compensated for by dissolving final metabolite residues in smaller volumes. Somewhat better sensitivity may be established if peak measurements are made by peak height or electronic integration and the limit of detection is defined as twice the noise level of the baseline.

LITERATURE CITED

Applied Science Labs. Inc., Technical Bulletin No. 2A, State College, Pa., 1967, p 5.
Church, D. D., Flint, D. R., Chemagro Report No. 32047, unpublished data, 1972.
Eue, L., Westphal, K., Dichore, K., Meister, W., *Symp. New Herbic., 3rd* 1, 125–132 (1969).
Gronberg, R. R., Flint, D. R., Shaw, H. R., Robinson, R. A., Chemagro Report No. 29800, unpublished data, 1971.
Hargroder, T. G., Rogers, R. L., *Weed Sci.* 22(3), 238–245 (1974).
Hilton, H. W., Nomura, N. S., Yauger, W. L., Kamada, S. S., *J. Agric. Food Chem.* 22, 578–582 (1974).
Pape, B. E., Zabik, M. J., *J. Agric. Food Chem.* 20, 72–75 (1972).
Stanley, C. W., Chemagro Report No. 40977, unpublished data, 1974.
Stanley, C. W., Schumann, S. A., Chemagro Report No. 25848, unpublished data, 1969.
Thornton, J. S., Schumann, S. A., Chemagro Report No. 30387, unpublished data, 1971.
Thornton, J. S., Stanley, C. W., Chemagro Report No. 33005, unpublished data, 1972.
Thornton, J. S., Stanley, C. W., Schumann, S. A., 164th National Meeting of the American Chemical Society, New York, N.Y., Aug 1972, Abstract PEST-006.
von Stryk, F. G., *J. Chromatogr.* 56, 348 (1971).
Webster, G. R. B., Macdonald, S. R., Sarna, F. P., *J. Agric. Food Chem.* 23, 74–76 (1975).

Received for review May 6, 1976. Accepted October 23, 1976.

Gas–Liquid Chromatographic/Mass Spectrometric Confirmatory Assay for Thiabendazole and 5-Hydroxythiabendazole

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A combined gas–liquid chromatographic/mass spectrometric confirmatory assay for thiabendazole and 5-hydroxythiabendazole at 0.1 ppm in animal tissue isolates has been developed. On-column methylation converts these compounds to their *N*-methyl and *N,O*-dimethyl derivatives, respectively. Identification and quantitation are achieved by selective ion monitoring of the M − 1, M, and M + 1 ions from *N*-methylthiabendazole and the M and M − 15 ions from *N,O*-dimethyl-5-hydroxythiabendazole.

The availability of analytical methods for the determination of possible residues in edible tissues of food animals is a necessity with respect to the use of animal health drugs. In addition, a method ("confirmatory assay") to validate positive findings in the primary routine assay for drug or metabolite is an FDA requirement. A confirmatory assay should be based upon characteristic structural features of the compounds(s) of interest, and it is for this reason that combined GLC–mass spectrometric (MS) techniques are well suited for validation of assay procedures based on other less specific methods.

Obtaining the appropriate mass spectrum at the retention time of the compound of interest is convincing evidence for the presence of that compound in the injected sample. However, a mass spectrum resulting from a single scan is of little value for quantitative purposes. The use of a mass spectrometer as a selective GLC detector is well established (Brooks and Middleditch, 1971; Jenden and Cho, 1973; Watson, 1973). Thus, Cala et al. (1972), Palmer and Kolmodin-Hedman (1972), and Mirocha et al. (1973) have employed GLC–MS techniques to validate other assays for pyrimethamine in chicken tissue, *p,p'*-DDE in human plasma, and diethylstilbestrol in swine feedstuff, respectively.

