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GAS CHROMATOGRAPHIC ANALYSIS OF URINARY DIMERCAPTOSUCCINIC ACID

JOHN J. KNUDSEN and EVELYN L. McGOWN*

Division of Biophysical Research, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129-6800 (U.S.A.)

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SUMMARY

The therapeutic use of disulfhydryl compounds such as 2,3-dimercaptosuccinic acid (DMSA) for the treatment of heavy metal poisoning has generated a requirement for specific and sensitive methods to determine those compounds in biological media. We have developed a gas chromatographic assay for DMSA in urine. The use of capillary column technology eliminates the requirement for a preliminary clean-up step. Samples are first reduced electrochemically to liberate DMSA present as disulfides. The reduced product is then extracted into ethyl acetate and the organic phase removed by evaporation. The residue is derivatized with N,O-bis(trimethylsilyl)acetamide for gas chromatography. The silylated DMSA derivative is then detected with a flame ionization detector. The detection limit for DMSA is 1.9 nmol per 1-μl aliquot of derivatized extract injected on column (detector sensitivity at $1 \cdot 10^{-11}$ A/mV). The utility of the method was demonstrated by analyzing the urine of rats orally dosed with DMSA.

INTRODUCTION

2,3-Dimercaptosuccinic acid (DMSA) is a promising new drug for the treatment of heavy metal poisoning, in particular lead, mercury, and arsenic [1–4]. It is an analogue of 2,3-dimercaptopropanol but is less toxic and is effective when taken orally. The Food and Drug Administration has classified DMSA as an orphan drug pending its approval for general use.

The projected therapeutic use of DMSA as a heavy metal-chelating agent has generated a requirement for quantitative methods to assay this disulfhydryl compound in biological media. Although many spectrophotometric procedures have been developed to quantify sulfhydryls (SH), e.g. Ellman's method [5], these assays are non-specific and generally inappropriate when applied to complex media where more than one species of SH compound might be present. A polarographic procedure has been described which quantifies DMSA in pharmaceutical

preparations [6], but this method of analysis is also non-specific when applied to sample matrices containing many potentially electroactive components.

We have developed a sensitive, specific gas chromatographic (GC) assay for DMSA in urine. The excellent resolution offered by capillary column technology eliminates the need for a preliminary sample clean-up step. For maximum recovery of DMSA, the samples are first reduced electrochemically to yield the free disulfhydryl compound. DMSA is then extracted into ethyl acetate. The organic phase is evaporated, and DMSA is derivatized by forming its trimethylsilyl (TMS) derivative. The DMSA-TMS derivative is then quantified by GC using a flame ionization detector. The utility of the method was demonstrated by quantitating DMSA found in the urine of rats orally dosed with this compound.

EXPERIMENTAL

Reagents

meso-2,3-Dimercaptosuccinic acid (DMSA) (98%), heptadecanoic acid (C_{17}) (99%), and ethylenediaminetetraacetic acid (EDTA) (99%) were purchased from Sigma (St. Louis, MO, U.S.A.); 4,4′-dithiodipyridine (98%) (Aldrithiol-4) and ethyl acetate (HPLC grade) were purchased from Aldrich (Milwaukee, WI, U.S.A.); N,O-bis(trimethylsilyl)acetamide (BSA) was purchased from Pierce (Rockford, IL, U.S.A.); all other reagents were AR grade. All reagents and standards were prepared in deionized, glass-distilled water.

Animal care, dosing, and urine collection

Sprague–Dawley rats (*Rattus norvegicus*) were purchased from Charles River (Wilmington, MA, U.S.A.). They were housed individually, maintained on a 12-h light–dark cycle, and given Purina lab chow and water ad libitum. Food was removed 18 h before they were given, by oral gavage, 1 mmol DMSA per kg body weight. The DMSA was prepared by suspending it in distilled water and adding 5% sodium bicarbonate dropwise until dissolution was complete. Each rat was placed in a glass-and-plastic metabolism cage which allowed separate collection of urine and fecal samples. Water was available ad libitum, and food was again offered approximately 6 h after dosing. The urine collection cups contained 250 μl of concentrated hydrochloric acid to retard degradation of urinary DMSA. Urine samples were collected immediately after voiding during the day or early the following morning and were electrochemically reduced, extracted, and derivatized for GC analysis.

