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DETERMINATION AND METABOLISM OF DITHIOL CHELATING AGENTS

IV. URINARY EXCRETION OF *MESO*-2,3-DIMERCAPTOSUCCINIC ACID AND MERCAPTOSUCCINIC ACID IN RABBITS GIVEN *MESO*-2,3-DIMERCAPTOSUCCINIC ACID*

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Abstract—The water-soluble dithiol chelating agents *meso*-2,3-dimercaptosuccinic acid (DMSA) and 2,3-dimercaptopropane-1-sulfonic acid (DMPS) are becoming of increasing importance for the treatment of lead, arsenic and mercury poisoning. There is, however, a paucity of data about their metabolic transformation. Male rabbits were given DMSA (0.20 mmol/kg) i.m., and urine was collected over a 6-hr period. Monobromobimane derivatization, HPLC separation, and fluorescence detection, along with [U-^{14}C]DMSA data, demonstrated that the total ^{14}C found in the urine was distributed as 73% unaltered DMSA, 7% mercaptosuccinic acid and 6 and 14% of two unknowns. Electrolytic reductive treatment of the urine did not increase the urinary content of DMSA, indicating that oxidative biotransformation is not a major pathway for DMSA in the rabbit. This latter result is strikingly different from that for DMPS in rabbit.

Meso-2,3-dimercaptosuccinic acid (DMSA‡) and 2,3-dimercaptopropane-1-sulfonic acid (DMPS) are water-soluble dithiols that are useful antidotes for the treatment of arsenic, mercury and lead intoxication [1, 2]. They are rapidly replacing their more toxic and lipophilic chemical analog, 2,3-dimercaptopropanol (British Anti-Lewisite, BAL). These dithiols remove the toxic metal from the body [3–12], presumably by forming water-soluble metal complexes or chelates [13–16]. Recent studies of men with high levels of lead in their blood have demonstrated the particular usefulness of DMSA as a deleading agent in humans [5, 12].

By 6 hr after DMPS administration i.m. to rabbits, 51 and 33% of the total DMPS found in the urine consisted of cyclic and acyclic polymeric disulfides of DMPS respectively [17]. After chemical reductive treatment of the urine, a 20-fold increase in DMPS was observed [17]. The disposition and biotransformation of DMSA, however, are not clear. Administration of [U-^{35}S]DMSA s.c. to rats showed that, by 5 hr, 59% and, by 24 hr, 95% of the administered ^{35}S were excreted in the urine [18]. When [U-^{14}C]DMSA was administered i.p. or p.o. to hamsters, the amount of DMSA recovered after electrolytic treatment of the urine was 87% of the radioactivity present in the urine [19].

Although the results of these previous studies indicated that the carbon chain and sulfhydryl groups of DMSA appear to remain mostly intact, the form in which DMSA is excreted was not determined. The purpose of the present research was to investigate the biotransformation of DMSA to determine if, like DMPS, acyclic and cyclic polymeric disulfides are the major metabolites excreted in the urine of rabbits given DMSA. The results clearly showed that in the rabbit, unlike DMPS, very little of the DMSA, given i.m., is biotransformed.

MATERIALS AND METHODS

Materials. *meso*-DMSA and [U-^{14}C]*meso*-DMSA (sp. act. 20.8 mCi/mmol) were gifts from Johnson & Johnson (Skillman, NJ). Elemental analysis of *meso*-DMSA gave 26.53% C, 3.35% H, 35.38% S and 35.32% O. The calculated values were 26.37% C, 3.32% H, 35.19% S and 35.12% O. The chemical purity was 99.9% as carboxyl and 94.9% as sulfhydryl. The radiolabel and chemical purities of [U-^{14}C]*meso*-DMSA were 95.0 and 93.7% respectively. The [U-^{14}C]DMSA was diluted with unlabeled DMSA to a specific activity of 143 μCi/mmol. Chemicals were obtained from the following sources: mercaptosuccinic acid (MSA), Sigma Chemical Co. (St. Louis, MO); cysteine HCl (CYSH, 99% purity), Eastman Kodak (Rochester,

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‡ Abbreviations: DMSA, *meso*-2,3-dimercaptosuccinic acid; MSA, mercaptosuccinic acid; DMPS, sodium salt of 2,3-dimercaptopropane-1-sulfonic acid; BAL, 2,3-dimercapto-1-propanol; CYSH, cysteine; mBBr, monobromobimane; TBAB, tetrabutylammonium bromide; and TBAOH, tetrabutylammonium hydroxide.

