

# An Evaluation of Preparation Methods and Storage Conditions of Tribromoethanol

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This reports the *in vitro* portion of a study designed to establish guidelines for the preparation, storage, and use of tribromoethanol (TBE). We evaluated: 1) the purity of TBE powder from three suppliers; 2) nine methods of preparation of a 25-mg/ml (working) solution for formation of particulates and breakdown products; 3) formation of particulates and breakdown products and pH change in 1-g/ml (stock) solutions and working solutions stored under four conditions (25°C and 5°C in light and in dark); and 4) stock and working solutions of TBE that caused lethal effects in mice. These objectives were met by using nuclear magnetic resonance spectroscopy, gas chromatography–mass spectroscopy, particle-size and turbidity analyses, and pH strips. TBE powder from three suppliers varied in purity. No significant differences in breakdown product formation, particle size, or turbidity were noted between the nine preparation methods evaluated. Stock solutions and the working solution stored at 5°C in the dark maintained a pH of 6.5 to 7.0, whereas the pH dropped for all other working solutions. A low level of dibromoacetaldehyde (DBA), a potential breakdown product reported to cause toxic effects, was detectable in all newly prepared solutions. Regardless of the storage condition or pH, DBA concentration did not increase measurably in any of the solutions after 8 weeks. The stock and working solutions that demonstrated lethal effects in mice had a pH of 6.5 and did not differ notably from newly prepared, non-lethal solutions, when evaluated for DBA. A decrease in pH could not be correlated to an increase in DBA or potential lethality, as suggested in the literature. The toxicity associated with the lethal TBE in this study appears to be a result of a chemical reaction or breakdown product that has not yet been reported.

Tribromoethanol (TBE), a non-pharmaceutical grade anesthetic, is often used for short surgical procedures in mice because it is a non-controlled substance with the ability to rapidly induce surgical anesthesia followed by a fast recovery. Although TBE is commonly used, there is much controversy in the literature concerning its use in laboratory animals. Several reports indicate a potential for adverse effects, including splenic lymphocyte injury, peritonitis, serositis, intestinal ileus, muscle necrosis, and death (1-6). Other reports point out the absence of adverse effects associated with TBE administration (7, 8). Because TBE is a non-pharmaceutical grade compound, these discrepancies could be attributed in part to differences in the preparation of TBE by different users. Moreover, the storage conditions under which TBE solutions are maintained also might account for these discrepancies, as these conditions were not described completely in previously published studies.

Adverse effects associated with TBE administration have been attributed to dibromoacetaldehyde (DBA; compound B in equation 1) and hydrobromic acid (HBr) (compound C), which are considered to be the breakdown products of TBE (A) (9, 10).



Multiple sources comment that the presence of these two compounds is signified by a drop in the pH of the solution to < 5 (7, 9, 11). A drop in pH could be a result of the formation of HBr but does not necessarily eliminate other possible breakdown mechanisms, nor does it constitute proof that breakdown occurs via formation of HBr and DBA. To the best of our knowledge, a thorough investigation has not been conducted to prove the formation of these breakdown products or to demonstrate that these products cause adverse effects in mice.

This is the first segment of a two-part study designed to establish guidelines for the preparation, storage, and use of TBE. This *in vitro* part of the study consisted of four objectives: 1) to evaluate the purity of TBE powder from three commercial suppliers; 2) to evaluate nine different methods reported in the literature for preparation of a 25-mg/ml (working) solution of TBE; 3) to evaluate crystal and breakdown product formation and pH change in 1-g/ml (stock) and working solutions under four storage conditions; and 4) to evaluate stock and working solutions that caused lethal effects in mice.

## Materials and Methods

**TBE stock solution preparation.** Stock solutions (1 g/ml) of TBE were prepared in Class B borosilicate glass vials (Fisher Scientific, Pittsburgh, Pa.) wrapped in aluminum foil to eliminate light. A glass pipette or Hamilton syringe was used to add 1 ml of *tert*-amyl alcohol (TAA, Sigma Aldrich, St. Louis, Mo.) to each 1-g aliquot of TBE powder (2,2,2-tribromoethanol, Acros Organics [Pittsburgh, Pa.]), as previously described (6, 12). The solution was mixed by vortexing for 2 min at room temperature (25°C). Gloves and a fume hood were used during preparation, glass vials were autoclaved, and all glassware was triple-rinsed with TAA before use.