Sulfhydryl assay and preparation of oxidized DMSA

SH concentration in distilled water solutions and urine samples was determined using the method proposed by Grassetti and Murray [7]. A 3.0 m*M* solution of 4,4′-dithiodipyridine (Aldrithiol-4) was prepared in 0.1 *M* phosphate buffer, pH 7.5, containing 0.01 *M* EDTA. SH concentrations were calculated from A_{324} values using the molar extinction coefficient of the reaction product, 4-thiopyridone ($\epsilon = 1.98 \cdot 10^4\ m^{-1}\ cm^{-1}$) [7,8].

The SH groups of DMSA are easily oxidized to disulfides. Oxidized species

include various intermolecular disulfides and possibly even intramolecular disulfides. None of the oxidized forms of DMSA are commercially available. To investigate the regeneration of reduced DMSA by electrochemical reduction, we prepared mixtures of DMSA and its disulfide products by exploiting the inherent instability of SH compounds in solution. In the presence of dissolved oxygen and trace metals, e.g. copper (II) or iron (II), compounds containing the SH functional group auto-oxidize to disulfides [9,10]. This process is pH-dependent and proceeds at a higher rate at alkaline pH values [11]. Solutions of DMSA (0.5, 1.0, 3.0, and 5.0 m*M*) were prepared in distilled water, and the pH was adjusted to 7.5 with 0.1 *M* sodium hydroxide. The solutions were allowed to air-oxidize at ambient temperature, and the auto-oxidation reaction was monitored by periodic spectrophotometric measurement of SH concentration. After 8 h the solutions were stored overnight at 4°C, and the final SH measurements were made the following morning. We made no attempt to determine the precise structures of the disulfide-containing oxidation products.

Electrochemical reduction

Mercury pool electrodes (MPEs) were constructed as specified by Saetre and Rubenstein [12]. Multiple samples were reduced simultaneously by connecting several cells in parallel. Sample volumes ranged from 0.5 to 2.0 ml. The pH of each solution was adjusted to 1.0 prior to reduction to insure that sufficient hydrogen ion was present for quantitative reduction. Samples containing more than 2 m*M* DMSA were diluted to minimize reduction time. The reduction process was monitored by removing 25-μl aliquots of sample (at 5-min intervals) from the MPE during electrolysis and assaying for SH concentration spectrophotometrically. Reduction was assumed to be complete for a given sample when SH concentration of the solution reached a plateau.

Sample preparation, gas chromatography, and mass spectrometry

DMSA standards in urine and reduced DMSA-dosed rat urine samples were diluted (if necessary) before extraction to contain a maximum of 2.0 m*M* SH, and the pH was adjusted to 1.0 with concentrated hydrochloric acid. Standards and samples were extracted and derivatized immediately upon preparation and/or collection. Sample aliquots (1.0 or 2.0 ml) were transferred into glass screw-capped tubes (13×100 mm) and extracted three times with 2-ml volumes of ethyl acetate. Mixtures were vortexed for 15 s and then centrifuged for 3 min to facilitate phase separation. The DMSA-containing organic phases from the three extraction steps were then combined. Internal standard (1 ml of 0.5 m*M* heptadecanoic acid in ethyl acetate) was added to each tube of combined organic phase. Ethyl acetate extracts were evaporated to dryness under a dry nitrogen stream at 60°C. To convert DMSA into a volatile form for GC analysis, the compound was converted to the TMS derivative using BSA. Distilled water extracts were derivatized by the addition of 50 μl BSA (neat), while urine extracts required 150 μl of the derivatizing reagent. A larger volume of BSA was required to dissolve urine extracts because of the greater quantity of residue remaining after evaporation of the extraction solvent.

A Varian (Palo Alto, CA, U.S.A.) Model 4600 gas chromatograph equipped with a flame ionization detector was coupled to a Varian Model CDS-401 integrator–plotter. A CP Sil 5 fused-silica capillary column (25 m×0.33 mm I.D.) (Chrompack, Middelburg, The Netherlands) was utilized for all separations. The flame ionization detector was operated at a sensitivity range of $1 \cdot 10^{-11}$ A/mV. Column temperature was 150°C at sample injection and immediately programmed at the following rates: 150 to 210°C at 5°C/min (initial temperature ramp) and from 210 to 320°C at 25°C/min (final temperature ramp). The final temperature ramp was included to bake out contaminants remaining on the column after elution of DMSA-TMS and the internal standard. Injector block and detector temperatures were maintained at 220 and 330°C, respectively. The column carrier gas was helium with a linear gas velocity of 30 cm/s. The injector splitter was adjusted to a split ratio of 1:5. Typically, 1.0-μl aliquots of derivatized sample (in BSA) were injected.

