppb levels of the compounds from 100-g tissue samples. Amber glassware must be used. The stock solutions may be kept for up to 1 week in the refrigerator.

Ipronidazole. Weigh exactly 10 mg of analytical standard and dissolve in 100 ml of methyl alcohol (Solution 1). A 1.0-ml aliquot of Solution 1 is diluted to 100 ml with glass-distilled benzene (Solution 2). A 5-ml aliquot of Solution 2 is diluted to 50 ml with glass-distilled benzene (Solution 3, 1×10^{-7} g/ml) and a 10 μl sample is injected in the gas chromatograph for 2 ppb. A 5-ml aliquot of Solution 2 is diluted to 25 ml with glass distilled benzene (Solution 4, 2×10^{-7} g/ml) and a 10 μl sample is injected in the gas chromatograph for 4 ppb.

1-α,α-Trimethyl-5-nitroimidazole-2-methanol. Weigh exactly 10 mg of the analytical standard and dissolve in 100 ml of methyl alcohol (Solution 5). A 1.0-ml aliquot of Solution 5 is diluted to 100 ml with glass-distilled benzene (Solution 6). A 5.0-ml aliquot of Solution 6 is diluted to 100 ml with glass-distilled benzene (Solution 7, 0.5×10^{-7} g/ml) and a 10 μl sample is injected in the gas chromatograph for 2 ppb. A 5.0-ml aliquot of Solution 6 is diluted to 50 ml with glass-distilled benzene (Solution 8, 1×10^{-7} g/ml) and a 10 μl sample is injected in the gas chromatograph for 4 ppb.

PREPARATION OF FORTIFIED SAMPLES

The standard solutions for fortification were prepared from the basic stock solutions of ipronidazole (Solution 1) and of 1-α,α-trimethyl-5-nitroimidazole-2-methanol (Solution 5) with distilled water as the diluent. Amber glassware must be used.

Ipronidazole. A 1.0-ml aliquot of Solution 1 is diluted to 100 ml with distilled water (Solution 9) and a 5-ml aliquot of Solution 9 is diluted to 50 ml with distilled water (Solution 10, 1×10^{-7} g/ml).

1-α,α-Trimethyl-5-nitroimidazole-2-methanol. A 1.0-ml aliquot of Solution 5 is diluted to 100 ml with distilled water (Solution 11) and a 5-ml aliquot of Solution 11 is diluted to 50 ml with distilled water (Solution 12, 1×10^{-7} g/ml). The following volumes of solutions were added to the 100-g tissue sample prior to initial homogenization: for a 2 ppb (2×10^{-9} g/g of tissue) spike for each compound; 2 ml of solution 10 (ipronidazole) and 2 ml of solution 12 (metabolite). Higher spike levels are prepared in a similar manner with proportionally larger volumes of solutions 10 and 12.

PREPARATION OF SILICA GEL COLUMN

The silica gel should be purified prior to use. Wash 20 g of silica gel with six portions of 75 ml of water-saturated ether.

Activate overnight at 110° C. Material not used the same day should be reactivated for 1 hr prior to use and cooled in desiccator.

The amber glass column is assembled as per manufacturer's instructions and 1.3–1.7 g of activated silica gel are poured dry into the column. The silica gel column should be 3 to 4 cm after gently tapping the outside of the column to insure close packing. The 500-ml amber reservoir (made from a 500-ml round-bottomed flask and a column end) is clamped in place and the silica gel is washed with 70 ml of anhydrous ether followed by 4 × 10 ml of benzene. The silica gel supply should be checked with a sample column using a 30-ml portion of H_2O-saturated ethyl ether as eluent and processing of the eluent as per the 1-α,α-trimethyl-5-nitroimidazole-2-methanol procedure and gas chromatographic analysis for possible interferences.

