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ANALYSIS, CHARACTERIZATION, AND PURIFICATION: REQUIREMENTS FOR TOXICOLOGICAL EVALUATION OF 1,4-DITHIANE

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SUMMARY

1,4-Dithiane is an organo-sulfur chemical associated with production and storage of munitions. Toxicological evaluation of this chemical was scheduled in rats because of the paucity of such information. Prerequisite for the evaluation was the development of gas chromatographic procedures using flame ionization detection to determine the purity of the test chemical and to certify the concentration and stability of the chemical in the dosage form, sesame oil. Mass and nuclear magnetic resonance spectrometry procedures are described for assessing the structural character and purity of the test chemical. A procedure for the purification of the test chemical and data concerning its solubility in various solvents are also presented.

INTRODUCTION

1,4-Dithiane (CAS No. 505-29-3) is a groundwater contaminant at certain military installations where munitions are stored.

There is a scarcity of toxicological data in the literature for 1,4-dithiane. The oral LD_{50} in rats is reported as 3500 mg/kg in unpublished results of a range-finding study by the U.S. Army Medical Bioengineering Research and Development Laboratory. In view of the lack of toxicity data, this chemical was proposed by the Department of the Army for 90-day sub-chronic toxicological evaluation at the National Center for Toxicological Research (NCTR).

However, before such toxicological tests could be initiated, analytical procedures were required for determining the purity, structural characterization, and stability of the test chemical in the dosage form used in the animal studies. Procedures were also needed for purification of the test chemical if deleterious impurities were found and for dosage form concentration certification assays.

Experiments to determine the toxicological effect of a test chemical require that the compound be examined for the presence of impurities prior to the initiation of animal tests. The presence of excessive amounts of impurities requires that the chemical be purified to minimize or eliminate the substances that might bias the experimental results[1]. Proper control of test substances must be maintained to ensure the validity of the experiment[2].

D'Agostino and Provost[3] determined gas chromatographic (GC) retention indices of chemical warfare agents and simulants, including dithiane, using GC–flame ionization detection (FID) with several different capillary columns. Adventitious trace organics, including 1,4-dithiane, have been identified in nitramine munitions recrystallization process samples using GC–mass spectrometry (MS)[4]. Neither of these methods was totally adequate for use in our proposed work.

This paper describes GC–FID procedures (packed and capillary columns) for determination of the purity of the test chemical and its stability in sesame oil. MS and nuclear magnetic resonance (NMR) spectrometry methodologies are also presented for structural characterization and purity determinations of the test chemical. Recrystallization techniques for purification of 1,4-dithiane are also described. Data concerning the approximate solubility of 1,4-dithiane in various solvents and its stability in sesame oil are also reported.

Data obtained in these experiments allowed the 90-day sub-chronic toxicological evaluation of 1,4-dithiane in cesarean derived (CD) rats to proceed.

EXPERIMENTAL

Test chemical and solvents

The test compound 1,4-dithiane was purchased from Fairfield (Blythewood, SC, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.). Pure cold-pressed sesame oil was purchased from Hain Pure Food (Los Angeles, CA, U.S.A.). All chromatographic solvents were CP grade, residue analyzed.

Gas chromatography

Purity and dose certification assays. Purity assays were performed by GC on packed and capillary columns using conditions described in Table I. Injections on packed columns were 2 μl, and those on capillary columns were 1 μl. Injections for headspace analysis were 50 μl. Samples for purity assays were prepared at a concentration of 1 mg/ml in dichloromethane. Gavage solutions for use in toxicological tests were prepared at concentrations of 35 and 2 mg/ml in sesame seed oil. These two solutions were diluted to 1 and 0.2 mg/ml, respectively with dichloromethane for dose certification analysis by GC–FID.

Stability assays. Stability assays were performed by GC on packed columns using conditions described in Table I.

Mass spectrometry

A Finnigan (San Jose, CA, U.S.A.) Model 4023 mass spectrometer operated in the electron impact (EI) mode was used to analyze solid samples of 1,4-dithiane via a solids probe. The dry sample was placed into a glass sample cup, which was placed in the solids probe. The probe was inserted into the solids probe inlet, cooled to 0°C and the inlet was evacuated. The probe was cooled to −30°C before insertion into the mass spectrometer. Data were collected as the probe was slowly heated. The ion source temperature was 250°C and the electron energy was 70 V. Representative spectra were compared to the NIH/EPA library, which contained the mass spectrum for 1,4-dithiane.