Thiabendazole, 2-(4-thiazolyl)benzimidazole (TBZ), is a widely used anthelmintic (Brown et al., 1961) and antifungal (Robinson et al., 1964) agent. A fluorescence

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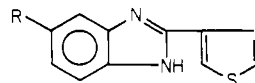

R = H (TBZ)
R = HO (HTBZ)

method is currently employed for the assay of TBZ in animal tissues (U.S. Department of Health, Education and Welfare, 1973). As this drug is polar and does not exhibit satisfactory GLC behavior at the submicrogram level, Jacob et al. (1975) converted it to the trimethylsilyl derivative for GLC–MS analysis in their study on the photolysis of TBZ. More recently Tanaka and Fujimoto (1976) employed methylation [dimethylformamide dimethyl acetal (Thenot et al., 1972; Thenot and Horning, 1972)] to achieve the GLC determination of TBZ in fruits.

A confirmatory assay for TBZ should be capable of adequate detection at $\leq$0.1 ppm (based on wet tissue sample), and at such a level derivatization of polar drug-related compounds is desirable so as to obtain reliable GLC results. We have chosen on-column methylation using trimethylanilinium hydroxide in methanol (Brochmann-Hanssen and Oke, 1969), a reagent shown to be useful in the GLC analysis of a number of drugs (Hammer et al., 1971; VandenHeuvel et al., 1975). This approach not only converts TBZ to its *N*-methyl derivative, but also yields the *N,O*-dimethyl derivative of 5-hydroxy-2-(4-thiazolyl)benzimidazole (HTBZ), a known metabolite of TBZ (Tocco et al., 1964).

Mass fragmentography (Hammar et al., 1968) or MF, a selective ion monitoring technique, in combination with

Table I. GLC-MS Results from Isolation Method I

	Intensity ratios		ppm found[a]	
	214/215/216 (TBZ)	230/245 (HTBZ)	TBZ	HTBZ
0.1 ppm of TBZ and HTBZ standards	32/100/14	42/100		
Control beef muscle			⩽0.015	⩽0.025
Control beef liver			⩽0.015	⩽0.025
Control beef kidney			⩽0.015	⩽0.025
Control beef fat			⩽0.015	⩽0.025
Control swine liver			⩽0.015	⩽0.025
0.1 ppm of beef muscle[b]	32/100/15	44/100	0.11	0.09
0.1 ppm of beef liver[b]	32/100/13	42/100	0.08	0.11
0.1 ppm of beef kidney[b]	32/100/14	40/100	0.11	0.09
0.1 ppm of beef fat[b]	32/100/15	40/100	0.09	0.11
0.1 ppm of swine liver[b]	31/100/14	42/100	0.10	0.10

[a] Quantification based on the intensity of the *m/e* 215 (TBZ) and 245 (HTBZ) ions. [b] Spikes were added to the control tissue residues after the isolation procedure, and were adjusted for the 60% recovery experienced with the isolation procedure. The 60% recovery figure was based on fortification experiments in the range 0.1–1 ppm. Recoveries from 21 tissue samples (based on flame ionization GLC) ranged from 51 to 68% (average 58%) for TBZ and from 49 to 71% (average 61%) for HTBZ.

Figure 1. Partial mass spectra of on-column methylation products of TBZ and HTBZ.

on-column methylation, was employed in our confirmatory assay for TBZ and HTBZ. As commonly practiced, MF involves use of a mass spectrometer as a selective detector, the spectrometer set to follow with time the production of certain characteristic ions. The simultaneous appearance of the chosen ions with the appropriate relative intensity relationships and at the retention time of the compound of interest demonstrates conclusively the presence of that compound in the injected sample.

The high mass regions of the electron impact (20 eV) mass spectra of the derivatives of TBZ and HTBZ are presented in Figure 1. The spectrum of *N*-methyl-2-(4-thiazolyl)benzimidazole is dominated by a characteristic cluster of ions at the molecular ion region, viz. *m/e* 214 (M − 1), 215 (M, the base peak), 216 (M + 1), whereas the most intense signals in the spectrum of *N,O*-Me_2HTBZ are the M and M − 15 ions (*m/e* 245 and 230). The intensity of the M − 1 ion of *N*-MeTBZ is unusually great, and thus the three ion cluster (M − 1, M, M + 1) is monitored to demonstrate the presence of TBZ in tissue isolates. The mass fragmentogram resulting from analysis of a 0.1-ppm TBZ standard (actual amount injected, 14.4 ng) via on-column methylation is shown in Figure 2. Because of sensitivity considerations two ions, *m/e* 230 and 245, are monitored for the presence of HTBZ.