**DBA preparation.** The DBA used as a reference in this study was synthesized by R. E. Forslund, PhD (Chemistry Department, University of Illinois at Chicago). Dibromacetaldehyde was prepared according to the procedure of Shchukina, as previously described (13). Briefly, a solution of acetaldehyde in ether was cooled to 0°C by an ice bath. To this, 18 g of bromine was added dropwise maintaining the temperature at 0°C, after which the reaction was allowed to warm to room temperature. After dropwise addition of another 18 g of bromine, the reaction was stirred for 24 h. The reaction was then purified by three short-path distillations at atmospheric pressure. The structure and purity of the product was demonstrated by nuclear magnetic resonance (NMR) spectroscopy and comparison of the gas chromatography–mass spectrum with the National Insti-

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tute of Standards and Technology (NIST98) mass spectral library reference database (14). The dibromoemethyl and aldehyde protons of DBA give rise to NMR doublet peaks ( $J_{\text{H-H}} = 5.0$  Hz) at 5.7 and 9.1 ppm, respectively.

**NMR spectroscopy.** NMR spectroscopy is an analytical technique that permits both the determination of chemical structure and purity of unknown compounds and the analysis of mixtures of compounds. This is done through the detection of atomic nuclei from any isotope that exhibits a magnetic moment, such as  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$ . In this investigation  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded to obtain specific structural information about the compounds of interest (i.e., TBE, TAA, and DBA). Reactions occurring during storage of solutions were characterized primarily by  $^1\text{H}$  NMR spectroscopy, which observes hydrogen whether rapidly exchanged, as with water in aqueous solutions, or covalently linked, as in DBA. It should be noted that the accepted international unit for the X axis of an NMR spectrum is called "chemical shift" and is expressed in parts per million (ppm). The unit does not refer to concentration; it is a dimensionless normalized unit that refers to variations in magnetic field strength.

Most NMR spectra for this study were obtained with a Bruker AVANCE 360 MHz NMR spectrometer with a 5-mm QNP probe set up to observe  $^1\text{H}$  in a sample of approximately 600  $\mu\text{l}$ . Some spectra were obtained with a Bruker AVANCE 500 MHz NMR spectrometer equipped with a 5-mm BBO probe.  $^1\text{H}$  NMR spectra were recorded under quantitative conditions with a  $30^\circ$  pulse angle, recycle delay of 2 sec, and 128 acquisitions. The lowest detectable limit for DBA with  $^1\text{H}$  NMR was 1.6 mg/ml, and as little as 5% conversion of TBE to DBA could be detected. Selected samples also were analyzed by  $^{13}\text{C}$  and 2-dimensional NMR methods to corroborate interpretations of  $^1\text{H}$  NMR spectra. The purity and composition of TBE powder were determined with solutions in perdeuteriochloroform. Aliquots of some samples were dissolved in perdeuteriochloroform or perdeuterioacetonitrile in order to permit more extensive NMR analysis of degradation products during storage.

**Particle size analysis.** The presence of particles was analyzed with a NICOMP 380 Submicron Particle Sizer (Particle Sizing System, Santa Barbara, Calif.) equipped with a helium–neon laser at 632.8 nm. The NICOMP is based on the concept of dynamic light scattering (quasi-elastic light scattering) that determines the change in mean particle size (range, 1 nm to 1  $\mu\text{m}$ ) as well as the distribution of particle sizes. The instrument calculates the mean particle diameter based on the principle of diffusion coefficients of particles in solution and estimates the particle size based on an autocorrelation function that uses the Stokes–Einstein equation for particle dynamics. This instrument is equipped with a neutral density filter that controls the intensity of light passing through the sample cell. This filter was initialized prior to each session of analysis, and each sample was run for two cycles of 10 min each with a channel width of 10.0  $\mu\text{sec}$ .

**Turbidity analysis.** Turbidity of the samples was analyzed with a Perkin–Elmer Lambda 35 UV-Vis spectrophotometer with scanning capabilities. Briefly, this machine measures absorbance of ultraviolet (UV) light and intensity of scattered visible light, thereby determining the turbidity level to provide a measurement of the concentration of solid particles in the solution. Each 1-ml sample was analyzed at wavelengths of 300 and 600 nm for the presence of particulates. The machine was zeroed with the diluent that was used during the preparation of working solutions and TAA for stock solutions.

**Gas chromatography–mass spectrometry (GC–MS).** GC–MS permits separation of individual components from a mixture by gas chromatography before each is introduced sequentially into the mass spectrometer. In most cases one does not need to know the identities of the compounds in advance in order to detect their presence with

GC–MS. Unknown compounds can be identified by a number of methods, such as comparison of the spectra with a database of reference compounds. Both TBE and DBA are readily detected and identified by GC–MS.