NY); monobromobimane (mBBr; Thiolyte, Behring Diagnostics (La Jolla, CA); tetrabutylammonium bromide (TBAB), J. T. Baker (Phillipsburg, NJ); tetrabutylammonium hydroxide (TBAOH), Aldrich (Milwaukee, WI); dichloromethane, acetonitrile, and methanol, Burdick & Jackson (Muskegon, MI). All other chemicals used were analytical reagent grade. Double-distilled, deionized water was used to prepare reagent and standard solutions. Glassware was rinsed with 25% HNO_3 to remove residual metals. Precoated silica gel TLC glass plates (20 × 20 cm; 250 μm) were obtained from J. T. Baker.

Animals. Male New Zealand white rabbits (1.5 to 2.0 kg) purchased from Blue Ribbon Ranch (Tucson, AZ) were quarantined and allowed to adapt for a 7-day period. They received Wayne Rabbit Ration and water *ad lib.* Prior to experimental use, rabbits (N = 4) were sedated with i.m. injections of ketamine (45 mg/kg) into the left gastrocnemius muscle. A catheter was inserted into the bladder. Physiological saline was supplied s.c. to replenish body fluids. After emptying the bladder, DMSA or [U-^{14}C]DMSA (0.20 mmol/kg) in 0.9% saline–5% $NaHCO_3$ (pH 7.4) was injected into the right gastrocnemius muscle, and urine was collected over a 6-hr period. At each collection time, a syringe was attached to the catheter and the bladder was emptied. This eliminated contact of the urine with metal parts of metabolic cages.

Treatment of urine. Immediately after collection, a 50-μl aliquot of urine was treated with mBBr to alkylate and prevent the oxidation of the sulfhydryl groups of DMSA and any other thiols [20]. Urine not treated with mBBr was frozen at −20° and was later treated with reductive electrolysis [21] to reduce any disulfide forms of DMSA and other thiols present in urine to their corresponding sulfhydryl forms. The reduced DMSA and other thiols were then treated with mBBr to form stable bimane derivatives. This procedure was used to determine total DMSA (unaltered + oxidized).

Incubation of DMSA. A control experiment was performed as follows: 0.15 ml of 0.10 M DMSA (pH 5.4) was added to 3 ml of rabbit urine and incubated at 37° for 6 hr. The final DMSA concentration was 5.0 mM. At various time points 40-μl aliquots were removed and treated with mBBr [20]. Aliquots (200 μl) also were removed and acidified to pH 1 with 0.4 M HCl for electrolytic reduction followed by mBBr derivatization [21].

Measurement of DMSA, MSA, CYSH and ^{14}C. The bimane derivatives of DMSA (B-DMSA), MSA (B-MSA) and CYSH (B-CYSH) in urine samples were determined by HPLC separation and fluorescence detection. *HPLC system I:* Separation was accomplished with a Spherisorb ODS RP column (4.6 mm × 150 mm; 5 μm particle size) using mobile phase conditions previously described [21]. Retention times of B-DMSA and B-MSA were 7.2 and 4.8 min respectively. *HPLC system II:* This system effectively separates the bimane derivatives of thiol zwitterions and anions. Separation was accomplished with an Altex Ultrasphere ODS column (4.6 mm × 250 mm; 5 μm particle size). The sample injection was 20 μl. Mobile phase A was 20 mM acetic acid and 20 mM TBAOH in methanol. Mobile phase B was 20 mM acetic acid in 25:75 methanol–water (v/v). After an isocratic period of 1 min (100% B), a stepwise change to 60% B was programmed and held for 14 min followed by a linear gradient programmed to 10% B over a 5-min period and then held for 5 min. The mobile phase conditions were then returned to 100% B over a 5-min linear gradient. The column was equilibrated with 100% B for 20 min before another sample injection. The flow rate was 1.0 ml/min. The HPLC instrumentation has been described previously [20]. Retention times of B-DMSA, B-MSA and B-CYSH were 15.2, 12.2 and 5.5 min respectively. Identification and quantification were accomplished with standards of B-DMSA, B-MSA and B-CYSH prepared by reacting DMSA, MSA or CYSH with mBBr.