Head space samples (100 μl) were obtained and injected via a Grob injector (splitless mode) into the GC–MS under conditions described in Table I.

TABLE I

INSTRUMENTAL CONDITIONS FOR VARIOUS ANALYSES OF 1,4-DITHIANE

	Column and temperature	*Detector temperature*[a]	*Injector temperature*	*Carrier gas and flow-rate*
GC Analysis				
Purity, dose certification, solubilities	3 ft. × 2 mm I.D. glass, 3% SP2250 on Supelcoport, 100–120 mesh; 80°C	280°C	120°C	N_2; 30 ml/min
Purity	30 m × 0.259 mm I.D. DB-1701; programmed 50–280°C at 20°C/min	300°C	programmed 50–280°C at 140°C/min	He; 1 ml/min
Purity (headspace)	15 m × 0.259 mm I.D. DB-1701; 34°C	280°C	110°C (split mode with 30:1 ratio)	He; 1 ml/min
Stability	240 cm × 4 mm I.D. glass, 10% OV-101 on Gas Chrom Q, 80–100 mesh; 150°C	270°C	160°C	He; 100 ml/min
GC–MS analysis				
Purity (headspace)	30 m × 0.259 mm I.D. DB5; programmed 40–150°C at 20°C/min		Grob splitless mode 255°C	He; 2 ml/min

[a] FID detector.

Nuclear magnetic resonance spectrometry

The 1,4-dithiane was dissolved in chloroform-d_2 (Merc Isotopes, Rahway, NJ, U.S.A.) at a concentration of 100 mg/ml for structure verification. The ^1H spectrum was obtained with a Model WM-500 NMR (Bruker Instruments, Bilerica, MA, U.S.A.) using standard acquisition parameters.

To estimate concentrations of impurities, proton NMR determinations were performed on a Model WM-500 NMR (Bruker Instruments) at room temperature in chloroform-d_2 (1 mg/ml). One transient was collected for each sample with a 78° flip angle (11.5 μs, 90° flip angle), 30K data points, 7024-Hz window, and a 20-s delay prior to pulse. The free induction decay data was treated with a 0.5-Hz line broadening prior to Fourier transformation. The spectra were analyzed for dichloromethane (singlet) and ethanol (methylene quartet and methyl triplet) by chemical shift using chloroform as a reference. Quantitation of the observed impurities was accomplished by integration of impurity resonances with respect to the 1,4-dithiane resonance.

Stability experiments

A stability study was performed to ensure that sesame oil solutions of 1,4-dithiane at the low (2 mg/ml) and high (35 mg/ml) dose levels could be stored and remain stable for the term of the study. Solutions of the chemical in sesame oil were prepared at the concentrations previously described, placed in 25 ml amber vials, sealed with Teflon™ faced septa and stored at −20°C. Triplicate vials of the high and low concentrations were assayed on days 0, 1, 2, 7, 14, 21, 28, 42, 56, and 90 after preparation, as described under *Stability assays*. Solutions were thoroughly mixed prior to taking a sample for analysis.

Purification of 1,4-dithiane

Approximately 100 g of 1,4-dithiane were dissolved in one liter of boiling absolute ethanol. After filtration the hot solution was rapidly cooled in an ice bath and the recrystallized dithiane was harvested. The recrystallized product was then allowed to air dry in a fume hood. This process was repeated several times to obtain sufficient amounts of the test chemical to perform the proposed toxicological tests.

Solubility determinations

The following are descriptions of the determination of approximate solubilities of 1,4-dithiane in the solvents listed.

Acetone, methanol and hexane. A 5-ml volume of each solvent was saturated with the test chemical. The mixture was centrifuged, a 100-μl aliquot was removed, diluted to 10 ml with dichloromethane and analyzed by GC–FID on a column containing 3% SP 2250 with GC conditions described in Table I. Quantitation was made by comparison to an appropriately diluted standard in dichloromethane.