Analysis was performed on a JEOL GCMate II high-resolution double-focusing magnetic sector benchtop GC–mass spectrometer. The mass spectrometer is interfaced with an Agilent 6890 gas chromatograph with an electronic pressure control and a split/splitless capillary column injector via a heated interface. The GC is equipped with an Agilent 7673 Automatic Liquid Sampler with 100-vial sample tray. A 30 m (length)  $\times$  0.25  $\mu\text{m}$  (inner diameter)  $\times$  0.5  $\mu\text{m}$  (film thickness) HP-5ms capillary GC column (Agilent Technologies, Palo Alto, Calif.) was used. The carrier gas was helium at a flow rate of 1 ml/min. Samples were prepared for GC–MS by diluting the 25-mg/ml working and 1-g/ml stock solutions 50-fold and 100-fold, respectively, with acetone (GC Resolve, Fisher Scientific). One  $\mu\text{l}$  of each acetone solution was injected in splitless mode at an injector temperature of  $230^\circ\text{C}$ . The GC oven temperature program had an initial temperature of  $40^\circ\text{C}$ , held for 1 min, followed by a  $10^\circ\text{C}/\text{min}$  heating rate to  $180^\circ\text{C}$ , where it was held for 5 min. The GC interface to the mass spectrometer was set at  $250^\circ\text{C}$ . Solvent delay was 3.5 min, with a total GC–MS run time of 20 min. The mass range was scanned from  $m/z$  10 to  $m/z$  400. Compounds were ionized under positive electron-impact conditions using electron energy of 70 eV. Slit settings were appropriate for a resolving power of 1000 (20% valley). Synthesized DBA and commercially available TBE (Sigma Aldrich, St. Louis, Mo.) were analyzed to determine retention times and establish reference mass spectra for these compounds. The retention time was 9.9 min for TBE and 4.6 min for DBA (10  $\mu\text{g}/\text{ml}$ ). Unknowns were searched against the NIST98 mass spectral library reference database (14).

**Experimental design. (i) Experiment 1—evaluation of TBE powder.** One sample each from three different suppliers of TBE powder (Acros Organics [Pittsburgh, Pa.], Sigma Aldrich, and Fluka [St. Louis, Mo.]) were analyzed for impurities by  $^1\text{H}$  NMR spectroscopy. Representative aliquots of the TBE powder were dissolved in separate NMR tubes by using 0.6 ml of perdeuteriochloroform from ampules of the same batch. The solvent alone was analyzed to establish the baseline for TBE analysis. The sample found to have the lowest concentration of impurities was used for subsequent *in vitro* and *in vivo* experiments.

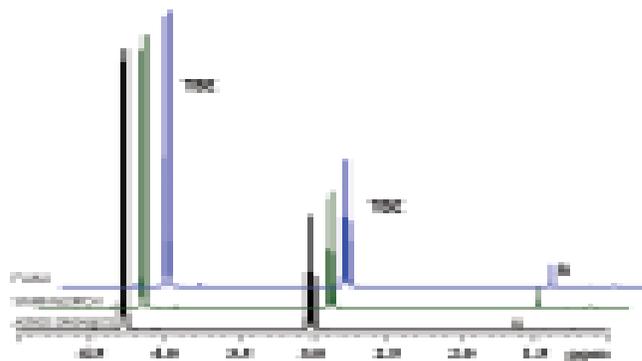
**(ii) Experiment 2—evaluation of preparation of working solution.** The preferred method for preparation of 25-mg/ml working solutions of TBE for subsequent studies was determined by diluting a newly prepared stock solution at each of three different temperatures (25, 37, and  $42^\circ\text{C}$ ) with three diluents for a total of nine samples. The three diluents were 0.9% NaCl (Baxter Healthcare Corporation, Deerfield, Ill.), Water for Embryo Transfer (ET) (Sigma Aldrich), and sterile water for injection (Abbott Laboratories, North Chicago, Ill.). These three diluents and temperatures were chosen because they reflect the various mixing temperatures and diluents that have been reported (2, 3, 15). To prepare a working solution, the diluent was added to the stock solution to produce a 2.5% aqueous solution. The solutions prepared at 37 and  $42^\circ\text{C}$  were stirred manually in a water bath at the desired temperature for 2 min after the diluent was added, and room temperature solutions were vortexed for 2 min after the diluent was added. Solutions were sterile filtered into a new vial with a 0.2- $\mu\text{m}$  Durapore polyvinylidene fluoride membrane Millex-GV syringe filter unit (Fisher Scientific). Each of the nine samples was evaluated by  $^1\text{H}$  NMR spectroscopy, UV turbidity analysis, and particle size analysis in order to determine the best method for preparation as defined by minimal turbidity and minimal formation of particles and breakdown products. The solutions

were also evaluated via S/P pH strips (Cardinal Health, Dublin, Ohio) with a range of 0.0 to 6.0 or 4.5 to 10.0 and a sensitivity of 0.5. Each of the nine samples was evaluated in triplicate for UV turbidity and particle size analysis and once for pH measurement and  $^1\text{H}$  NMR analysis. This set of experiments was repeated a total of three times, on three separate days, with nine samples on each day. For each day of preparation, all working solutions were prepared from a single, newly prepared, stock solution. The preparation and diluent that was determined to be the most ideal, using the criteria described, was used to prepare working solutions for all subsequent studies. Gloves and a fume hood were used during preparation, glass vials were autoclaved, and all glassware was triple-rinsed with the respective diluent.