Quantitation of TBZ and HTBZ is based upon the intensities of the molecular ions, *m/e* 215 and 245. A polysulfone stationary phase (Mathews et al., 1974) was chosen for GLC because its background bleed and adsorption characteristics were more desirable than those of others (e.g., SE-30, OV-1) tested.

Two isolation schemes have been developed to use in combination with the GLC–MS confirmation for the presence of TBZ and HTBZ. Both schemes involve initial enzymatic conversion of any HTBZ glucuronide and HTBZ sulfate (known metabolites of TBZ) to HTBZ as a preliminary step to the isolation process. The simplest

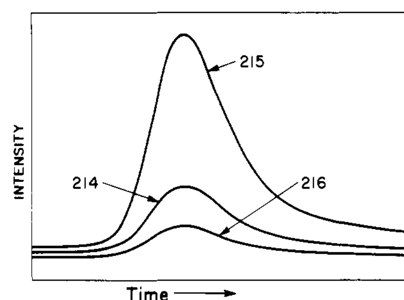

Figure 2. Mass fragmentogram resulting from analysis of a 0.1-ppm TBZ standard via on-column methylation.

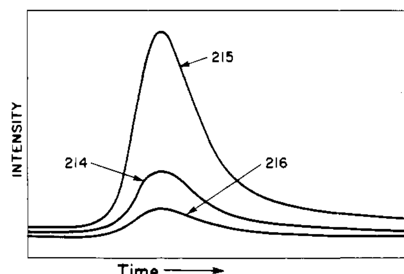

Figure 3. Mass fragmentogram resulting from analysis of a control beef kidney isolate spiked at the 0.1-ppm level with TBZ.

starting point for the confirmatory assay for these substances is at the finish of the original, fluorescence assay. Assuming that the final acid solution is available, a few simple steps provide dry material suitable for the proposed validation. Method I combines the original assay procedure with these few additional steps.

If the acid solution is not available, a second scheme allows improved recoveries in providing a new isolate with little or no additional effort required as compared to repeating method I. Method II should be used where the confirmatory assay must start with the tissue itself. Use of procedure II yields the equivalent of approximately three times as much tissue isolate (compared to procedure I).

The mass fragmentographic results from control beef and swine tissue isolates and control isolates spiked with 0.1 ppm of TBZ and HTBZ, obtained through use of isolation procedure I, are presented in Table I. The parts per million values and intensity ratios found at the appropriate retention times match those for authentic *N*-MeTBZ and *N,O*-Me_2HTBZ, thus fulfilling the requirements of confirmation by MF. The mass fragmentogram

Table II. GLC-MS Results from Isolation Method II

Sample	ppm found[a]	
	TBZ	HTBZ
0.1 ppm of TBZ and HTBZ standards		
Control beef muscle	≤0.015	≤0.025
Control beef liver	≤0.015	≤0.025
0.1 ppm in beef muscle[b]	0.09	0.10
0.1 ppm in beef liver[b]	0.11	0.10

[a] Quantification based on the intensity of the m/e 215 (TBZ) and 245 (HTBZ) ions. [b] Spikes were added to the control tissue residues after the isolation procedure. No adjustment was made for recovery. Flame ionization GLC experiments gave a range of recoveries of 84-112% (average 96%) for TBZ and 81-102% (average 91% for HTBZ).

resulting from analysis of a control beef kidney isolate (procedure I) spiked at the 0.1-ppm level with TBZ is presented in Figure 3. A comparison of Figures 2 and 3 indicates that the two mass fragmentograms are virtually indistinguishable.