Urine samples from a rabbit given [U-^{14}C]DMSA were treated with mBBr, and 100 μl was analyzed by HPLC with the fluorescence detector using HPLC system I. Fractions (0.3 ml) were collected, scintillation fluid [22] was added, and ^{14}C was measured by scintillation counting. Urine samples (50 μl) were added to scintillation fluid and counted for total ^{14}C. Recovery of total ^{14}C from the column was 100.8 ± 3.6% (mean ± SD, N = 6 time points).

Isolation and identification of MSA by TLC. A 2-hr urine sample or a 2-hr urine sample spiked with MSA was treated with reductive electrolysis followed immediately with mBBr derivatization. The sample was streaked (0.2 ml) onto a silica gel plate and developed with *n*-butanol–acetic acid–water (3:1:1) for a distance of 15 cm. The bimane derivatives were revealed on the plates as fluorescent yellow bands when viewed under long wavelength UV light. After the plate was thoroughly dried, the band corresponding to the R_f of B-MSA was scraped off the plate into a 1.5-ml Eppendorf polypropylene microcentrifuge tube, and 0.5 ml of 20 mM acetic acid in 25:75 methanol–water was added. After vortex mixing for 2 min and centrifugation at 3000 rpm for 2 min, the supernatant fraction (0.2 ml) was streaked onto another plate and developed in chloroform–methanol–acetic acid (55:36:9) for a distance of 15 cm. The band corresponding to the R_f of B-MSA was removed, and 0.5 ml of 20 mM acetic acid in 25:75 methanol–water was added. After mixing and centrifugation, the supernatant fraction was analyzed using HPLC system II with fluorescence detection. The R_f values of B-MSA, B-DMSA and B-CYSH in the urine sample were compared to authentic standards added to a 0 time urine sample (before DMSA administration).

RESULTS

Urinary excretion of DMSA and ^{14}C. Within 6 hr after i.m. administration of 0.20 mmol DMSA/kg to rabbits, 46.8 ± 6.1% (mean ± SE) of the DMSA given was excreted as unaltered drug in the urine (Fig. 1). The mean maximum amount of unaltered DMSA was detected within 2 hr after administration. After reductive electrolytic treatment of the urine, the excretion profile was not changed significantly (Fig. 1), indicating that the reductive treatment did not cause an increase in DMSA. But within 6 hr after

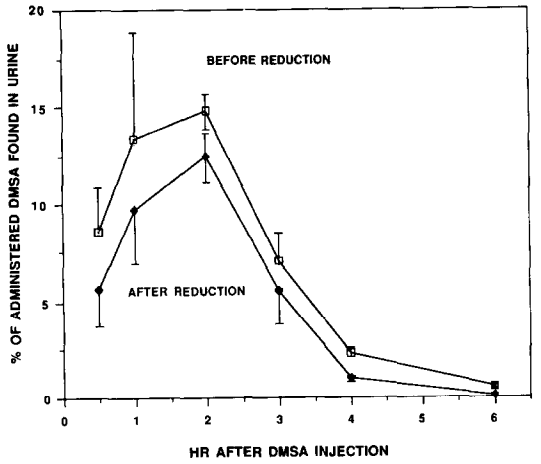

Fig. 1. Urinary DMSA before and after electrolytic reduction. Rabbits received 0.20 mmol DMSA/kg i.m. HPLC system I was used for the analysis. Each point is the mean ± SE for four rabbits. The amount of DMSA excreted at 2 hr was 57.5 ± 4.7 μmol (mean ± SE) before reduction and 48.6 ± 7.0 μmol after reduction. The mean dose of DMSA was 0.388 ± 0.023 mmol.