Sesame oil. 1,4-Dithiane (100 mg) was placed in a tube and 2 ml of sesame oil were added. The mixture was sonicated causing the oil temperature to increase, which resulted in dissolution of the dithiane. Upon cooling to 25°C crystallization of a portion of the dithiane occurred. The suspension was centrifuged and a 100-μl aliquot taken and diluted to 5 ml with dichloromethane. GC–FID analysis was performed as described in the previous section.

Water. A 44-mg amount of 1,4-dithiane was placed in a tube, and 40 ml of deionized water were added. The tube was capped, vortexed for 20 min and filtered through a 0.45-μm filter. A 3-ml sample was removed and partitioned against an equal volume of dichloromethane. The dichloromethane fraction was analyzed by GC–FID as described for acetone, methanol and hexane.

DMSO and Tween 80. Amounts of 120 mg and 100 mg of dithiane were dissolved in 1 ml of DMSO and 1 ml of Tween 80, respectively, at 65°C. Upon cooling each solution to 35°C, crystallization of a portion of the dithiane occurred. Each suspension was centrifuged and a 100-μl aliquot taken and diluted to 10 ml with dichloromethane. GC–FID analysis was performed as described for acetone, methanol and hexane.

RESULTS AND DISCUSSION

When a chemical is proposed for toxicological evaluation it is imperative that preliminary experiments be performed to ensure the validity of the toxicology experiments. The chemical structure of the test compound must be verified and its purity determined. The impurities detected (if any) must be identified, if possible, to ensure that these do not bias the results of the toxicological experiment. Verification of the chemical structure of 1,4-dithiane was made using the Bruker Model WM-500 NMR. The ^1H spectrum was obtained from a solution of the chemical in chloroform-d_2 (100 mg/ml), and a singlet at 2.8 ppm[3] was observed, which matches the literature spectrum for this compound. The structure of 1,4-dithiane was also verified by EI-MS (solids probe), and the mass spectra are illustrated in Fig. 1. The library search produced 1,4-dithiane as the best match (94%), with a spectral purity of 72% indicating possible impurity ions. These impurity ions were determined to be from the

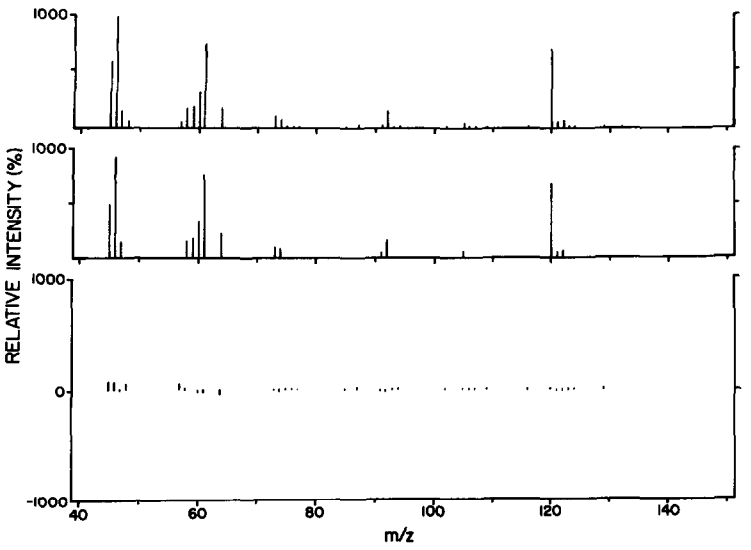

Fig. 1. Mass spectra of 1,4-dithiane. Upper is sample spectrum, middle is library spectrum for 1,4-dithiane, lower is sample spectrum subtracted from library spectrum.