TISSUE SAMPLE PREPARATION

Muscle, liver, or kidney tissue is allowed to come to room temperature, grossly subdivided, and ground using a meat grinder. Size of tissue sample dictates the size grinder to be used, Hobart K5-A (small samples) or Intedge C-2 (large samples). Fat and skin tissue samples are ground in a semi-frozen condition after gross subdivision of the sample.

EXTRACTION PROCEDURE

A 100-g sample of ground tissue is weighed into a 500-ml amber centrifuge bottle and 10 g each of borax and salt is added. The sample is homogenized with the Virtis for 1 min to provide a homogeneous mixture, and 100 ml of glass-distilled benzene is added to the mixture and homogenized at moderate speed for 2 min. The use of high homogenizing speeds after benzene is added sometimes results in emulsions that are difficult to break. Special caution is needed with liver, where it may be preferable to use manual shaking only.

The bottle is stoppered and shaken by hand for 2 min and the sample is then centrifuged for 15 min at 1500 rpm. The use of a refrigerated centrifuge may be helpful in breaking emulsions. Following centrifugation, the benzene layer is decanted into a storage 500-ml amber Virtis flask and 100 ml of glass-distilled benzene is added to the tissue in the 500-ml bottle. The compacted tissue is broken up with a spatula, the bottle stoppered, shaken by hand for 2 min, and the mixture is then centrifuged for 15 min at 1500 rpm.

A third extraction with another 100 ml of benzene is done in the same manner and the extract is pooled with the first and second extracts. At least 270 ml of benzene should be recovered; 250 ml of the total pooled benzene extract is then transferred to the reservoir of the previously prepared silica gel column, allowed to run through the column (followed by 20 ml of benzene as a wash), and the benzene discarded.

The silica gel column is then stripped in a hood by the addition of 25 ml of water-saturated ethyl ether (prepared fresh daily using an unopened can of anhydrous ether and distilled water), allowing the ether to pass through the column. Wash column with an additional 5 ml of water-saturated ether. The column is pressurized using a hand bulb or nitrogen to insure that all the ether goes through the column and is caught in the 40-ml amber centrifuge tube.

The combined ether eluent is mixed well on a Vortex mixer and divided into two equal portions designated A and B in 15-ml amber centrifuge tubes. Portion A is used for the analysis of ipronidazole and portion B is used for the analysis of 1-methyl-5-nitroimidazole-2-isopropanol.

PROCEDURE FOR IPRONIDAZOLE

Reduce volume of ether portion A to approximately 10 ml in a stream of molecular sieve filtered: dry prepurified nitrogen in a hood. Add 3 ml of 3 *N* HCl to the ether, stopper, and shake for 30 sec on a Vortex mixer. Allow the layers to separate and discard the ether layer making sure that none of the aqueous layer is removed. Wash the aqueous layer with 2 × 2-ml portions of glass-distilled benzene. Care must be taken during this washing step that none of the aqueous layer is removed. To the HCl layer add 0.5 g of borax and adjust the pH of the solution to approximately 8 with 6 *N* NaOH using a pH meter. The compounds should not be left at alkaline pH any longer than necessary, each sample being extracted with benzene as soon as the pH has been adjusted (2–5 min). Add 1 ml of glass-distilled benzene, shake well, and allow the layers to separate. The benzene layer is removed and a 10-μl sample is used for glc analysis of ipronidazole.