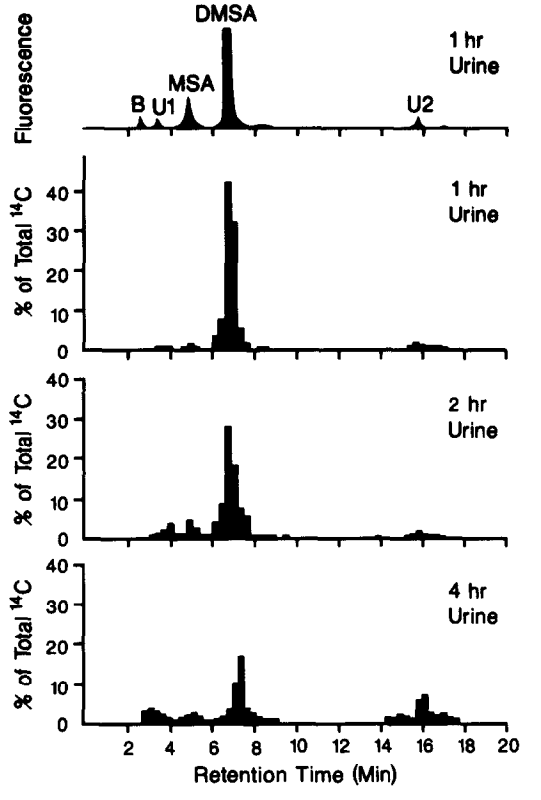

Fig. 2. HPLC separation of DMSA and its biotransformed products in rabbit urine. Dose of [U-^{14}C]DMSA was the same as in Fig. 1. Urine was treated with mBBr and analyzed for unaltered and altered DMSA by HPLC system I with fluorescence detection and scintillation counting. Percent of total ^{14}C equals ^{14}C in individual HPLC fractions divided by the sum of the ^{14}C found in all of the fractions times 100. The total ^{14}C in dpm for 1, 2 and 4 hr was 15,974, 6,488 and 5,246 respectively. Peaks in chromatograms are: (B) bimane hydrolysis product, (U1, U2) unknowns, (MSA) mercaptosuccinic acid, and (DMSA) dimercaptosuccinic acid.

administration of [U-^{14}C]DMSA (0.20 mmol/kg), 79.7% of the ^{14}C given was excreted in the urine, suggesting the presence of ^{14}C-containing biotransformed compounds other than disulfides of DMSA in the urine.

HPLC separation. HPLC analysis of mBBr-treated urine from a rabbit given [U-^{14}C]DMSA showed the presence of ^{14}C-peaks other than unaltered DMSA (Fig. 2). Two small ^{14}C-peaks with retention times of 3.5 and 4.8 min exhibited fluorescence. The 4.8-min peak matched the retention time of the bimane derivative of authentic MSA. ^{14}C-Peaks (U2) that eluted in a cluster between 14 and 18 min were also fluorescent. The major peak of the U2 cluster had a retention time of approximately 16 min. These peaks increased with time, while DMSA decreased with time. The 3.5 and 4.8 min peaks were not seen in the chromatogram of a mBBr-treated [U-^{14}C]DMSA standard. The latter chromatogram showed a major radioactive peak (93%) with a 7.2-min retention time for unaltered DMSA and a minor peak (7%) with a 16-min retention time. These peaks also exhibited fluorescence.

When the 4-hr urine was treated by reductive electrolysis, HPLC analysis showed little change in the DMSA peak (Fig. 3), indicating that any disulfide species of DMSA were present in minute amounts if at all.

TLC identification and isolation of MSA. The R_f value of B-MSA found in urine matched the R_f of B-MSA in urine spiked with MSA and the R_f of

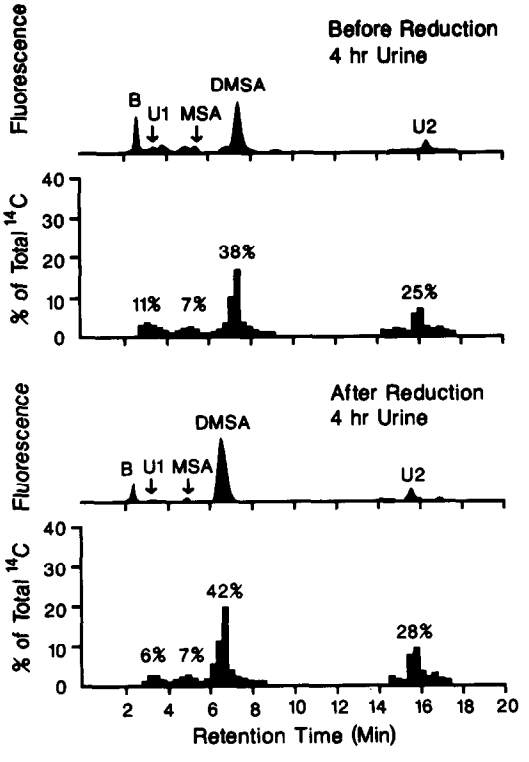

Fig. 3. HPLC separation of DMSA and its biotransformed products in rabbit 4-hr urine before and after electrolytic reduction. Conditions and procedures are as described for Fig. 2. The percentage for U2 refers to the major peak of the cluster of peaks.

