Gas Chromatographic Determination of Pyrimethamine in Tissue

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A procedure has been developed for the isolation and gas chromatographic determination of the coccidiostat pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) in tissue. Analysis can be accomplished with a sensitivity of 0.1 ppm through the employment of electron capture detection with an average recovery of 86 ± 20%. The specificity of the method has been demonstrated in studies with ^{14}C-labeled drug and by use of combined gas chromatography–mass spectrometry.

A study of the metabolic fate and tissue distribution in the chicken of the coccidiostat pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) necessitated the availability of a sensitive and reliable chemical assay for the drug. A number of methods for the determination of pyrimethamine, mainly in connection with its use as an antimalarial, have been published. These include both photometric techniques (Schmidt *et al.*, 1953; Clyde *et al.*, 1956; Smith and Ihrig, 1959; Gaudette *et al.*, 1961; Smith and Schmidt, 1963) and bioassay procedures (Clyde *et al.*, 1956; Smith and Ihrig, 1959; Kaufman, 1961). Only the methyl orange method of Brodie *et al.* (1947), as applied to pyrimethamine by Schmidt *et al.* (1953), appeared to offer the necessary sensitivity. This method, however, evidently does not possess the desired specificity; previous workers in this laboratory encountered high blank values in using this approach. Gas chromatography (gc), especially when detection is *via* electron capture (ecd), has proven to be a powerful method for the determination of nanogram amounts of biologically active compounds (Gudzinowicz, 1967; de Silva and Puglisi, 1970). Because of its sensitivity and possible specificity, the gc–ecd approach was investigated as the measurement step in the assay. We now wish to report on the successful application of gc combined with ecd to the analysis of pyrimethamine in tissue with a sensitivity of 0.1 ppm and recovery of 86 ± 20%.

EXPERIMENTAL

Assay Procedure. The detailed procedure devised for isolation of the drug in a form suitable for gc analysis is presented below. The main steps are summarized as follows: 1, extraction into benzene; 2, back extraction into acid; 3, benzene wash of the acid layer; 4, adjustment to pH 7 and extraction into benzene; 5, evaporation of the extract to dryness; 6, dissolution of the residue in ethyl acetate; 7, gc–ecd.

Two grams of sample were homogenized for 2 min with a Sorvall omnimixer in a 50-ml capacity stainless steel cup with 8 ml of pesticide-quality benzene containing 5% pesticide quality isobutyl alcohol and centrifuged. The benzene layer was decanted into a 15-ml centrifuge tube and the residue again homogenized for 2 min with the benzene/alcohol solvent. The process was repeated for a total of three extractions. (Addition of the alcohol prevented adsorption of the drug onto glassware.) The combined extracts were evaporated to 5 ml under a stream of nitrogen in a 50°C water bath, and the drug was then extracted twice into 5 ml of 0.1 *M* hydrochloric acid. The acid solution in a 50 ml centrifuge tube was washed with 10 ml of benzene and 5 ml of ethyl acetate in the case of kidney, or three times with 10 ml of isooctane for skin fat, to remove most of the interfering substances. Each organic wash was sucked off the aqueous layer with a disposable pipet using a water aspirator to waste. The sample was then neutralized with 10 ml of 0.1 *M* sodium hydroxide and 4 ml of 0.2 *M* pH 7 phosphate buffer. The pH of the sample was then 7.0 ± 0.2. The drug was extracted back into three times 15 ml of the benzene/alcohol solvent. The sample was evaporated to *ca.* 0.5 ml in a 250-ml round-bottomed standard taper flask on a Rotovac using a water aspirator and a water bath of *ca.* 50°C around the flask. The sample was quantitatively transferred to a 15-ml centrifuge tube and evaporated to dryness in a water bath (40°C) under a stream of nitrogen. The residue was dissolved in 100 μl of ethyl acetate and a 1-μl sample immediately injected into the gas chromatograph. A process standard was prepared by adding 0.1 ml of a 10 μg/ml solution of pyrimethamine in ethyl acetate to 25 ml of the benzene–alcohol solvent; this was then carried through the isolation procedure.