Structure of the DMSA-TMS derivative was confirmed using a Carlo Erba (Milan, Italy) gas chromatograph coupled to a Kratos (Manchester, U.K.) MS25RFA double-focusing mass spectrometer (MS). The MS operating conditions used to obtain a low-resolution electron-impact (EI) spectrum of the derivative were an electron ionization energy of 30 eV, emission current of 100 μA, and an ion source temperature of 80°C.

RESULTS

Reduction of oxidized DMSA

To determine the optimum conditions for recovery of reduced DMSA from solutions containing disulfides of this compound, we allowed DMSA solutions in distilled water to air-oxidize and then reduced them electrolytically. In a typical experiment, 30% of the SH content was lost from a 1.0 m*M* solution of DMSA within 8 h at ambient temperature and an additional 15% during overnight refrigeration at 4°C (Fig. 1A). We empirically found that an applied current of 6 mA was required to achieve maximum SH recovery in the minimum amount of time. The time required to reach maximum yield was proportional to disulfide concentration. As shown in Fig. 1B, maximum recovery of reduced DMSA was obtained by applying a current of 6 mA for ca. 30 min to a 2.0-ml aliquot of the air-oxidized solution (described in Fig. 1A). Yields obtained (percentage of original DMSA concentration) were 98.2 ± 2.1% (mean ± S.D., $n=4$) by spectrophotometric SH assay and 97.8 ± 1.8% (mean ± S.D., $n=4$) by GC assay.

Extraction and derivatization

DMSA was extracted from aqueous solutions with ethyl acetate. Extraction efficiency (partition of DMSA between organic and aqueous phases, according to SH assay) was ca. 98% for distilled water solutions and ca. 95% for urine samples containing 2.0 m*M* DMSA or less.

Silylation of DMSA was effected by adding BSA directly to the anhydrous residue remaining after evaporation of the extraction solvent. Derivative formation was complete as soon as the residue had dissolved completely in BSA (ca. 5

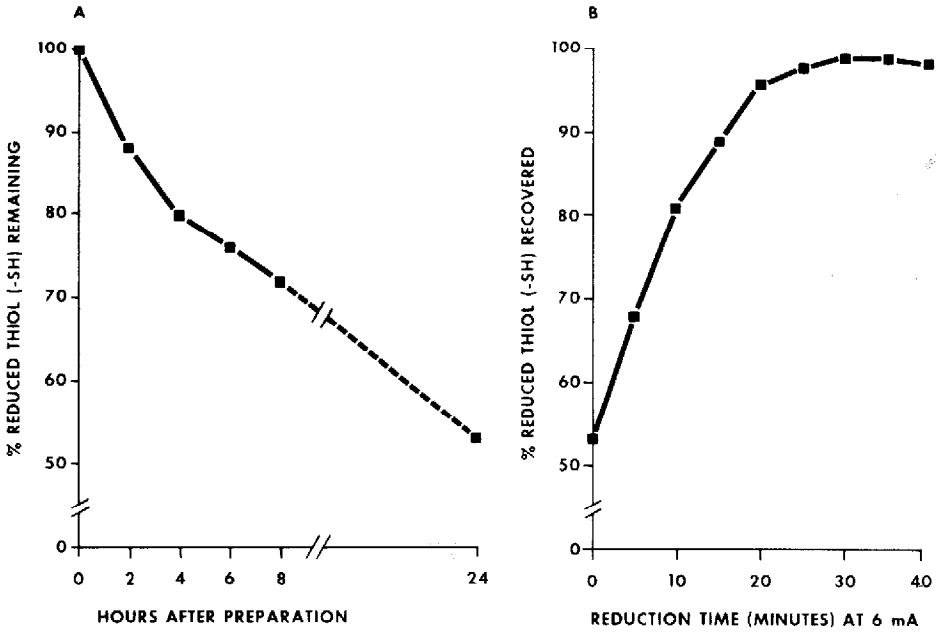

Fig. 1. (A) Auto-oxidation of DMSA in a distilled water solution (1.0 m*M*, pH 7.5) and (B) its subsequent recovery by electrochemical reduction at 6 mA.

min at ambient temperature or ca. 1 min at 60 °C). BSA solutions containing the DMSA-TMS derivative were stable for several hours at ambient temperature when protected from atmospheric moisture and indefinitely when stored under nitrogen at −70 °C.