Replicate analysis of 0.1 ppm of TBZ and HTBZ samples from isolation method I gave peak intensity values for m/e 215 and 245 of 8.3 ± 0.9 cm (multiplier voltage, 2.1 kV) and 9.1 ± 0.9 cm (multiplier voltage, 2.3 kV), respectively. Lower limits of detection are approximately 0.015 ppm of TBZ and 0.025 ppb of HTBZ with this isolation procedure.

Mass fragmentographic results from samples prepared by isolation method II are given in Table II. As with isolation method I, the observed retention times and intensity ratios for the spiked samples were the same as those noted for the reference compounds, and no interference occurred with unspiked isolates.

Although this combined GLC–MS method has been designed to serve as a confirmatory assay, it could also function as a sensitive, specific assay for TBZ and HTBZ per se. Further, the isolation, derivatization, and GLC aspects need not be interfaced with a mass spectrometer, but could be combined with a simpler, e.g., flame ionization, detection system.

EXPERIMENTAL SECTION

Apparatus. (a) An LKB Model 9000 combined gas chromatograph–mass spectrometer was used. The column was a 45 cm × 2 mm i.d. coiled glass tube packed with 3% polysulfone on 60–80 mesh Gas-Chrom Q (Applied Science Labs., Inc., State College, Pa.). Operating conditions were as follows: temperatures, column 238 °C for TBZ and 260 °C for HTBZ, flash heater 270 °C, molecular separator 250 °C, and ion source 250 °C; helium carrier gas flow rate, 30 ml/min; 20-eV ionizing potential, 60-μA trap current, and accelerating voltage alternator; and retention times ~1 min for both TBZ and HTBZ. (b) The homogenizer unit was a Sorval Omni-Mixer equipped with 200-ml cups.

Reagents. (a) Burdick and Jackson glass distilled solvents were employed. (b) Glusulase stock was supplied by Endo Laboratories, Garden City, N.Y., and contained 100 000 units of glucuronidase and 50 000 units of sulfatase/ml. A working dilution was made fresh daily of 1 ml of stock to 9 ml of pH 4.5 buffer. (c) Trimethylanilinium hydroxide stock was 0.5 M as supplied by Southwestern Analytical Chemicals, Inc., Austin, Tex. A working dilution of 1 ml of stock plus 4 ml of methanol was made. (d) The pH 6 buffer was made by adding 133.8 g of sodium pyrophosphate decahydrate to enough distilled water to make a total of 1 l. and adjusting the pH to 6.0 with 6 N HCl. The solid only completely dissolved as the acid was added. (e) The pH 4.5 buffer was made by *dissolving* 8.2 g of anhydrous sodium acetate in enough distilled water to make 1 l. and adjusting the pH to 4.5 with 6 N HCl. (f) Thiabendazole and 5-hydroxythiabendazole were Merck reference standards. Solutions were: (i) stock solution—25 mg of standard was added to a 50-ml volumetric flask and dissolved in methanol; (ii) method II working solution—5 ml of the stock solution was added to a 100-ml volumetric flask and made to volume with methanol; (iii) method I working solution—7.2 ml of the method II working solution was added to a 25-ml volumetric flask and made to volume with methanol; (iv) 0.1 ppm of dry GLC standards—0.5-ml aliquots of the working standard solutions were evaporated to dryness at 50 °C with nitrogen purge in 15-ml centrifuge tubes.

Method I: Sample Preparation, Incubation, and Extraction. Each isolate was prepared as in the fluorescence procedure (U.S. Department of Health, Education and Welfare, 1973). Standards and blanks described in said method were not used. The final 2-ml acid phase from the fluorescence method was washed with 2 ml of ethyl acetate. To the washed acid was added 1 drop of 27% NH_4OH and 1 ml of pH 6 buffer. The resulting solution (pH 6–10) was extracted twice with 5 ml each of ethyl acetate. The combined acetate layers were blown to dryness in a 15-ml centrifuge tube at 50 °C with nitrogen purge.

