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.

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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

William J. A. VandenHeuvel, III,* Virginia F. Gruber, Robert W. Walker, and Frank J. Wolf

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

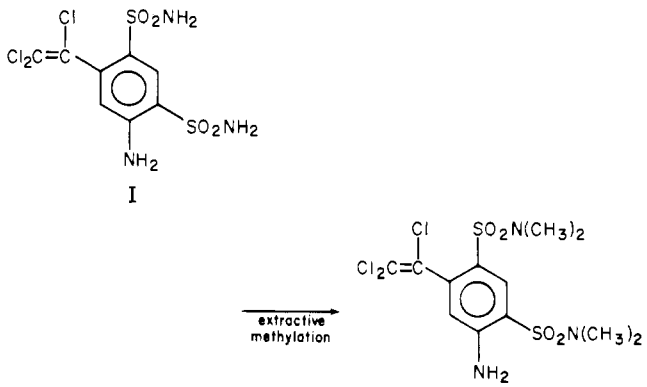
Table I. Levels of 4-Amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide in Bovine Milk and Bull Urine[a]

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.

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.

Tilly, 1973), barbiturates (Ehrsson, 1974), and the primary sulfonamides chlorthalidone (Ervik and Gustavii, 1974) and hydrochlorothiazide (Lindstrom et al., 1975). In our continuing studies to analyze sulfonamides in biological fluids (VandenHeuvel et al., 1975; VandenHeuvel and Gruber, 1975) we have now investigated the use of extractive methylation and found it a suitable derivatization technique for the assay of 4-amino-6-trichloroethenylbenzene-1,3-disulfonamide (I), a new anthelmintic effective against *Fasciola hepatica* (Mrozik, 1976; Ostlind and Mrozik, 1976), in bovine milk and urine and canine blood.



I

EXPERIMENTAL SECTION

Isolation of I from bovine milk is accomplished by adding 0.15 ml of glacial acetic acid to 1 ml of milk in a 15-ml glass centrifuge tube. After mixing, the sample is extracted with 2 ml of ethyl acetate, and the organic phase is transferred to a clean 15-ml glass centrifuge tube and taken to dryness with a stream of nitrogen. The residue is equilibrated with 1 ml of 0.1 N NaOH; following centrifugation 0.8 ml of the basic solution is transferred to a clean 15-ml centrifuge tube. The sample is derivatized by adding 0.05 ml of 0.1 M tetra-*n*-butylammonium hydroxide and 2 ml of 0.5 M methyl iodide in methylene chloride to the above solution. Stoppers are taped into position and the samples heated at 50 °C for 15 min with vigorous shaking for 15 s every 5 min. Samples are then centrifuged and the methylene chloride phase transferred to a clean centrifuge tube and blown to dryness with a stream of nitrogen. Samples are taken up in appropriate amounts of ethyl acetate and analyzed by automated injection in a Hewlett-Packard 7610A gas chromatograph using the following conditions: 4 ft × 2.5 mm i.d. column containing 3.8% W-98 on 100–120 mesh H-P Chromosorb at 225 °C; 60 ml/min of 95:5 argon/methane carrier gas; injection port temperature, 280 °C; 8 mCi of ^{63}Ni electron-capture detector, 310 °C, 50-μs pulse.

Urine and blood samples are assayed in similar fashion. Urine samples (0.1 ml of urine diluted with distilled water to 1.0 ml) are extracted as above. Blood samples are assayed as follows: to 0.5 ml of blood add 0.15 ml of glacial acetic acid. Extract twice with 1-ml portions of ethyl acetate, employing vigorous agitation of the two-phase system. The organic layers are combined in a clean 15-ml centrifuge tube and reduced to dryness as above. One-half milliliter of 0.1 N NaOH is added to the residue and the resulting solution is derivatized as above (i.e., tetra-*n*-butylammonium hydroxide/methyl iodide/methylene chloride) for milk, with the exception that the reaction period is extended to 20 min.