Low-resolution EI-MS analysis of the DMSA-TMS derivative (Fig. 2) gave a molecular ion of mass 470.1. This is consistent with a molecular formula of $C_{16}H_{38}O_4S_2Si_4$ for the derivative. This fact, coupled with interpretation of the observed fragmentation pattern, confirms that the acidic hydrogen of both carboxyl and SH groups of the parent DMSA molecule are replaced by TMS upon reaction with the derivatizing reagent (BSA).

Internal standard and gas chromatography

Several commercially available SH and disulfhydryl compounds were investigated as potential internal standards [e.g. mercaptosuccinic acid, dithiothreitol (DTT), and thiosalicylic acid]. The TMS derivatives of these compounds either possessed unsuitable retention times, exhibited thermal instability, or were potential biotransformation products of DMSA. We ultimately chose an aliphatic fatty acid because it formed a stable TMS derivative that eluted in a region of the chromatogram free of interfering peaks produced by compounds which co-extracted with DMSA from urine. Because of the insolubility of heptadecanoic acid in aqueous media, we could not introduce it to the sample at the beginning of the analytical procedure and carry it through all sample manipulation steps. We therefore added the internal standard to the sample after extraction. This was

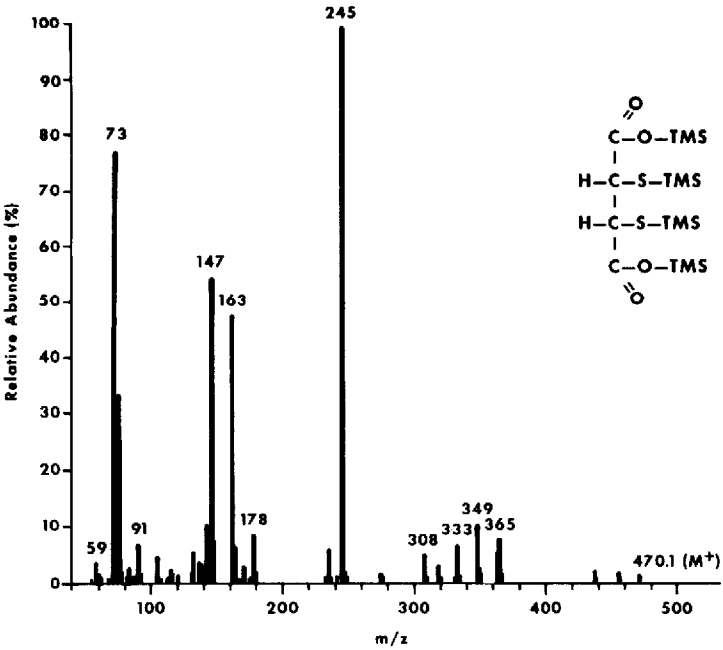

Fig. 2. Electron-impact (30 eV) mass spectrum of DMSA-TMS derivative.

justified on the basis of the consistently high extraction efficiency of DMSA from aqueous solutions.