Calculation of Results. Peak areas were determined from height and width at half-height. Sample peak area was compared to a working curve determined each day from a series of standard solutions of pyrimethamine. Recovery through the assay of the process standard was calculated, and the sample results were corrected by this factor (normally ~73%).

Instrumentation. Gc was carried out with a Glowall instrument equipped with a Lovelock argon ionization detector (22.5 μCi ^{226}Ra) operated at 8 V in the ECD mode (300°C). Commercially available acid-washed support, 80–100 mesh Gas Chrom P, was re-acid washed and silanized (Horning *et al.*, 1968), and this material coated with 1.5% SE-30 *via* the filtration coating technique (Horning *et al.*, 1968). The dried packing was then subjected to a second deposition of stationary phase, 6% SE-30. This approach furnished a packing suitable for the quantitative gc of underivatized pyrimethamine at levels adequate for our purposes. Column conditions: 2-ft × 3-mm i.d. glass spiral column; 190°C; nitrogen carrier gas, 42 ml/min; flash heater 275°C. The retention time for pyrimethamine is approximately 2 min.

Combined gc–mass spectrometry (ms) (Horning *et al.*, 1968; McCloskey, 1969) was effected with the LKB combination gas chromatograph–mass spectrometer. Column conditions were essentially the same as above. Spectrometer conditions: 70 eV ionizing potential, 270°C ion source temperature, 60 μA filament current, 3.5 kV accelerating voltage. For the repetitive scan experiments, the voltage ramp output of a Tektronix Model 162 Waveform generator was connected across a resistor in series with the mass spe-

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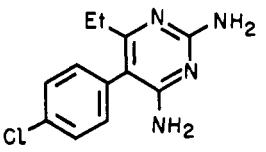

Figure 1. Structural formula of pyrimethamine

Table I. Percent Recovery of Pyrimethamine Added to Chicken Tissues[a]

Spike level, ppm	Plasma	Muscle	Kidney
1	100	81	91
0.5	86	77	92
0.25	85	74	88
0.1	81	77	79

[a] Corrected for process standard recovery.

trometer accelerating voltage supply. The sum of these voltages, one periodically increasing and returning to zero and the other fixed, results in a repeating mass scan over a small interval, at a rate determined by the generator (2.5 sec/scan). The spectrometer controls are set for the mass range of interest and the output is viewed on the oscillograph in the usual way.

RESULTS AND DISCUSSION

Tissue residues in the chicken were found to consist of only unchanged drug, as established by reversed isotope dilution assay after dosing with ^{14}C-labeled pyrimethamine (a small amount of acid-labile conjugate was present in urine) (Buhs and Trenner, 1971). The requirement for a satisfactory assay was therefore a suitably sensitive and quantitative determination for pyrimethamine itself. Preliminary experiments indicated that pyrimethamine (Figure 1) undergoes gc satisfactorily at the microgram level (flame ionization detection). The requirements of the tissue residue assay were such that a sensitivity of ~0.1 ppm was needed, necessitating successful chromatography and detection of nanogram quantities of drug. The presence of a chlorine atom in pyrimethamine would be expected to result in good ecd sensitivity for this drug. The gas chromatogram resulting from analysis of 10 ng of pyrimethamine is shown in Figure 2. Linear response was obtained with this gc–ecd system over the range 2–20 ng, but was observed to drop off below 2 ng (Figure 3). This latter effect is probably the result of irreversible adsorption, most readily observed as sample size diminishes (Horning *et al.*, 1963; VandenHeuvel *et al.*, 1963).

Average process standard recoveries were 73%. Losses were ~15% because of incomplete liquid–liquid extractions, and ~12% in the chromatography. Contaminants present both from the solvents used and from the tissues themselves were found to decrease the response of the detector to a given amount of pyrimethamine.