Demonstration of the presence of a "conjugate" of I in 4-days-off drug bull urine which had previously been extracted with ethyl acetate was accomplished as follows. The spent aqueous phase was acidified (pH 1) with HCl, refluxed for 1 h, neutralized (pH 8) with NaOH, and extracted with ethyl acetate. Evaporation of the extract gave a radioactive product which was examined by TLC (silica gel G, $CHCl_3$/MeOH, 4:1); the observed radioactive compound was eluted from the silica gel with ethyl acetate.

Mass spectrometric data were obtained with an LKB 9000 instrument equipped with an accelerating voltage alternator and employing a GLC column and conditions similar to those described above. Spectrometer settings were 3.5-kV accelerating potential, 270 °C source temperature, 60-μA trap current, and 70-eV ionizing potential (20 eV for mass fragmentography).

RESULTS AND DISCUSSION

Because of the highly polar nature of I this drug must be derivatized to allow its GLC analysis. A number of approaches were investigated. On-column methylation with trimethylanilinium hydroxide yielded multiple products in low yield, and trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide was also unfruitful. Reaction with dimethylformamide dimethyl acetal formed the expected tri-*N*-DMAM derivative (demonstrated by direct probe mass spectrometry (MS)). Attempts to achieve satisfactory GLC conditions for this product were unsuccessful, probably because conversion of primary sulfonamides and amines to *N*-DMAM derivatives leads to large increases in retention time (VandenHeuvel and Gruber, 1975). The success of Ervik and Gustavii (1974) and Lindstrom et al. (1975) in applying extractive alkylation to the GLC determination of sulfonamide drugs encouraged us to attempt this derivatization approach with I. Initial experiments using the appropriate reagents—methyl iodide, sodium hydroxide, tetra-*n*-butylammonium hydroxide, and methylene chloride—resulted in the conversion of I to a tetramethyl derivative plus a minor amount of pentamethyl product, as demonstrated by combined GLC–MS; the mass spectrum of the former is presented in Figure 1. GLC monitoring of the reaction products arising from a variety of reaction conditions (proportions and concentrations of the methyl iodide and tetra-*n*-butylammonium hydroxide, and temperature and duration of the reaction) allowed the establishment of conditions suitable for conversion to the tetramethyl derivative, which exhibits satisfactory GLC properties. Inspection of the functional group content of the derivative suggests that it should be readily detectable by electron capture, and this is the case; with normal operating conditions a value of 5.3×10^3 C/mol is observed, permitting detection of 0.02 ng. Comparison of detector response for known injected quantities of the derivative vs. aliquots of known amounts of I carried through the derivatization procedure indicated a yield of ~90%. An overall recovery of ~45% is found for drug added to milk, urine, or blood and carried through the isolation (and derivatization) procedure described in the Experimental Section. Control fluid spiked with I at various levels and carried through the entire procedure is used to establish a working curve (see Figure 2) for assay of samples from treated animals.

The chromatograms resulting from analysis of milk samples from a cow dosed with 6.6 mg/kg of I are presented in Figure 3. The drug levels at various times postdose as determined by GLC are presented in Table I. As the drug was labeled with ^{35}S, radiochemical techniques were employed for the determination of all drug-related species in the milk. The similarity of the two sets of data (parts per million levels) strongly suggests that only parent drug is present in the milk.

The urinary levels of I (GLC assay) for a bull dosed at the same level are significantly lower than the total residue

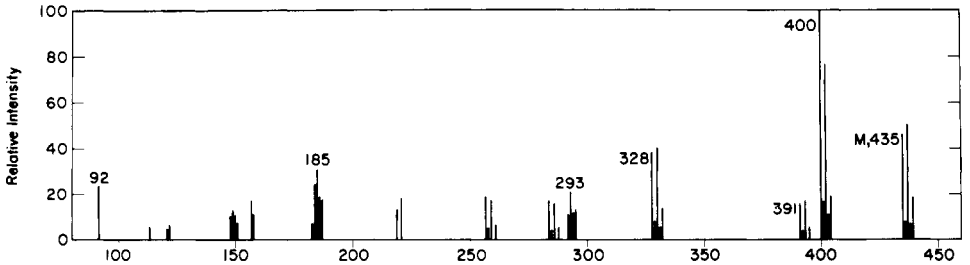


Figure 1. Mass spectrum of tetramethyl-4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide.