PROCEDURE FOR 1-α,α-TRIMETHYL-5-NITROIMIDAZOLE-2-METHANOL

Reduce volume of ether portion B to approximately 10 ml in a stream of nitrogen in a hood. Add 2 ml of glass-distilled benzene to the ether in the 15-ml amber centrifuge tube. Evaporate the ethyl ether from the tube using a stream of filtered dry prepurified nitrogen passed through an all-glass manifold at room temperature until a volume of approximately 2 cm^3 remains in the tube. Add 1.4 ml of 3 *N* HCl to the tube, shake for 30 sec on a Vortex mixer, and allow the layers to separate. Discard the upper benzene layer, ensuring that none of the aqueous layer is removed. Wash the aqueous layer with 2 × 2-ml portion of glass-distilled benzene. Care must be taken that, during the washing step, none of the aqueous layer is removed. To the aqueous layer, add 0.5 g of borax and adjust the pH of the solution to approximately 8 with 6 *N* NaOH using a pH meter. The compounds should not be left at alkaline pH any longer than necessary, each sample being extracted with benzene as soon as the pH has been adjusted (2–5 min). Add 2 ml of glass-distilled benzene, shake well, and allow the layers to separate. The benzene layer is removed and a 10-μl sample is used for glc analysis of 1-α,α-trimethyl-5-nitroimidazole-2-methanol.

GAS CHROMATOGRAPHIC ANALYSIS

The following conditions were used for ipronidazole and 1-α,α-trimethyl-5-nitroimidazole-2-methanol for a sensitivity of 1.0 and 0.5 ng, respectively.

Temperatures. Column, 190° C; injection port, 225° C; detector, 265° C.

Carrier Gas. Prepurified nitrogen at 60 ml per min.

Instrument Parameters. Electrometer, 1×10^{-9}; attenuation, $10^2 \times 16$; detector voltage as per Micro-Tek manual for optimum.

Column and Packing. Ipronidazole column is stainless steel, 4 ft × 1/4 in. o.d., packed with 4.2% OV-17 on Anakrom ABS 90–100 mesh. The packing is prepared using the filtration–fluidization technique (Applied Science Labs, 1967) and the packed column conditioned at 250° C for 2 days. The retention time for ipronidazole is approximately 90 sec. The 1-α,α-trimethyl-5-nitroimidazole-2-methanol column is silanized glass, 6 ft × 1/4 in. o.d., packed with 6% SE-30 Ultraphase on Gas Chrom Q, 80–100 mesh. The packing is prepared using the filtration–fluidization technique and the packed column conditioned overnight at 300° C. The reten-

Table I. Ipronidazole Levels in Turkey Tissues (ppb)

Day[a]	Blood		Muscle	
	Ipronidazole	Metabolite	Ipronidazole	Metabolite
0	0	224	0	64
1	0	97	0	22
2	0	17	0	0
3	0	4	0	0
4	0	0	0	0
	Kidney		Liver	
	Ipronidazole	Metabolite	Ipronidazole	Metabolite
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
	Skin and fat			
	Ipronidazole	Metabolite		
0	0	90		
1	0	8		
2	0	4		
3	0	2		
4	0	0		

Metabolite = 1-α,α-trimethyl-5-nitroimidazole-2-methanol.

[a] After drug withdrawal.

tion time for the metabolite is 2 min. A 10-μl sample is used for injection. Area of the peak is used for the determination and is obtained as the product of the peak width at the half height and the peak height.

TISSUE RESIDUE STUDY

Nine-week-old large white turkeys were placed on feed medicated with ipronidazole at the recommended use level of 0.00625% for a period of 12 weeks. Ten birds each were sacrificed at 0, 1, 2, 3, 4, 5, and 6 days of drug withdrawal and their tissues were assayed for ipronidazole and its metabolite by the present method. The results are summarized in Table I. Ipronidazole could not be detected in any tissues, even at 0 day, indicating a rapid conversion to its metabolite. The metabolite was easily detected in muscle, skin and fat, and blood at 0 day, but not in kidney or liver. The metabolite levels dropped rapidly in the tissues and could not be detected after 3 days.

Table II. % Recovery of Turkey Tissue

Level ppb added	Blood		Muscle	
	Ipronidazole	Metabolite	Ipronidazole	Metabolite
2	83.3	72.8	90.4	74.3
4	63.2	65.6	91.5	76.6
6	75.1	69.0	106.3	71.5
	Kidney		Liver	
	Ipronidazole	Metabolite	Ipronidazole	Metabolite
2	46.8	82.8	76.3	80.7
4	50.1	53.3	73.5	73.8
6	46.0	67.5	84.2	92.5
	Skin and fat			
	Ipronidazole	Metabolite		
2	71.3	81.9		
4	72.6	81.9		
6	86.2	75.9		

Metabolite = 1-α,α-trimethyl-5-nitroimidazole-2-methanol.