(iii) **Experiment 3—evaluation of stock and working solutions after storage.** The four storage conditions evaluated were room temperature ( $25^\circ\text{C}$ ) in light (25L) and dark (25D) and refrigeration ( $5^\circ\text{C}$ ) in light (5L) and dark (5D). Light levels were monitored using a Foot Candle/Lux Meter (Extech Instruments, Waltham, Mass.) and were maintained at 100 to 130 lx. To achieve dark conditions, vials were wrapped in aluminum foil and placed under a light-impermeable box. Newly prepared stock and working solutions (day 0) were analyzed prior to placement into one of the four storage conditions. Stock solutions were tested via  $^1\text{H}$  NMR spectroscopy, UV turbidity analysis, and pH measurement on day 0, day 8, week 4, and week 8. In addition, both working and stock solutions were evaluated via GC–MS on day 0, week 4, and week 8. Working solutions were evaluated by  $^1\text{H}$  NMR, UV turbidity analysis, particle size analysis, and pH measurement at days 0, 1, 2, 3, and 4 and then twice weekly for a total of 8 weeks. On each day of analysis, a sample from each storage condition was evaluated twice for UV turbidity and particle size analysis and once for pH measurement and  $^1\text{H}$  NMR analysis. Stock and working solutions were monitored for bacterial contamination, with negative results throughout the duration of the study.

(iv) **Experiment 4—analysis of a lethal solution.** During the *in vivo* portion of this study, a working solution that was prepared by dilution of newly made stock solution and used immediately without storage was demonstrated to have toxic effects on 10 of 17 female ICR mice (16). The working solution that was administered was prepared aseptically with sterile water and was cultured on a Tryptic Soy Agar with 5% sheep blood plate (Remel, Lenexa, Kans.) to assess bacterial contamination. In addition, the working solution was submitted to Charles River Laboratories, Endosafe Division (Charleston, S.C.) and assessed for the presence of endotoxins (Kinetic LAL analysis, ES-SOP-502-06). The stock and working solutions and TBE powder from which the toxic solutions were made were evaluated via  $^1\text{H}$  NMR spectroscopy,  $^{13}\text{C}$  NMR spectroscopy, and GC/MS.

**Statistical analysis.** A two-way analysis of variance (ANOVA) was used to evaluate mean turbidity values at 300 and 600 nm for diluent, temperature, and the temperature–diluent interaction for newly prepared solutions in experiment 2. For experiment 3, turbidity values at 300 and 600 nm were evaluated for stock and working solutions between groups on each day as well as across the different time points for that condition. A two-way ANOVA with a Bonferroni correction was used to evaluate data generated from NICOMP particle size analysis. For experiment 2, the preparation conditions were evaluated on each day, as well as across the three experimental trials. For experiment 3, storage conditions for working solutions were evaluated between groups on each day as well as across the different time-points for each condition. Significance was inferred at the  $P < 0.05$  level. GC–MS and NMR spectroscopy were not analyzed by conventional statistical methods; reliability of the data was confirmed by running experiments under different instrumental and incubation conditions with more than one analytical technique (in this case, both NMR spectroscopy and GC–MS).



**Figure 1.** Offset and stacked  $^1\text{H}$  NMR spectra of TBE powder (2,2,2-tribromoethanol) from three suppliers dissolved in perdeuteriochloroform. The two resonances of dibromoacetaldehyde (DBA), which would appear at 5.7 ppm and 9.1 ppm, were not detectable in these spectra. Therefore, peak A arises from a contaminant other than DBA. Comparison of relative peak intensities indicates that the sample from Acros Organics contains the lowest level of contaminants. Spectral interpretations are given in Results.