This "quenching" can be seen in reagent blanks and control tissue extracts that were spiked with drug immediately prior to gc. Both responses were lower than would be expected from the standard curve; the reagent blank was 12% lower and the tissue extract was 15% lower. Working-up of control tissues to which ^{14}C-labeled drug was added at levels of 0.5 ppm or lower showed a difference of ~15% between recoveries calculated from radioactive data and from gc data.

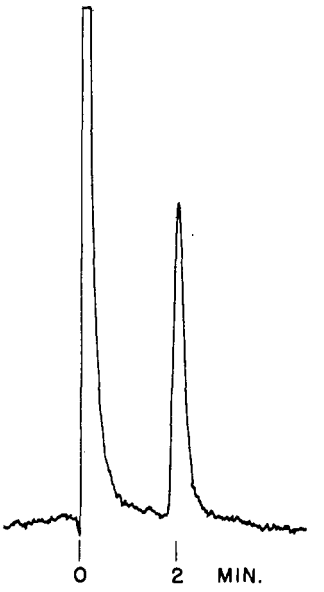

Figure 2. Gas chromatogram resulting from the analysis of 10 ng of pyrimethamine (ECD). Column conditions: 2-ft × 3-mm i.d. glass spiral column; 6% SE-30 coated over 1.5% SE-30 on 80–100 mesh acid-washed and silanized Gas Chrom P; 190°C; 42 ml/min nitrogen carrier gas

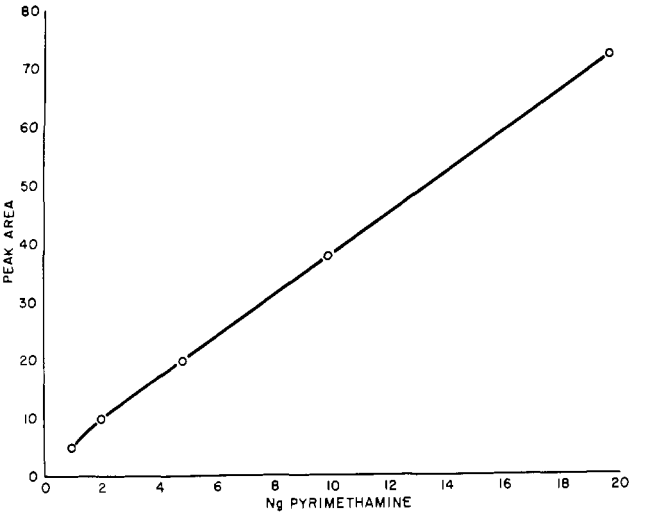

Figure 3. Plot of detector response (tenths of inches)^2 *vs.* ng of pyrimethamine. Data obtained with column conditions described for Figure 2

The source of this "gc quenching" is not known, but a correction was made for it by use of a process standard.

Data (gc) on recovery of pyrimethamine added to several types of chicken tissue are presented in Table I. Average recoveries in the initial extraction are 88% (muscle, 89%; kidney, 87%; liver, 88%; skin fat, 85%) based on a study carried out on chickens fed radioactive pyrimethamine. All losses except that resulting from nonextractability from the tissue and additional "gc quenching" from the tissue components were corrected by the process standard. No blank corrections were necessary, as gc of control tissue showed no interfering peaks.

Preliminary experiments with tissues from animals fed ^{14}C-labeled pyrimethamine indicated that the drug residue levels determined by radioactivity and by gc were gratifyingly similar. For example, in one experiment "4-day-on-drug"

Figure 4. Gas chromatogram resulting from the analysis of the extract (obtained by following assay procedure) from a 3-day-off-drug liver (0.2 ppm). Column conditions same as for Figure 2

Table II. Residue Levels (ppm) in Chicken Tissue[a]