Freon 12 used to cool the probe. The spectrum of the major constituent matched well with 1,4-dithiane after subtraction of the Freon 12 peaks. The isotope ratios of the M, M + 1, and M + 2 ions (*m/z* 120, 121, and 122) indicated a compound with molecular weight 120 containing two sulfur atoms, which is consistent with the 1,4-dithiane structure. The purity of the compound as received was determined by GC–FID with both packed and capillary columns. The chromatogram in Fig. 2 is the result of a

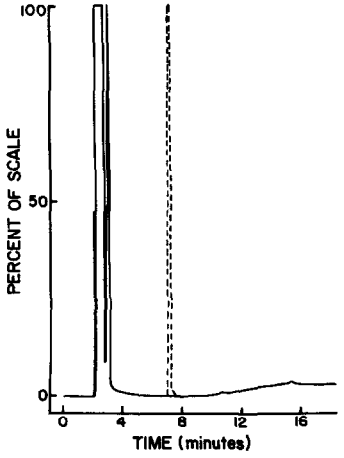

Fig. 2. Gas chromatograms of purity determination of 1,4-dithiane. Solid line is blank assay. Dotted line superimposed represents the 1,4-dithiane peak. Column, 30 m × 0.259 mm I.D. DB-1701; He carrier gas (1 ml/min); programmed from 50°C to 280°C at 20°C/min; FID detector temperature, 300°C, injection port programmed from 50°C to 280°C at 140°C/min; injection volume, 1 μl.

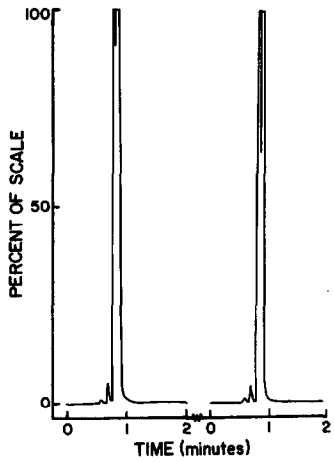

Fig. 3. Successive injections of headspace samples. Column, 30 m × 0.259 mm I.D. DB-1701; He carrier gas (1 ml/min); column and injector temperatures, 34°C, FID detector temperature, 300°C.

temperature-programmed determination on the DB-1701 capillary column. The retention time for 1,4-dithiane was 7.15 min, using conditions described in Table I. The GC–FID assays indicated that the compound was essentially pure. However, these GC conditions are not amenable to detection of more volatile impurities.

In an attempt to detect more volatile impurities in the test compound, qualitative determinations were performed by sampling the headspace above the solid compound in sealed containers with a gas tight syringe and chromatographing the samples on a DB-1701 capillary column at 34°C with FID. Fig. 3. illustrates two successive injections, which show two major volatile impurities. A headspace sample was procured in like manner for determination by GC–MS in an attempt to identify these impurities. Three peaks were observed while the column oven temperature was 40°C. One peak consisted of a single ion of m/z 44 and was determined to be carbon dioxide. The second peak exhibited ions at m/z 43, 46, and 58. This second component appeared to be acetone since its spectrum contains two of the major ions found in the standard mass spectrum of acetone (43 and 58) in the proper ratio (100:30). The presence of acetone may be from the manufacturer using this solvent in synthesis or purification schemes. The third peak had major ions at m/z 84,, 86, and 88 with abundances similar to those contributed by a dichloro compound. This spectrum was subjected to a library search, which produced a match with dichloromethane (95% fit). The spectra are shown in Fig. 4 along with the library spectra for dichloromethane. The concentration of dichloromethane in the test compound was estimated by NMR, as previously described in the Experimental section, to be 0.2%. Based on the techniques used, the purity of the test chemical was 99.8%. As dichloromethane is a known carcinogen, its presence in the test compound could bias any toxicological results obtained. Dichloromethane in the test chemical resulted from the manufacturer's recrystallization and purification of the product before sale. Its presence necessitated purification of the test chemical prior to the initiation of the toxicoligical experiment.