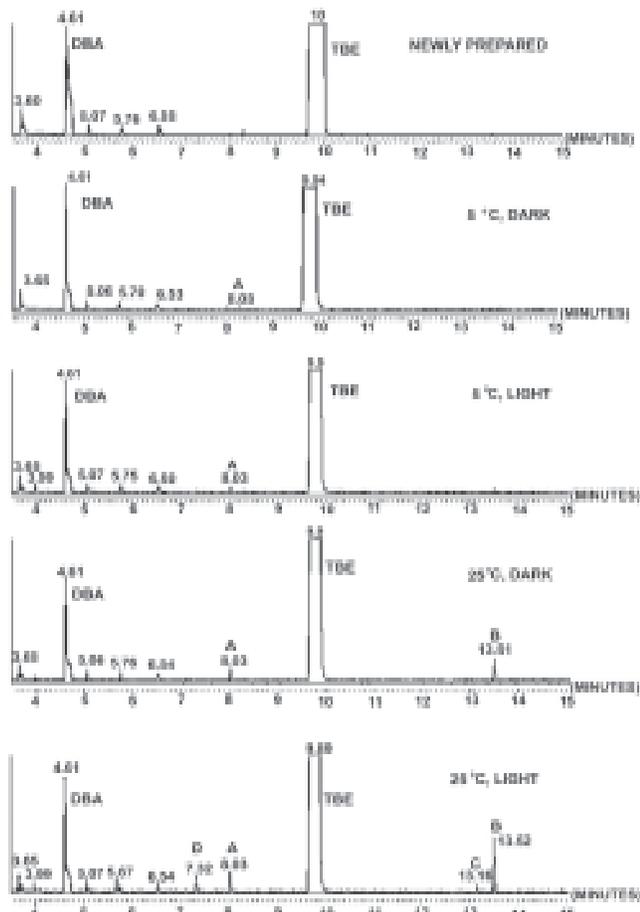
## Results

**Evaluation of TBE powder.**  $^1\text{H}$  NMR spectra of TBE powder from different sources (Fig. 1) indicated a variation in purity among suppliers (Acros Organics, 99.5%; Sigma Aldrich, 99%; Fluka, 98%). The purity was calculated by integrating the areas of each peak. The largest peak of TBE was set to a value of 100, and the value of peak A was measured at 2 for Fluka, 1 for Sigma Aldrich, and 0.5 for Acros Organics (Fig. 1). The doublet peak at 4.3 ppm arises from the two protons of the methylene group of TBE, whereas the triplet peak at 3.1 ppm arises from the proton of the hydroxyl group. The splitting constants for these two multiplets were the same (i.e.,  $J_{\text{H-H}} = 7.8$  Hz). The very narrow peaks of the hydroxyl proton indicate that the TBE and perdeuteriochloroform were very dry. The  $^1\text{H}$  NMR resonances of DBA were not detected in any sample of TBE. The multiplet peak labeled A in the spectra of commercial TBE samples (Fig. 1) arises from a contaminant other than DBA. The TBE from Acros Organics was used for all subsequent studies because it exhibited the least contamination.

**Evaluation of working solution preparation.** UV turbidity analysis identified no significant differences among the nine conditions under which working solutions were prepared. Statistical analysis of particle size analysis could not be performed because of extremely large standard deviations; however, based on raw data, no visible differences were seen. No new or different peaks were observed in the  $^1\text{H}$  NMR spectra of these solutions. The pH was 6.5 for all solutions. For both the *in vivo* studies and the following experiments on sample aging, sterile water was chosen as the diluent because of the availability of sterile, single-use vials. Room temperature was chosen as the mixing temperature because of ease of use.

**Evaluation of stock solutions after storage.** Results of turbidity analysis for each storage condition were not significantly different among the four storage conditions, nor did it change significantly for each condition over time. Throughout the 8-week storage period, all stock solutions had a slightly yellow tint and exhibited grossly visible particles when shaken. The pH readings for all four storage conditions were 6.0 to 7.0 after 8 weeks.

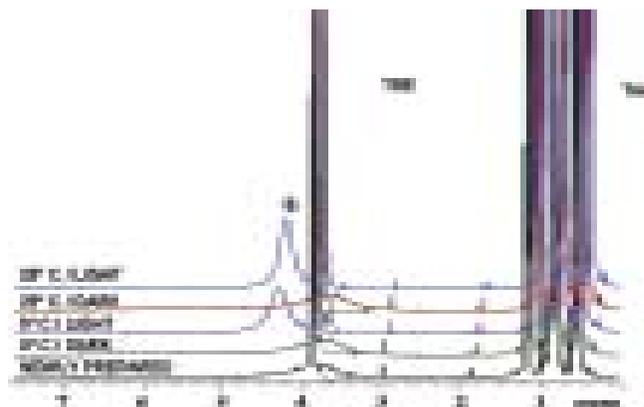
Because chemical changes in the solutions during various stages of storage were minor, we report only the results from the final time point, at which the changes were most pronounced. GC–MS detected DBA as a minor contaminant at similar concentration in all TBE powder samples (chromatograms not shown) and stock solutions, even when newly prepared (Fig. 2). The possibility that the small amount of DBA in the GC–MS chromatogram in Fig. 2 might