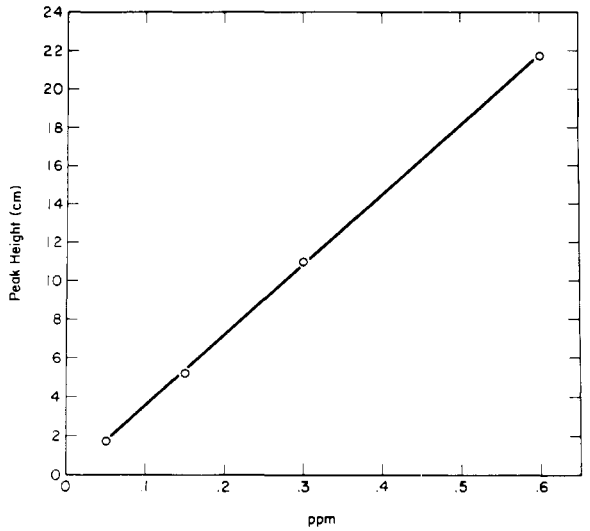


Figure 2. Typical working curve established by carrying control bovine milk spiked with 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide at 0.05, 0.15, 0.3, and 0.6 ppm through the assay procedure.

based on ^{35}S determinations (see Table I), indicating the presence of a metabolite or metabolites in urine. This has been found to be the case (Wolf et al., 1976). Bull urine preextracted with ethyl acetate to remove I was hydrolyzed with HCl, and a radioactive product which exhibited the same TLC behavior (R_f 0.73; see Experimental Section) as parent drug was obtained; the TLC zone was eluted and found by direct probe MS to contain I. GLC assay and radiochemical analysis of aliquots of the TLC isolate gave values of 17 and 19 μg of I, respectively. These data conclusively demonstrate that the urine contains a

Table II. Levels of 4-Amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide in Dog Blood 3 h Postdose

Dog	Dose, mg/kg	ppm
74-0592, M	Control	0.0
74-0569, F	Control	0.0
74-0582, M	25	29
74-0001, F	25	39
74-0584, M	50	43
74-0637, F	50	48
74-0010, M	100	64
74-0629, F	100	91

"conjugate" of I which is hydrolyzed to parent drug under acidic conditions.

In another study, male and female dogs were dosed with unlabeled I at three different levels and blood (3 h postdose) assayed for parent drug. The results are presented in Table II.

It is desirable to prove conclusively that the eluted substance quantitated in a GLC assay is truly the compound of interest. MS in combination with GLC can provide compelling evidence for such a confirmation. Unfortunately little quantitative information can be obtained from a single scan spectrum. The mass spectrometer can be used, however, as a selective GLC detector (Brooks and Middleditch, 1971) by monitoring the intensity of an ion or ions characteristic of the compound of interest. Such an approach, known by a variety of terms such as multiple ion detection or mass fragmentography, possesses the sensitivity and selectivity necessary to make it an attractive means for demonstrating the validity of GLC assays. This is especially true when the drug is not radioactive, precluding a radiochemical confirmation of the GLC assay. Such a mass spectrometric approach for a "confirmatory assay" for I as its tetramethyl derivative