Method II: Tissue Sample Preparation and Incubation. A 30-g tissue sample was weighed into a homogenizer cup, 60 ml of pH 4.5 buffer was pipetted into the cup, and the resulting mixture was homogenized at the highest speed for 2 min. An aliquot of 60 g of the well-mixed homogenate was weighed into a 250-ml centrifuge bottle. Two milliliters of diluted glusulase was added and the resulting mix shaken for 5 min. A drop of toluene was added and the bottle was incubated unshaken for 18 h at 37 °C. With fat samples, the homogenizer cup was placed in a beaker of water (70 °C) to yield a smooth, milky homogenate which was aliquoted while still warm.

Extraction and Partition. The mix was shaken with exactly 80 ml of ethyl acetate for 15 min and centrifuged for 10 min. A 50-ml aliquot of the upper phase was pipetted into a fresh bottle and extracted by shaking for 1 min each three times with 5 ml each of 0.1 N HCl. The combined acid layers were washed twice with 5 ml each of *n*-hexane. To the washed acid 0.35 ml of 27% NH_4OH and 7 ml of pH 6 buffer were added (resulting pH range 8–10). Two extractions with 15 ml each of ethyl acetate were performed and the combined ethyl acetate layers were blown to a volume of 2–3 ml at 50 °C with nitrogen purge. The resulting solution was transferred into a 15-ml graduated centrifuge tube with 3 × 2 ml of ethyl acetate using a disposable pipet. The ethyl acetate was blown down to 5 ml or less and made back up to a volume of 5 ml using the tube markings. Three extractions were performed with 1 ml each of 0.1 N HCl. To the combined extracts 1 drop of 27% NH_4OH and 1 ml of pH 6 buffer were added and the resulting solution (pH 6–10) extracted twice with 5 ml each of ethyl acetate. The combined extracts were blown to dryness at 50 °C in a 15-ml centrifuge tube using a nitrogen purge.

GLC–MS Validation. A dry 0.1-ppm standard was carefully and completely dissolved in 500 μl of trimethylanilinium hydroxide in methanol (0.125 M) immediately before injecting 2 μl into the combined gas chromatograph–mass spectrometer. The latter was focused on m/e 214 for TBZ (and m/e 230 for HTBZ) with the main manual controls. The instrument was also focused on m/e 215 and 216 for TBZ (and m/e 245 for HTBZ)

using the additional channels provided by the accelerating voltage alternator. The intensities of the ions were recorded on the recording oscillograph as the appropriate peak eluted from the column. The unknown sample was carefully dissolved in 50 μl of the hydroxide solution and 2 μl injected. The same measurements were made at the elution time determined from the standard run. A residue of 0.1 ppm or more was confirmed for an unknown whose signal intensity (peak height) at m/e 215 for TBZ and m/e 245 for HTBZ was equal to or greater than the equivalent standard. To complete the validation the ratio of the intensities was found to be equal for unknown and standard.