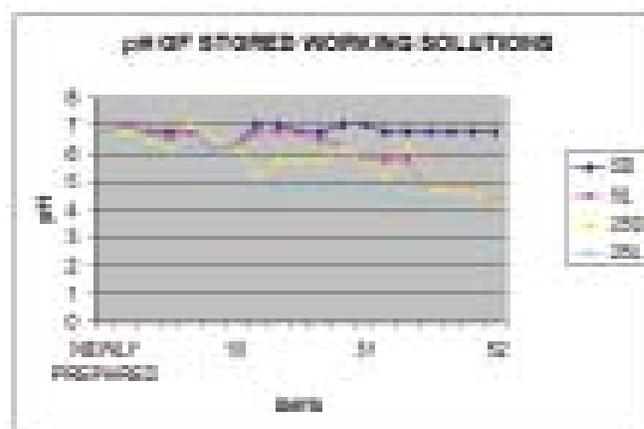
**Figure 2.** GC-MS chromatograms of newly prepared (day 0) stock solution and stock solutions stored for 8 weeks at four different conditions. DBA and TBE represent peaks produced by dibromoacetaldehyde and 2,2,2-tribromoethanol, respectively. Peaks A, B, C, and D represent contaminants that increase in concentration during storage.

have arisen during analysis as a result of thermal degradation of TBE in the heated GC inlet and column cannot be ruled out. Other trace contaminants, some with a molecular weight greater than TBE, were demonstrated via GC-MS to appear or to increase in concentration in all solutions except those that were stored at 5°C in the dark (Fig. 2, peaks A, B, C, and D). The solution stored in the light at the higher temperature (25L) had the largest number and quantity of unknown trace contaminants. The exact concentrations of these minor and trace contaminants could not be measured. The estimated concentrations of DBA and other compounds present in a solution were determined by integrating the areas of each peak, with TBE set to a value of 100. We estimate that as little as 0.5% to 1.0% conversion of TBE to DBA can be detected by GC-MS and that DBA can be detected at levels above 1 µg/ml. Based on these calculations, DBA represents less than 3.0% of the sample. The trace contaminants are proportionately less (approximately 0.5%, estimated to be 50 µg/ml), on the basis of relative peak intensities. The fragmentation patterns in the mass spectra of the trace contaminants were not found in the NIST98 mass spectral library reference database (14).

DBA was not detected in NMR spectra of newly prepared or stored solutions of TBE. However, NMR evaluation did detect a new peak (Fig. 3, peak B) in both solutions that were exposed to light. The 25L and 5L solutions also lacked a peak that was present in both baseline solutions and solutions that were stored in the dark (Fig. 3, peak C). The apparent broadening of peaks B and C indicates weakly bound protons in chemical exchange, and the chemical



**Figure 3.** <sup>1</sup>H NMR spectra of newly prepared (day 0) stock solution and stock solutions stored for 8 weeks at four conditions. TBE and TAA represent multiplet peaks produced by 2,2,2-tribromoethanol and *tert*-amyl alcohol, respectively. The broad resonances labeled B and C arise from exchangeable protons.

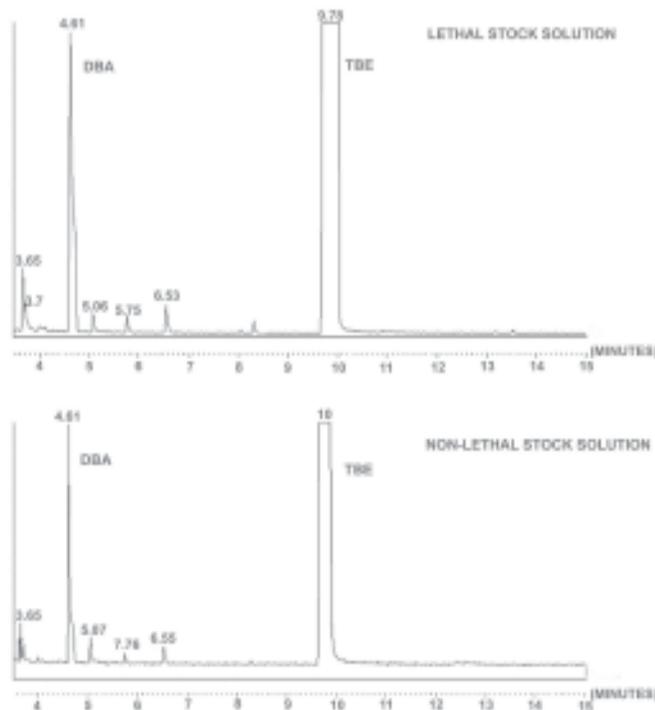


**Figure 4.** The pH of TBE working solutions stored at 5°C in dark (5D), 5°C in light (5L), 25°C in dark (25D), and 25°C in light (25L). Solutions were stored for 8 weeks.

shift range of both peaks suggests that they might represent hydroxyl groups in somewhat different chemical environments. In separate experiments, it was demonstrated that the difference in chemical shifts of peaks B and C was not affected by small additions of water or acid to the samples. Subsequent *in vivo* experiments (16) demonstrated no correlation between these chemical changes and lethality of the samples; therefore, we did not proceed to determine the chemical structures of the contaminants detected by GC-MS and NMR spectroscopy.