Table 1. Isolation and identification of MSA in rabbit urine by TLC

Sample	R_f value	
	Solvent system 1	Solvent system 2
Urine	0.42	0.59
	0.33	0.51
Urine + MSA	0.42	0.58
	0.33	0.51
Standards		
B-MSA	0.43	0.58
B-DMSA	0.33	0.49
B-CYSH	0.23	0.19

A 2-hr urine sample or 2-hr urine sample spiked with MSA was electrolytically reduced and treated with mBBr. The standards (1 mM bimane derivatives of MSA, DMSA and CYSH) were added to 0-hr urine. A silica gel plate was spotted with the samples and standards and developed in solvent system 1 or in solvent system 2. The bimane derivatives fluoresced under long wavelength UV light. Solvent system 1: *n*-butanol–acetic acid–water (3:1:1). Solvent system 2: chloroform–methanol–acetic acid (55:36:9).

an authentic standard of B-MSA (Table 1) in both solvent systems. No corresponding band was seen when a 0-hr urine sample (before administration of DMSA) was analyzed. HPLC analysis showed that the retention time of the putative B-MSA recovered by TLC matched that of B-MSA from the same urine sample spiked with MSA (Fig. 4). The 10.8 min peak (labeled B) represents a bimane hydrolysis product that was also seen in TLC solvent system 2 as a streak. Note the excellent isolation of B-MSA after TLC as compared to before TLC (Fig. 4).

Metabolic profiles. The majority of the [U-^{14}C]DMSA administered to a rabbit was excreted in the urine as unaltered DMSA (Fig. 5A). By 6 hr, the total ^{14}C found in the urine was distributed as follows: 73% unaltered DMSA, 6% unknown 1 (peak R_t 3.5 min), 7% MSA (peak R_t 4.8 min) and 14% unknown 2 (peak cluster). Note that most of the altered [U-^{14}C]DMSA (MSA, U1 and U2) accumulated at the later time points (Fig. 5B), while most of the unaltered [U-^{14}C]DMSA was excreted earlier.

In a control experiment in which DMSA was incubated in rabbit urine, however, a different picture emerged (Fig. 6). After 6 hr no DMSA remained. After electrolytic reduction, 68% of the added DMSA was found as DMSA and 9% of the added DMSA was found as MSA. The remaining 23% DMSA may have oxidized to species such as sulfonic acids that would not react with mBBr. It is pertinent to note that the bladder was catheterized for rabbits given DMSA. A syringe was attached to the catheter to empty the bladder at the given time points. An aliquot of the collected urine was immediately reacted with mBBr to minimize non-metabolic oxidation.

Urinary excretion of cysteine and MSA. Cysteine was detected after reductive electrolytic treatment of the urine. By 6 hr, the amount of DMSA found in the urine exceeded cysteine by 17-fold. The excretion profiles of the two compounds, however, were similar (Fig. 7). A linear regression comparison of their excretion profiles yielded a correlation coefficient of 0.776 and a level of significance of $P < 0.04$ (df = 6). The amount of DMSA was 11-fold greater than the amount of MSA found in the urine by 6 hr. Their excretion profiles (Fig. 7) were very similar ($r = 0.879$; $P < 0.009$; df = 6).