Bird		Skin/fat	Liver	Muscle
4261	Control	0	0	0
4262		0	0	0
4263		0	0	0
4341		0	0	0
4342		0	0	0
4343		0	0	0
Avg		0	0	0
4291	On-drug	0.14	1.2	0.10
4292		0.16	0.8	0.10
4293		0.14	0.9	0.15
4371		0.16	1.3	0.14
4372		0.13	0.9	0.16
4373		0.14	1.1	0.09
Avg		0.16	1.0	0.12
4301	3-day-off-drug	0	0.13	0
4302		0	0.18	0
4303		0	0.17	0
4381		0	0.10	0
4383		0	0.12	0
4384		0	0.11	0
Avg		0	0.14	0
4311	5-day-off drug	0	0	0
4312		0	0	0
4313		0	0	0
4391		0	0	0
4392		0	0	0
4393		0	0	0
Avg		0	0	0

[a] 0 signifies that there was no indication of a peak with the retention time of pyrimethamine. The sensitivity limit of the assay is estimated to be 0.05 ppm, thus "0" is equivalent to <0.05 ppm.

liver was found to contain 0.73 ppm based on radioactivity and 0.75 ppm based on gc. For a "1-day-off-drug" liver, residues were found to be 0.26 ppm (radioactivity) and 0.25 ppm (gc). These experiments indicate the specificity of the gc assay procedure.

Tissues from chickens on feed containing 0.0005% pyrimethamine were assayed according to the herein described procedure. Four groups of birds (three males, three females per group) were studied: nonmedicated controls; on-drug; 3-day and 5-day withdrawal. The results for skin fat, liver, and muscle are presented in Table II. The gas chromatogram of the extract from a typical "3-day-off-drug" liver sample is presented in Figure 4.

The method mentioned above (comparison of radioactivity and gc data) for confirming the specificity of the gc assay is

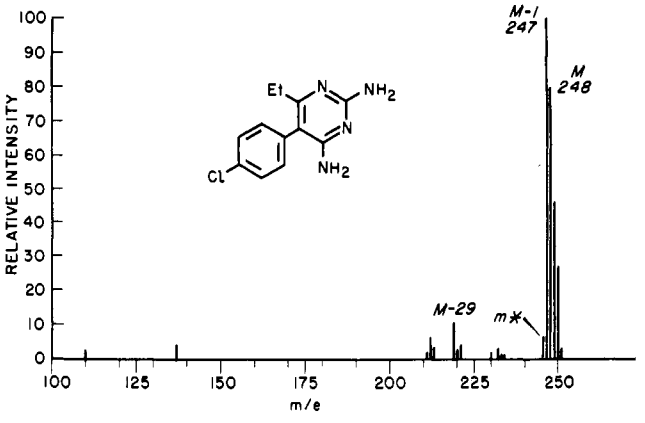

Figure 5. Mass spectrum (m/e 100–250) of pyrimethamine. Spectrometer conditions: 70 eV ionizing potential; 270°C ion source temperature; 60 μA filament current; 3.5 kV accelerating potential