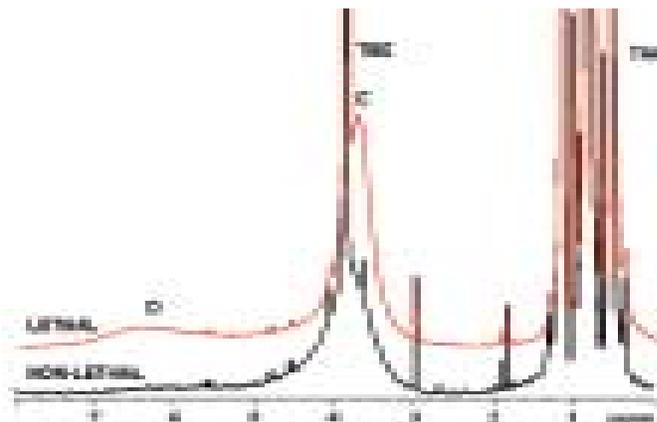
**Evaluation of working solutions after storage.** No significant differences in particle size or turbidity were noted among the four storage conditions over time. The pH of the solution stored at 5°C in the dark (5D) remained at 6.5 to 7.0, whereas the pH dropped for all other storage conditions (Fig. 4). The most dramatic drop in pH was observed at 25°C in the presence of light (25L). However, no commensurate increase in the concentration of DBA, which is hypothesized to be associated with a drop in pH, was observed. <sup>1</sup>H NMR spectroscopy, which can detect DBA quantities as low as 1.6 mg/ml, did not detect DBA in any of the working solutions tested (spectra not shown). The low level of DBA that was detected by the more sensitive GC-MS method (chromatograms not shown) did not change under these storage conditions.

**Analysis of a lethal solution.** The GC-MS chromatograms of a newly prepared working solution that caused morbidity and mortality in mice and of the stock solution used in its preparation were compared to previously recorded chromatograms of newly prepared



**Figure 5.** GC-MS chromatograms of lethal and non-lethal stock solutions. DBA and TBE represent peaks produced by dibromoacetaldehyde and 2,2,2-tribromoethanol, respectively.

TBE working and stock solutions. The pH of the working solution was 6.5, and the solution was determined to be free from bacterial contamination and endotoxins. GC-MS did not detect any new compounds in the lethal stock or working solutions when compared with non-lethal stock (Fig. 5) and working solutions (chromatogram not shown). Because the possibility could not be ruled out that the lethal compound might be non-volatile, thermally unstable, or too polar to pass through the GC inlet and column, samples also were analyzed by NMR.  $^1\text{H}$  NMR spectra of the lethal working solution and of TBE powder dissolved in perdeuteriochloroform exhibited no new peaks compared with the spectrum recorded at the start of this investigation (spectra not shown). However,  $^1\text{H}$  NMR analysis of the lethal stock solution detected a broad, low-intensity signal at 6.5 ppm, which was not seen in other solutions tested (Fig. 6, peak D). The width of the peak suggests that it arises from an exchange mechanism involving weakly bound protons. The possibility of an exchange mechanism is supported by two experiments. First, the two broad peaks at 3.6 ppm (Fig. 6, peak C) and at 6.5 ppm (Fig. 6, D) could be made to coalesce by adding microliter quantities of water to the stock solution. This was not the case with peak B at about 4.2 ppm in the non-lethal stock solutions stored in the light (Fig. 3). The two peaks also could be made to coalesce when the sample was heated during NMR data acquisition. The two broad peaks reappeared when the temperature was lowered. Thus, a reversible chemical exchange appears to be occurring. However, in the absence of evidence from GC-MS for the presence of a new compound, there is insufficient information to provide a definitive interpretation. However, it can be stated that no measurable differences were noted in the concentration of DBA present when evaluated via GC-MS (Fig. 5),  $^1\text{H}$  NMR spectroscopy (Fig. 6), or  $^{13}\text{C}$  NMR spectroscopy (spectra not shown). Furthermore, addition of DBA to the lethal stock solution demonstrated that DBA was not the source of either of the broad peaks at 3.6 ppm and 6.5 ppm (Fig. 6, peaks C and D, respectively). Therefore, the hypothesized correlation between lethality and the presence of increased DBA concentration was not found in this investigation.



**Figure 6.**  $^1\text{H}$  NMR spectra of lethal and non-lethal stock solutions. TBE and TAA represent multiplet peaks produced by 2,2,2-tribromoethanol and *tert*-amyl alcohol, respectively. The broad resonances labeled C and D likely arise from an exchange mechanism involving weakly bound protons.