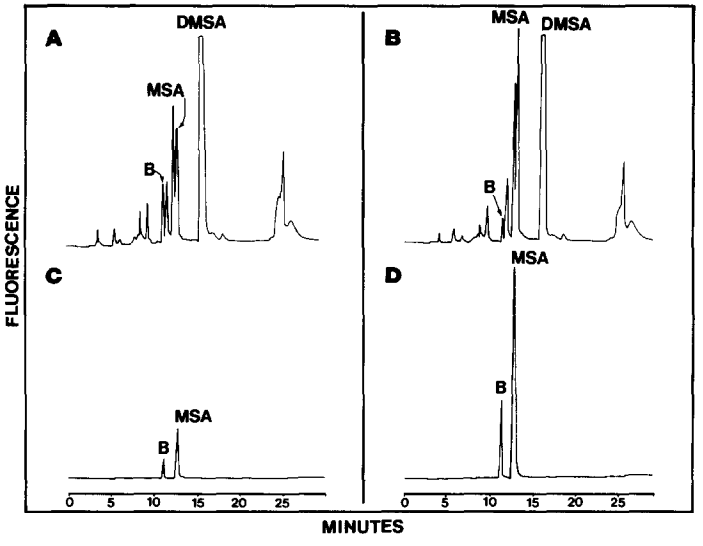

Fig. 4. HPLC separation of MSA before and after TLC isolation from rabbit 2-hr urine. Urine samples without and with added MSA were treated with mBBr after electrolytic reduction. (A) and (B) are HPLC chromatograms of the samples without and with added MSA respectively. (C) and (D) are HPLC chromatograms of the same samples after isolation of MSA by TLC. HPLC system II was used for analysis.

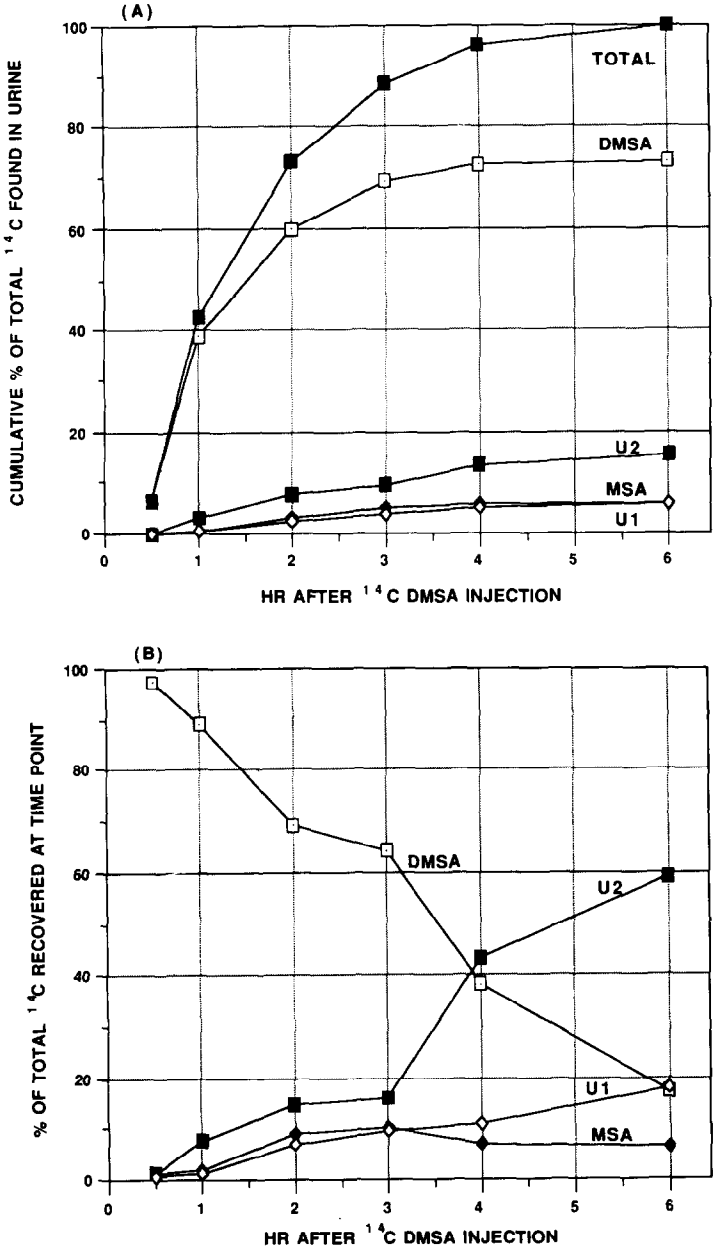

Fig. 5. Excretion of DMSA and its biotransformed products in the urine of a rabbit given [U-^{14}C]DMSA. Time profiles of DMSA, MSA and unknowns are expressed in (A) as percent of cumulative ^{14}C found in the urine and in (B) as percent of total ^{14}C recovered at that time point. Dose and abbreviations are described as in Fig. 2. The 6-hr cumulative amount of total ^{14}C found in urine was 1.00×10^8 dpm or 0.319 mmol.