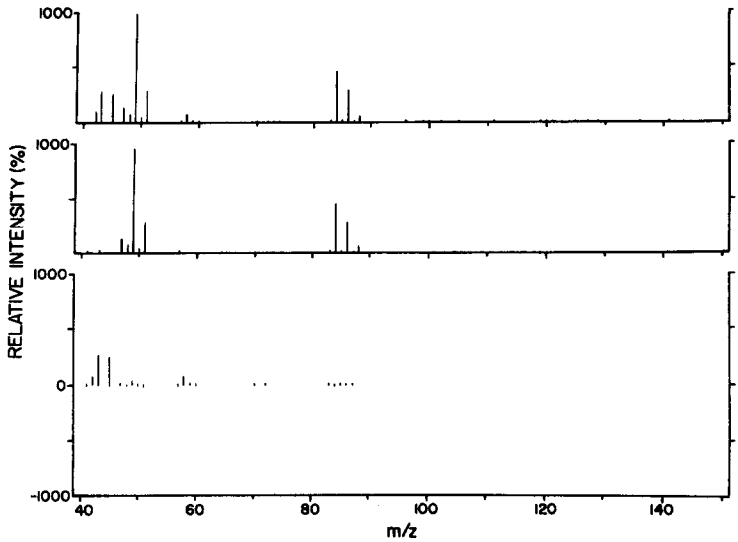

Fig. 4. Mass spectra of headspace sample. Upper is sample spectrum, middle is library spectrum for dichloromethane, lower is sample spectrum subtracted from library spectrum.

There were two obvious procedures for eliminating dichloromethane from the 1,4-dithiane — sublimation and recrystallization. Preliminary experiments were initiated to compare the efficiency of each technique. Sublimation worked well but the apparatus available was quite small and, considering the amount of compound required for this study, would have been very time-consuming and probably would have delayed the start of the experiment. It was determined that the compound could be recrystallized from absolute ethyl alcohol with a recovery of *ca.* 65%, and this method was used to purify all of the test compound. Residual ethanol in the recrystallized chemical was estimated by NMR, as described in the experimental section, to be *ca.* 0.5% and was deemed acceptable. The purity of the recrystallized product was 99.5% based on the NMR techniques used and contained no detectable dichloromethane. Samples of recrystallized and sublimed dithiane gave identical responses

TABLE II

APPROXIMATE SOLUBILITIES OF 1,4-DITHIANE IN VARIOUS SOLVENTS

Solvent	*Solubility (mg/ml)*
Sesame oil	40[a]
DMSO	98[b]
Tween 80	99[b]
Acetone	79[a]
Methanol	21[a]
Hexane	17[a]
Water	0.6[a]

[a] At 25°C.
[b] At 35°C.

TABLE III

STABILITY OF 1,4-DITHIANE IN SESAME OIL
Mean and standard deviation of triplicate assays.

Day	*Low concentration 2 mg/ml*	*High concentration 35 mg/ml*
0	1.77 ± 0.01	32.7 ± 0.94
1	1.68 ± 0.04	33.4 ± 1.36
2	1.64 ± 0.04	33.0 ± 1.23
7	1.62 ± 0.03	33.6 ± 0.40
14	1.63 ± 0.10	33.0 ± 0.59
21	1.64 ± 0.04	33.8 ± 0.58
28	1.67 ± 0.03	32.7 ± 0.77
42	1.59 ± 0.08	34.3 ± 1.01
56	1.77 ± 0.07	33.0 ± 0.75
90	1.81 ± 0.06	34.4 ± 0.86

when analyzed by GC–FID. Triplicate injections of each purified sample indicated a purity of 100 ± 1%. These data along with NMR and MS data indicate that the dithiane was essentially pure with the exception of the 0.5% ethanol.

The lack of information in the literature on 1,4-dithiane required experiments be performed to determine a suitable medium in which to administer the chemical to test animals. Due to the volatility and stench of the chemical, it was unlikely that it could be administered in feed. Initial experiments, however, showed that the animals would eat feed mixed with the compound. Unfortunately, even after pelletizing the mixed feed, stabilities of the chemical at the concentrations proposed for the study were not adequate. In an attempt to find a suitable solvent for use as a possible vehicle for delivery of the test chemical to the test animals by gavage, the approximate solubility of 1,4-dithiane in several solvents was determined. Table II lists the solubilities. Sesame oil provided adequate solubility to allow solutions of desired concentration to be prepared for dosing test animals in the proposed toxicological tests and was chosen over other solvents listed. Solutions of 1,4-dithiane in sesame oil at 2 and 35 mg/ml were prepared and subjected to a 90-day stability experiment at −20°C as previously described. The results of that experiment are presented in Table III, which demonstrate that the test chemical is stable in sesame oil stored at −20°C for 90 days.

With information developed in this study, it was then possible to prepare an experimental protocol for conducting the 90-day sub-chronic toxicological evaluation of 1,4-dithiane in rats.

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