## Discussion

TBE stock solution, previously marketed as Avertin, is no longer available as a pharmaceutical-grade anesthetic agent. Therefore, the variability of previously reported side effects could arise from differences in the source or lot number of TBE powder and various methods of preparation and storage of TBE powder and/or of the stock and working solutions. The purpose of this project was to establish guidelines for the preparation, storage, and use of TBE. Moreover, we attempted to determine the cause for toxicity in a solution that was demonstrated to cause morbidity and mortality in female ICR mice.

On the basis of the 1952 edition of *The Merck Index of Chemicals and Drugs* (10), previous reports cite DBA and HBr as TBE breakdown products, which result in a decrease of pH (7, 9, 11) over time. The products of this reaction have been assumed to cause the pathologic changes and adverse side effects associated with the intraperitoneal administration of TBE (7, 9). To the best of our knowledge, a thorough investigation to identify these breakdown products, with a causal relationship to pH change, had not been conducted previously; therefore our research methodology was chosen to permit the detection of these compounds.

Both TBE and DBA are readily detected and identified by GC-MS and NMR spectroscopy, therefore we used a combination of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy and GC-MS to confirm the structures of chemicals and to detect the presence of contaminants. GC-MS is more sensitive than is NMR for most organic compounds and can separate chemical mixtures and be used to identify a compound based on the mass spectrum, also without prior knowledge of the compounds that might be present. In vitro and in vivo studies have demonstrated that crystals, particulates, and even talc from surgical gloves can promote macrophage survival and contribute to inflammation and granuloma formation (17-19). In light of these reports, we deduced that particulates are not a desirable feature for a solution that is administered intraperitoneally. Therefore our evaluations included UV turbidity and particle size analyses in an attempt to determine whether a particular diluent or temperature would predispose a solution to form these substances.

In this project, we evaluated TBE powder from three commercial suppliers to identify the most pure starting product. We then evaluated nine commonly used methods for TBE solution preparation (2, 3, 15) for formation of breakdown products and particulates. Samples from all three TBE suppliers were found to contain contaminants. This finding demonstrated that the purity of TBE can vary by supplier, and it is likely to vary by lot number as well. We did not find a difference associated with the diluent used or the temperature at which

the solution was prepared.

GC-MS data indicated that unknown products were present in the stock solutions that were stored at elevated temperature or in the light, with the greatest quantity seen in the solution stored at 25°C in the light (25L; Fig. 2). Some of these products have molecular weights greater than TBE, suggesting that bromination and/or other bimolecular reactions (i.e., reaction with other TBE or solvent molecules) could be taking place. The pH of the 25L solution was < 5 after 8 weeks of storage; however, neither NMR nor GC-MS detected a measurable increase in DBA concentration above that detected prior to storage. During the in vivo study, no significant differences were noted when anesthetic efficacy and pathology were compared in mice that received 25L solution stored for 8 weeks and those that received newly prepared TBE (16). On the basis of the present study and our related in vivo studies, a decrease in pH does not appear to signify a measurable increase of DBA quantities, nor is it indicative of a lethal solution (16).

During a related clinical trial, we experienced pronounced morbidity and mortality in female ICR mice associated with TBE administration (16). The development of toxicity in this case appeared to be associated with storage of the solid TBE powder in the bottle from the manufacturer, not from storage of a TBE solution, as the TBE stock and working solutions were prepared on the day of administration. The sudden appearance of toxicity was not associated with any increased concentration of DBA. GC-MS chromatograms did not provide evidence for the appearance of a new contaminant (Fig. 5). <sup>1</sup>H NMR spectra of the lethal TBE powder dissolved in perdeuteriochloroform exhibited no new peaks compared with the spectrum (Fig. 1) recorded at the start of this investigation. We were able to appreciate only an indirect indication from <sup>1</sup>H NMR of the lethal stock solution (Fig. 6) a low concentration of something with a slowly exchangeable proton, but there are not enough data to permit a full interpretation. Future investigations of the potential cause(s) of toxicity of aged TBE should consider the effects of storage conditions on the stability of TBE powder. We find no evidence to support the long-standing hypothesis that toxicity is caused by breakdown of TBE to DBA in solution. The observed drop in pH during storage of TBE solutions need not necessarily be indicative of reaction(s) that give rise to toxic products, nor is a pH > 5 indicative of a TBE solution that is safe for administration (16).

In conclusion, the purity of commercially available TBE powder is variable. A measurable difference in terms of DBA formation, particle size, or turbidity was not detected in a comparison of commonly used methods for preparation and storage of stock and working solutions. A decrease in pH could not be correlated with a measurable increase in either DBA concentration or lethality, as has been suggested in the literature. More work will be needed to demonstrate a causal relationship between toxicity of the lethal TBE solution evaluated in this study and the small spectral differences observed in its <sup>1</sup>H NMR spectrum. However, it should not go unnoticed that toxicity appears to have arisen during storage of the TBE powder in the absence of any solvent, not after preparation of the stock and working solutions.

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