DISCUSSION

A number of chelating agents have been used in the past for various therapeutic reasons [23]. Examples of these are $CaNa_2EDTA$, D-penicillamine and British Anti-Lewisite (BAL). All of these have serious side reactions or toxicities [23]. BAL is now being replaced by DMSA and DMPS [1, 2]. These two compounds are analogous in chemical structure to BAL. They are crystalline, relatively stable, orally useful compounds. BAL is an oily liquid and very susceptible to oxidation. The LD_{50} values of these compounds when given to mice i.p. are 13.7 mmol DMSA/kg, 6.5 mmol DMPS/kg and 1.48 mmol BAL/kg [10].

There are only four quantitative studies on the therapeutic usefulness of DMSA or DMPS in humans. DMPS was found to be the most effective chelating agent for treating humans in Iraq poisoned by ingestion of bread and other food, prepared using grain seeds impregnated with mercurial fungicide [24]. A number of different treatments were used

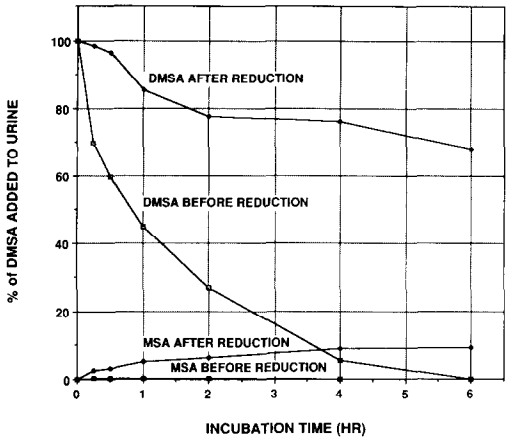

Fig. 6. Incubation of DMSA in rabbit urine at 37°. The initial concentration of DMSA was 5 mM. Aliquots were analyzed for DMSA and MSA before and after electrolytic reduction using HPLC system II.

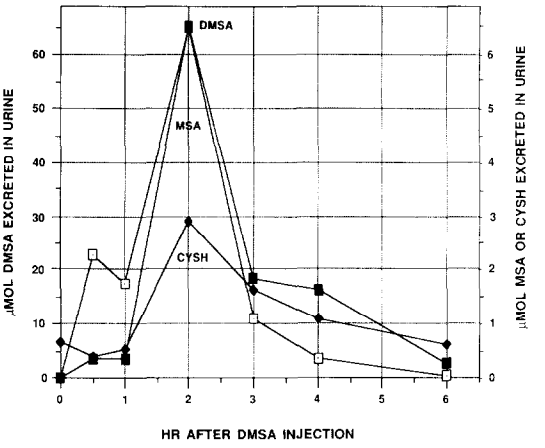

Figure 7. Urinary excretion of DMSA, MSA and cysteine measured after electrolytic reduction of the urine. Dose of DMSA given to a rabbit as described in Fig. 1. Key: (□) DMSA, (■) MSA, and (◆) CYSH. HPLC system II was used for the analysis.

and compared. The $T_{1/2}$ of methylmercury in the blood was used as a measure of efficacy. The mean $T_{1/2}$ values were: no treatment, 63 days; DMPS, 10 days; thiolated resin, 20 days; D-penicillamine, 26 days; and *N*-acetyl-DL-penicillamine, 24 days.

The efficacy of DMSA and DMPS in the treatment of children with lead intoxication has been demonstrated in quantitative studies by Graziano *et al.* [24] and Chisolm and Thomas [26] respectively. DMSA has been classified as an orphan drug by the FDA.

A major advantage of *meso*-DMSA appears to be that it does not increase significantly urinary copper or zinc excretion compared to the corresponding effect of $CaNa_2EDTA$ [12, 25]. With DMPS there is a relatively small increase in urinary copper and zinc excretion [26] in children treated for lead intoxication. DMPS, however, does not affect the plasma levels of copper or zinc during the 5-day regimen of DMPS treatment [26].