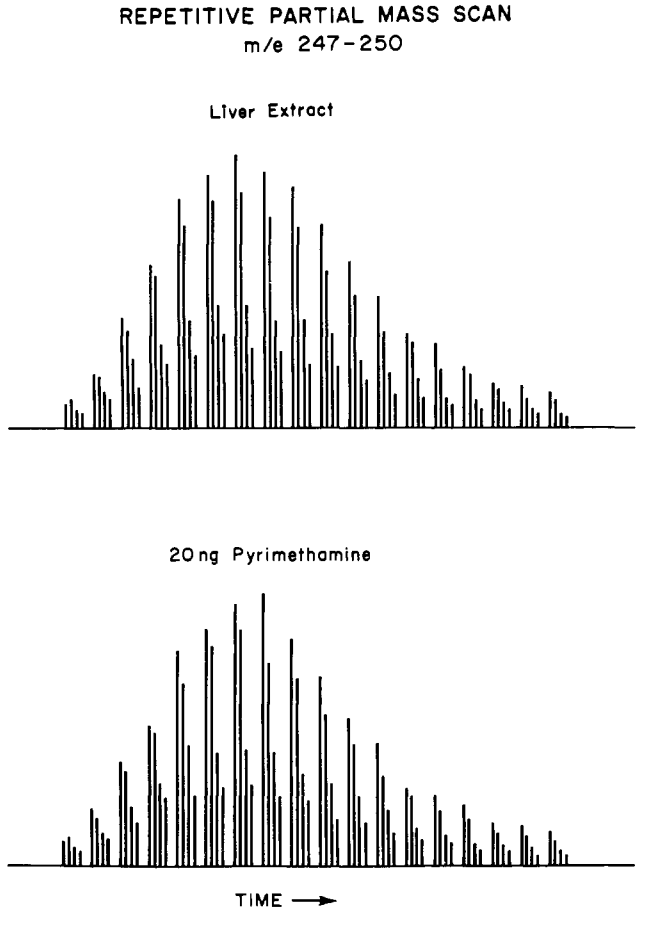

Figure 6. Signals observed on the mass spectrometer at m/e 247–250 (scanned repetitively) during the elution from the gc column of (a) 20 ng of pyrimethamine (lower plot), and (b) an aliquot of a 1-day-off-drug liver extract shown by gc–ecd to contain 20 ng of drug (upper plot)

very useful but would not be applicable if only samples from animals treated with nonlabeled drug were available. An alternate approach for establishing specificity with normal drug, *i.e.*, that the gc peak measured indeed represents pyrimethamine and only pyrimethamine, would be most desirable. Retention time and peak shape are useful but are no guarantee of identity and purity. Combined gc–mass spectrometry (ms) (Horning *et al.*, 1968; McCloskey, 1969) has proven to be a superior method for the identification of gc effluent peaks and the indication of their homogeneity. The mass spectrum of pyrimethamine is presented in Figure 5. The highly aromatic nature of this compound results in a relatively small number of fragment ions, and the major signals are found in the region of the molecular ion, *i.e.*, *m/e* 247–250. Pyrimethamine possesses a molecular weight of 248, but undergoes a metastable transition to lose a proton ($M \rightarrow M - 1$), with the result that the $M - 1$ ion is the base peak (most intense ion) in the spectrum. Because of this the characteristic isotope cluster usually observed with monochloro-substituted compounds is obscured, but is noted clearly with the fragment ion $M - 29$ (loss of the ethyl group) at *m/e* 219–221. Combined gc–ms was employed in several cases to prove that the component observed in biological extracts at the retention time of pyrimethamine was authentic drug.

A mass spectrometer combined with a gas chromatograph can function as a very selective detector for the chromatograph (Zinbo and Sherman, 1970; Samuelsson *et al.*, 1970; Hammar and Hessling, 1971); that is, the spectrometer can be set to respond only to one (or several) ion(s) of preselected *m/e* value, rather than producing complete spectra (as in normal mass spectrometry) or responding to a compound on a mass basis (as does a normal gc detector). Further, if the ion or ions chosen to be monitored are also of high abundance or intensity, the great sensitivity of the combined instrument makes this approach extremely attractive for the characterization and quantitation of trace amounts of compounds. Similarly, a small range of the spectrum can be scanned repetitively across a gc peak (Bergstedt and Widmark, 1970; VandenHeuvel *et al.*, 1970). Pyrimethamine is a compound which exhibits a mass spectrum particularly suited to this type of analysis—the molecular ion region (*m/e* 247–250) is a characteristic set of intense ions.

The specificity and accuracy of the gc–ecd assay was further demonstrated by employing the LKB gas chromatograph–mass spectrometer as a selective gc detector. The gc–ms instrument was set to scan repetitively over the region of the molecular ion. An aliquot (20 ng on the basis of gc–ecd) of the extract from a sample of 1-day-off-drug liver was subjected to gc–ms and found to give the same response, both qualitatively and quantitatively and at the same retention time as 20 ng of pyrimethamine (Figure 6). Techniques of this type should prove applicable to other drug residue and related problems.

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Received for review April 19, 1971. Accepted September 15, 1971.