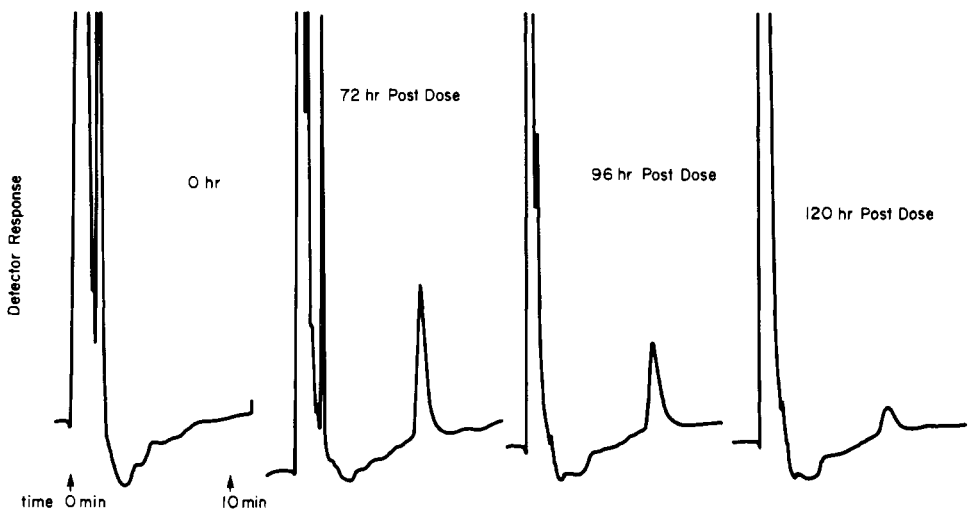


Figure 3. Gas chromatograms resulting from assay of milk (time 0, 72, 96, and 120 h postdose) from a cow dosed with 6.6 mg/kg 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide.

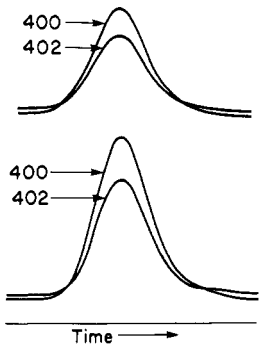


Figure 4. Mass fragmentograms (ions of m/e 400 and 402) resulting from analysis of 40 ng of tetramethyl-4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide (top) and an aliquot of the methylated isolate from the blood of a dog dosed with 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide (bottom).

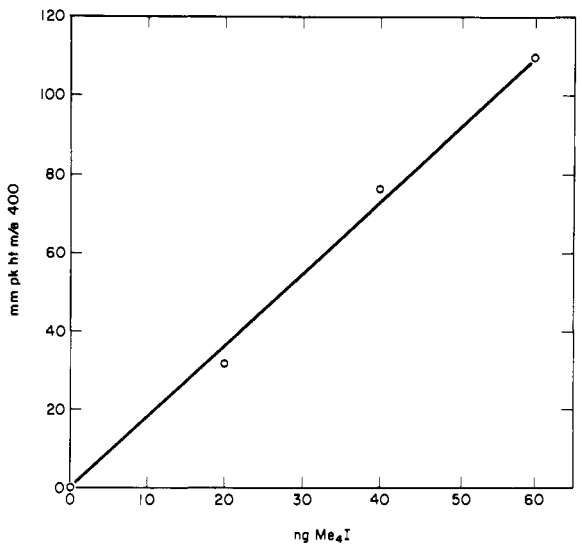


Figure 5. Plot of intensity of the ion of m/e 400 vs. nanograms of 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide as its tetramethyl derivative.

involves monitoring the intense ions (Figure 1) of m/e 400 and 402, M – Cl. The mass fragmentogram resulting from injection of 40 ng of tetramethyl-I gave the result shown in Figure 4 (lower). Analysis of an aliquot of the methylated isolate from dog 74-0637, F, yielded the mass fragmentogram shown in Figure 4 (upper). It is clear that in each case both monitored ions exhibited their maxima at the same retention time and with the same characteristic intensity ratio, fulfilling the requirements of confirmation by mass fragmentography (Hammar et al., 1968). Use of a calibration curve (Figure 5) established by monitoring the intensity of the ion of m/e 400 demonstrated that this aliquot contained 24 ng of I, equivalent to 48 ppm, the same value obtained for this blood by GLC.

ACKNOWLEDGMENT

The authors wish to thank F. R. Koniuszy and D. E. Wolf for the urinary metabolite, T. A. Jacob for the ^{35}S radiochemical values, R. E. Stoll for the blood samples, and D. A. Ostlind for the milk and urine samples.

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Received for review July 22, 1976. Accepted December 8, 1976.