A comparison of the activities of DMSA and DMPS in mobilizing arsenic in rabbits previously given ^{74}As is available [10]. DMPS was found to be superior in mobilizing ^{74}As from liver, lung and brain tissues, while both DMSA and DMPS were equally effective in mobilizing ^{74}As from kidney. DMPS also causes the urinary excretion of total arsenic (inorganic + organic) at a greater rate than DMSA [11]. Several studies of the mobilization of methylmercury from rat and mice tissues by DMSA and DMPS are available. DMSA was found to be more effective than DMPS in mobilizing methylmercury from blood, kidney, liver and especially brain of the mouse [27]. In the rat, DMSA was most effective in removing the mercurial from all organs and blood except the kidneys, for which DMPS was better [6]. DMSA removed more of the organic mercury while DMPS removed more of the inorganic mercury. The influence of DMPS and DMSA on the distribution and excretion of inorganic mercury in the rat has been compared [28]. DMPS was more efficient in removing inorganic mercury from the body. If a maximum tolerated dose is used as the criterion, however, DMSA is more effective than DMPS for increasing the urinary excretion of inorganic mercury [29].

DMSA, administered i.m. to rabbits, was rapidly excreted in the urine, predominantly in its unaltered form (Figs. 1 and 5A). After electrolytic reductive treatment of the urine, no significant change in unaltered DMSA was found (Fig. 1). This indicates that oxidized forms of DMSA such as disulfides were not formed and that DMSA has a different metabolic behavior than that reported previously [17] for the chemically analogous chelating agent DMPS. The majority (84%) of DMPS administered i.m. to rabbits was found in the urine as cyclic and acyclic polymeric disulfides of DMPS [17]. After chemical reductive treatment of the urine from rabbits given DMPS, a 20-fold increase in DMPS was observed by the end of the 6-hr urine collection period [17]. The differences in the biotransformation and excretion of these two useful therapeutic dithiols may be related to their different susceptibilities to oxidation [30]. DMSA is more stable than DMPS in water at pH 7.0. After 7 days at 24°, 82% of the sulfhydryl groups of DMSA remained while, by the same time, none of the sulfhydryl groups of DMPS remained [30]. When DMSA or DMPS was given p.o. to humans, however, disulfide species of both DMSA or DMPS were excreted in the urine [21]. After reductive treatment of the human urines, a 4-fold increase in DMSA and a 20-fold increase in DMPS were found by the end of the 12-hr urine collection period [21]. Either DMSA disulfide formation occurs in the GI tract, before or during absorption across the gastrointestinal mucosa, or there is a species difference between rabbit and human.

Within 6 hr after [U-^{14}C]DMSA administration to a rabbit, only 73% of the total ^{14}C found in the urine represented unaltered DMSA, indicating the presence of DMSA metabolites other than DMSA disulfides. HPLC analysis of urine indicated the presence of at least three metabolites. One of the metabolites has been identified as MSA based on TLC and

HPLC analyses (Figs. 3 and 4, Table 1). Therefore, the desulfuration of DMSA occurs to a limited extent.

MSA was also found after DMSA was incubated in rabbit urine as a control. The pK_a values of the sulfhydryl groups of *meso*-DMSA are approximately 9 and 11 [13, 15, 31]. The more reactive sulfhydryl (pK_1) would be expected to be more labile and more susceptible to removal by trace metals. Although desulfuration can occur in the presence of endogenous copper or iron, it may also occur via nonspecific thiol transsulfurases [32]. These enzymes located in erythrocytes and hepatocytes catalyze the desulfuration of 3-mercaptopyruvate [32].

The other minor metabolites found in urine have not been identified. They may represent sulfinic or sulfonic forms of DMSA or MSA since they would be expected to be resistant to electrolytic reduction. It is only at the later time points that these minor metabolites appear in significant amounts.

In addition, incubation of DMSA in rabbit urine (Fig. 6) as a control showed that all of the DMSA was oxidized since most of the DMSA could be detected only after electrolytic reduction. This is in stark contrast to the results obtained when urine from rabbits given DMSA was examined (Fig. 1). In the latter case, no significant difference was found in the urinary DMSA before and after electrolytic reduction. This indicates that treatment of the urine with bromabimone immediately upon collection avoided autooxidation of DMSA.

Cysteine was found only after electrolytic reductive treatment of urine. The amount of cysteine, found after electrolytic treatment of the urine from rabbits given DMSA was 17 times less than that of DMSA over the 6-hr period.

The results of this investigation demonstrate that the majority of the therapeutically important chelating agent DMSA was excreted in the urine as unaltered DMSA when administered i.m. to rabbits. The biotransformation of DMSA was much different from that of DMPS.

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