DMSA-TMS and C_{17}-TMS internal standard eluted at 8.6 and 10.8 min, re-

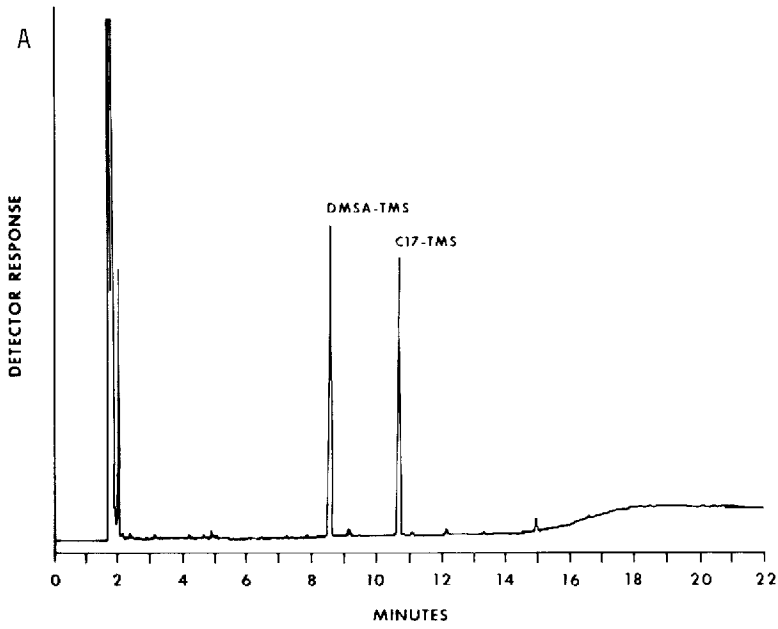

Fig. 3.

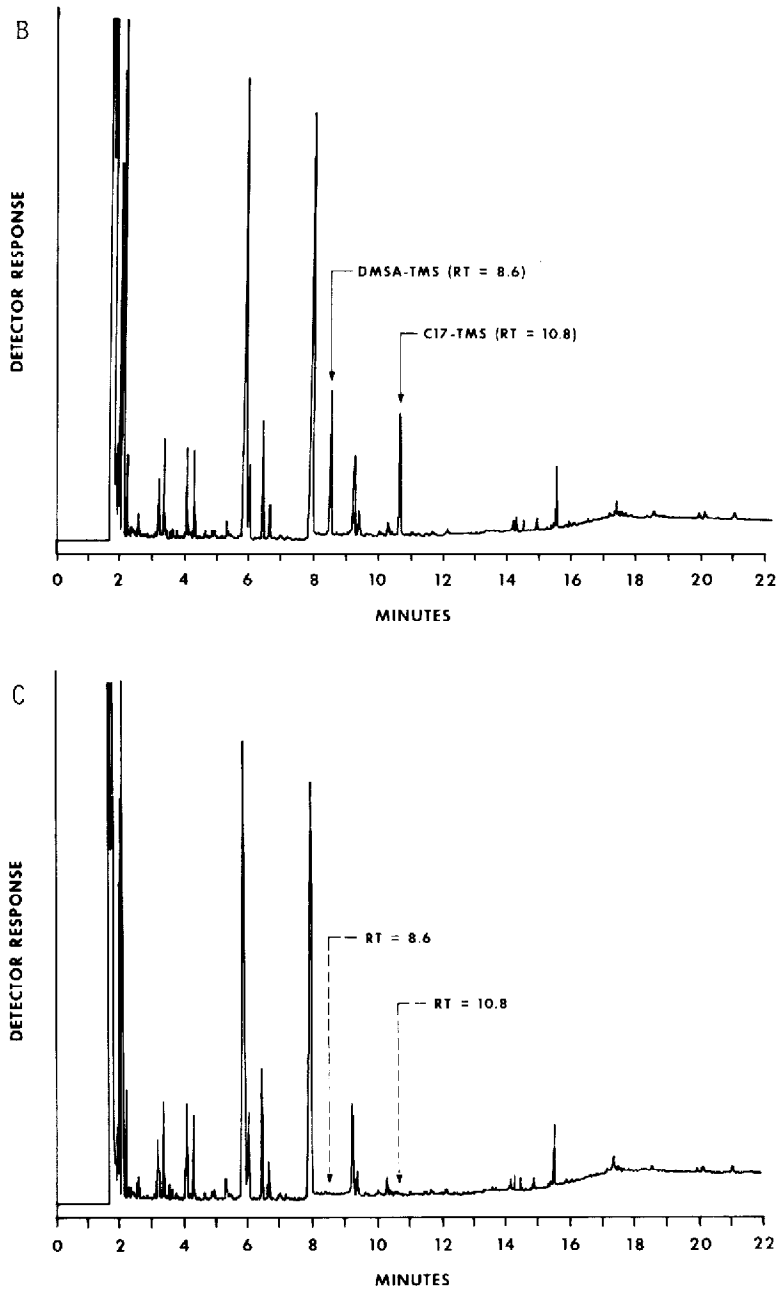

Fig. 3. Representative gas chromatograms of (A) aqueous DMSA standard (1.0 μmol/ml) and internal standard (C_{17}) (0.5 μmol/ml), (B) urine (after electrochemical reduction) from DMSA-dosed rat, and (C) urine from untreated rat. RT = retention time. Derivatization and chromatographic conditions are described in Experimental.