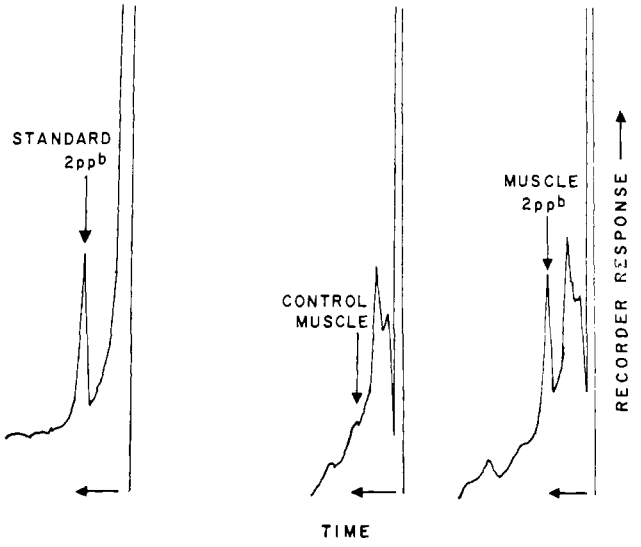


Figure 4. Chromatograms for ipronidazole samples

RESULTS AND CONCLUSIONS

The percent recovery of ipronidazole and its metabolite is summarized in Table II. Each value shown is the average of three or more determinations and the recovery is reasonable for all tissues involved with a standard deviation of 6%.

Typical gas chromatograms for ipronidazole fortified tissue, control tissue, and standard are shown in Figure 4 with metabolite fortified tissue, control tissue, and standard gas chromatograms shown in Figure 5.

The methodology is suitable for quantitatively determining both compounds at levels of 2 ppb and higher in tissue. Levels between 1 and 2 ppb can be estimated as being in this range but not quantitatively determined. Levels less than 1 ppb can only be reported as such.

The following aspects of the prodcedure must be carefully

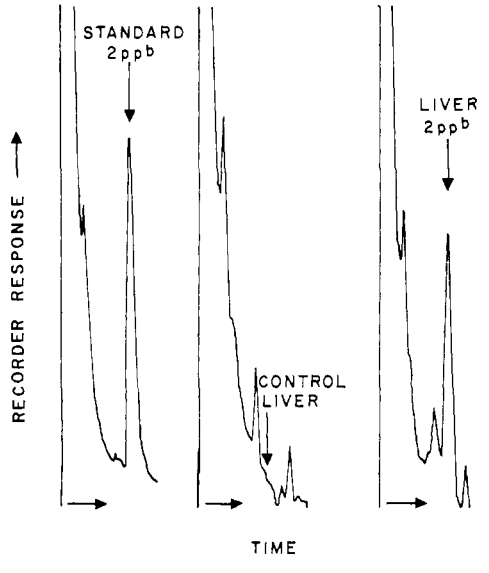


Figure 5. Chromatograms for 1-α,α-trimethyl-5-nitroimidazole-2-methanol solutions

observed to ensure good recoveries and reproducible results. The sample in solution must be protected from light at all times. The ether eluent from the column must be shaken before division and the division must be performed carefully to ensure two equal portions. No solution should be allowed to go to dryness during an evaporation step. The compounds should not stand in or in contact with a basic solution or phase for any prolonged period of time. The electron-capture detector should be standardized daily for both compounds, and for best results the assay procedure must be completed in one working day.

Tissue clearance data show that turkeys fed a 0.00625% ipronidazole ration for 12 weeks are free of the drug and its metabolite 4 days after cessation of treatment.

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