LITERATURE CITED

Brochmann-Hanssen, E., Oke, T. O., *J. Pharm. Sci.* **58**, 370 (1969).
Brooks, C. J. W., Middleditch, B. S., *Clin. Chim. Acta* **34**, 145 (1971).
Brown, H. D., Matzuk, A. R., Ilves, I. R., Peterson, L. H., Harris, S. A., Sarett, L. H., Egerton, J. R., Yakstis, J. J., Campbell, W. C., Cuckler, A. C., *J. Am. Chem. Soc.* **83**, 1764 (1961).
Cala, P. C., Trenner, N. R., Buhs, R. P., Downing, G. V., Jr., Smith, J. L., VandenHeuvel, W. J. A., *J. Agric. Food Chem.* **20**, 337 (1972).
Hammar, C. G., Holmstedt, B., Ryhage, R., *Anal. Biochem.* **25**, 532 (1968).
Hammer, R. H., Wilder, B. J., Streiff, R. R., Mayersdorf, A., *J. Pharm. Sci.* **60**, 327 (1971).
Jacob, T. A., Carlin, J. R., Walker, R. W., Wolf, F. J., VandenHeuvel, W. J. A., *J. Agric. Food Chem.* **23**, 704 (1975).
Jenden, D. J., Cho, A. K., *Annu. Rev. Pharmacol.* **13**, 371 (1973).
Mathews, R. G., Schwartz, R. D., Pfaffenberger, C. D., Lin, S. N., Horning, E. C., *J. Chromatogr.* **99**, 51 (1974).
Mirocha, C. J., Christensen, C. M., Davis, G., Nelson, G. H., *J. Agric. Food Chem.* **21**, 135 (1973).
Palmer, L., Kolmodin-Hedman, B., *J. Chromatogr.* **74**, 21 (1972).
Robinson, H. J., Phares, H. F., Graessle, O. E., *J. Invest. Dermatol.* **42**, 479 (1964).
Tanaka, A., Fujimoto, Y., *J. Chromatogr.* **117**, 149 (1976).
Thenot, J-P., Horning, E. C., *Anal. Lett.* **5**, 519 (1972).
Thenot, J-P., Horning, E. C., Stafford, M., Horning, M. G., *Anal. Lett.* **5**, 217 (1972).
Tocco, D. J., Buhs, R. P., Brown, H. D., Matzuk, A. R., Mertel, H. E., Harman, R. E., Trenner, N. R., *J. Med. Chem.* **7**, 399 (1964).
U.S. Department of Health, Education and Welfare, FDA, "Food Additives Analytical Manual", Revised Edition, 1973.
VandenHeuvel, W. J. A., Gruber, V. F., Walker, R. W., Wolf, F. J., *J. Pharm. Sci.* **64**, 1309 (1975).
Watson, J. T., *Annu. Rev. Pharmacol.* **13**, 391 (1973).

Received for review October 12, 1976. Accepted December 13, 1976.

Gas–Liquid Chromatographic Determination of 4-Amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide, a New Anthelmintic, in Biological Fluids

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Extractive methylation (methyl iodide/sodium hydroxide/tetra-*n*-butylammonium hydroxide/methylene chloride) has been employed in an electron-capture GLC method for determination of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide, a new anthelmintic, in several biological fluids including milk, blood, and urine. Comparison of GLC and radiochemical data obtained from animals treated with ^{35}S-labeled drug demonstrated the presence of drug in milk, and drug plus a metabolite (which can be hydrolyzed to parent drug) in urine. A GLC–mass spectrometric technique has been developed to confirm the presence of the drug in biological extracts.

Successful gas–liquid chromatography (GLC) of many drugs at the submicrogram level requires that they be converted to more volatile, less polar derivatives. This is true of primary sulfonamides, for example, and on-column methylation (Brochmann-Hanssen and Oke, 1969; MacGee, 1970) has been employed to convert bumetanide (Fiet et al., 1973) and hydrochlorothiazide (VandenHeuvel et al., 1975) to derivatives suitable for GLC. Dimethylformamide dimethyl acetal, reported to be a useful GLC derivatization reagent for carboxyl (Thenot et al., 1972) and amino (Thenot and Horning, 1972) groups, has been employed to convert barbiturates and glutethimide to the corresponding acetals (Venturella et al., 1973). More recently it has been found to react readily with primary sulfonamides to form *N*-dimethylaminomethylene (*N*-DMAM) derivatives which possess excellent GLC properties (VandenHeuvel and Gruber, 1975). Extractive

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Table I. Levels of 4-Amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide in Bovine Milk and Bull Urine[a]

Milk			Urine		
Time post-dose, h	ppm GLC	ppm RA[b]	Time post-dose, days	ppm GLC	ppm RA
12	0.36	0.44	1	6.82	7.81
24	0.56	0.53	2	21.9	36.0
36	0.29	0.35	3	19.9	32.7
48	0.20	0.21	4	15.1	25.5
58	0.15	0.13	5	11.8	17.1
72	0.06	0.07	6	5.78	10.9
82	0.03	0.04	7	3.26	6.24
106	<0.03	0.01			
120	<0.03	0.01			

[a] Animals dosed at 6.6 mg/kg of ^{35}S-labeled I. [b] Liquid scintillation counting.

alkylation has been used for the methylation and subsequent GLC determination of nitrazepan (Ehrsson and