spectively (Fig. 3A). Peaks appeared with the same retention values in chromatograms obtained from the extraction of urine from DMSA-dosed rats (Fig. 3B). Chromatograms of extracts of urine samples from untreated rats had little back-

ground noise in these regions (Fig. 3C). Similar chromatograms were obtained from human, hamster, and rhesus monkey urine spiked with DMSA (data not shown). Figs. 3B and C clearly show that both the DMSA-TMS and C_{17}-TMS peaks are well separated from potentially interfering substances in the urine sample. DMSA-TMS and C_{17}-TMS were identified in all chromatograms by comparison of their retention times to known standards. We detected no peaks which could be associated with DMSA disulfides in any chromatogram obtained while developing this assay. Apparently, DMSA-TMS disulfide derivatives are unstable or possess boiling points which preclude their elution from the column using the GC parameters employed.

Calibration, precision, and detection limits

The GC assay was calibrated by correlating the ratio of peak areas of DMSA-TMS and C_{17}-TMS (R_A) to the ratio of the concentrations of DMSA and C_{17} (R_Q) for a set of standards prepared in rat urine. The C_{17} internal standard concentration was 0.5 μmol/ml while the DMSA concentration varied from 0.1 to 2.0 μmol/ml. Calibration curves were linear and reproducible over the above concentration range. Calibration was confirmed on a daily basis by assaying a rat urine standard containing 1.0 μmol/ml DMSA along with unknown rat urine samples. Urine was diluted, if necessary, after the reduction procedure but before extraction and derivatization to insure that assay values fell within the calibration range of the method. The within-run precision of the assay was determined by assaying six replicates of rat urine containing 0.5 and 1.5 μmol/ml DMSA. The coefficients of variation (C.V.) were 8.3 and 6.3%, respectively. Likewise, the between-run precision of the method was determined by analyzing rat urine containing 0.5 and 1.5 μmol/ml DMSA on six different days, and the C.V. values were 9.2 and 7.0%, respectively. The detection limit of the assay (at a flame ionization detector sensitivity of $1 \cdot 10^{-11}$ A/mV) was approximately 1.9 nmol DMSA-TMS per 1.0 μl of derivatized sample injected onto the gas chromatograph (signal-to-noise ratio of 3). This corresponded to a DMSA concentration in the original urine sample of 0.05 μmol/ml per 1.0 μmol derivatized sample injected.

Stability of DMSA in urine

When urine samples were spiked with DMSA and assayed promptly, recovery of DMSA was virtually quantitative. For example, recovery (mean ± S.D., $n=4$) of 2 m*M* DMSA from urine was 99.2 ± 1.3% by spectrophotometric assay (after correction for endogenous SH content) and 100.3 ± 1.1% by GC assay. However, DMSA was not stable in urine and disappeared in a temperature- and pH-dependent manner. Some, but not all, of the DMSA was recovered upon electrochemical reduction. Table I summarizes the results obtained with DMSA-spiked urines held for 5 h at two temperatures (ambient or 4°C) and at pH 1.0 or 7.5. DMSA loss (as ascertained by GC analysis) was retarded by lowering pH and temperature. By acidifying the samples to pH 1.0 and maintaining them at 4°C, most of the 1m*M* DMSA was recovered after 5 h (95.1 ± 1.7%; mean ± S.D., $n=4$). Recovery was poor (30.7 ± 3.2%; mean ± S.D., $n=4$) from pH 7.5 solutions held at ambient temperature. Presumably the fraction of DMSA not recovered by elec-

TABLE I

RECOVERY OF DMSA FROM SPIKED RAT URINE

Rat urine samples containing 2 m*M* DMSA were adjusted to pH 1.0 or pH 7.5 and maintained for 5 h at ambient temperature (ca. 25 °C) or 4 °C before GC assay.

Treatment	DMSA spike recovered (mean ± S.D., $n=4$) (%)	
	Before reduction	After reduction
pH 1.0		
Ambient	65.3 ± 1.9	81.2 ± 1.9
4 °C	72.1 ± 2.1	95.2 ± 1.7
pH 7.5		
Ambient	15.8 ± 2.5	30.7 ± 3.2
4 °C	48.2 ± 2.9	70.6 ± 2.3

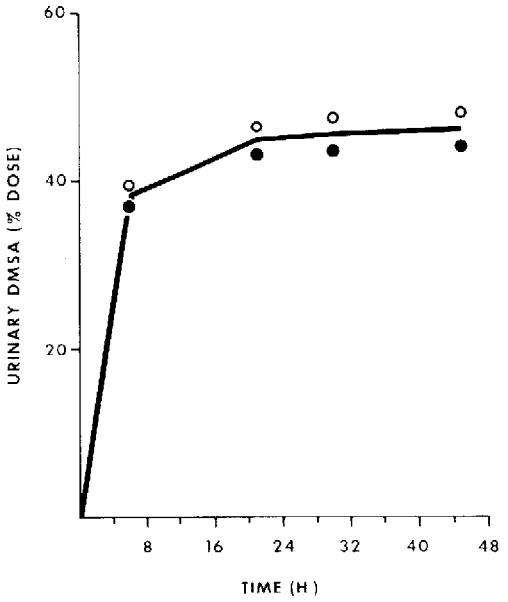

Fig. 4. Cumulative percentage of dose found (after electrochemical reduction) in urine of rats dosed by oral gavage with 1 mmol DMSA per kg body weight. The solid and hollow circles represent two different rats.

trochemical reduction represented material that had transformed beyond disulfide bond formation.

Recovery of reduced DMSA from 2 m*M* solutions stored at −20 °C for 24 h immediately after preparation was 83.2 ± 2.6% (mean ± S.D., $n=4$) in pH 1.0 and 74.3 ± 3.1% (mean ± S.D., $n=4$) in pH 7.5. We did not evaluate DMSA stability in urines stored longer than 24 h.

Recovery of DMSA from the urine of orally dosed rats

We used the assay described above to study the excretion of DMSA by two rats which had been orally dosed with 1 mmol DMSA per kg body weight. Urine samples were collected over a 48-h period, electrochemically reduced, and assayed. Results from the two rats were remarkably similar (Fig. 4).

Nearly 40% of the dose (as DMSA) was excreted in the urine within 6 h. Additional DMSA continued to be excreted but much more slowly. By 48 h approximately 50% of the dose (as DMSA) was accounted for in the urine.

DISCUSSION

DMSA readily undergoes auto-oxidation to form intermolecular disulfides. In a biological milieu, mixed disulfide formation with cysteine, glutathione, and other endogenous thiols is also likely. Such transformations can occur both in vivo and in vitro. To devise an assay for DMSA in urine which quantifies both the parent compound as well as any DMSA present as disulfides, it was necessary first to reduce disulfides in the sample. Chemical techniques such as the sodium borohydride ($NaBH_4$) method of Bir et al. [13] and the DTT method of Zahler and Cleland [14] have been employed to reduce urinary disulfides. $NaBH_4$ gave us unsatisfactory yields of reduced DMSA when used to treat both distilled water solutions and urine samples containing DMSA disulfides. Reduction with DTT was not investigated because addition of another SH compound to the sample matrix would have unnecessarily complicated both the non-specific spectrophotometric SH assay used to quantify reduced SH in urine before sample extraction and, potentially, the GC analysis as well. We obtained excellent recoveries of reduced DMSA from distilled water solutions containing DMSA disulfides by reducing them electrochemically over an MPE. By taking appropriate precautions we also recovered most of the DMSA added to rat urine, even when it was partially oxidized.

Approximately half of the DMSA given orally to rats was excreted in the urine within 48 h as a mixture of parent compound and its disulfides. In view of the temperature- and pH-dependent irreversible degradation of DMSA in urine (Table I), it is likely that additional breakdown products (or even metabolites) were also excreted, but were not recovered by electrochemical reduction.

Studies with radiolabeled DMSA in many animal species have demonstrated that this promising heavy metal chelator is rapidly eliminated from the body by urinary excretion [1,15]. The nature of the excretion products has, until recently, remained largely unknown. On the basis of [^{14}C]DMSA experiments, coupled with a specific packed-column GC method, we have previously reported that DMSA is excreted in urine by the hamster apparently completely as a mixture of the parent compound only and its intermolecular or mixed disulfides [15]. Other radiolabeled metabolites of DMSA were not observed in hamster urine. The sensitive capillary GC method described in this paper is specific for DMSA in urine, convenient, and reliable. Coupled with electrochemical reduction, it offers a valuable tool for use in future studies of DMSA metabolism.

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