Journal of Chromatography, 374 (1986) 297—310
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2876

FLUOROMETRIC DETERMINATION OF 2,3-DIMERCAPTOPROPANE-1-SULFONIC ACID AND OTHER DITHIOLS BY PRECOLUMN DERIVATIZATION WITH BROMOBIMANE AND COLUMN LIQUID CHROMATOGRAPHY

RICHARD M. MAIORINO, GREGORY L. WEBER and H. VASKEN APOSHIAN*

University Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721 (U.S.A.)

(Received August 16th, 1985)

SUMMARY

The increasing therapeutic use of dithiol metal binding agents, such as 2,3-dimercaptopropane-1-sulfonic acid (DMPS), has stimulated the need for a sensitive and selective method for their determination in biological fluids. A method has now been developed in which DMPS was converted to a highly fluorescent and stable derivative by reaction with bromobimane in aqueous solution at pH 8.3. The reaction was complete within 5 min. The derivative was separated by ion-pair reversed-phase column liquid chromatography. The mass spectrum of the derivative showed that two bromobimane molecules reacted with one DMPS molecule. This method is also applicable to the determination of other dithiols. The detection limit for DMPS in urine is 10 pmol per 20-μl injection and the precision is 7.4% at the 100-pmol level. The fluorescence response was linear from 1 to 400 μM. This method was used to determine DMPS in the urine of rabbits treated with this metal binding agent. In addition, total DMPS was determined by adding sodium tetrahydridoborate to the same urine to reduce biotransformed and oxidized DMPS.

INTRODUCTION

Water-soluble dithiols such as the sodium salt of 2,3-dimercaptopropane-1-sulfonic acid (DMPS) and 2,3-dimercaptosuccinic acid (DMSA) are replacing the more toxic and lipophilic 2,3-dimercaptopropanol (BAL) in the treatment of arsenic, mercury and lead poisoning of humans and experimental animals [1—3]. These dithiols rapidly remove the toxic metal or metalloid from body tissues via urinary excretion presumably by forming more water-soluble metal complexes or chelates [3—5].

0378-4347/86/$03.50 © 1986 Elsevier Science Publishers B.V.

Despite the usefulness of these drugs in treating children and adults poisoned with lead [6—8] or other metals [9], definitive biotransformation studies of them are lacking because of the limitations of present analytical methods. The lack of such studies has also brought into question the appropriateness and interpretation of pharmacokinetic investigations. Analytical methods having both sufficient sensitivity and selectivity such as fluorescence detection and high-performance liquid chromatography (HPLC) are not available for the determination of dithiols in urine and serum. Although a colorimetric assay using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid), has been reported for the quantitative determination of DMSA in whole blood [10], it has limited sensitivity and selectivity. Since the sulfhydryl groups of these dithiols are susceptible to oxidation, the development of analytical techniques that are capable of detecting and distinguishing between the parent dithiol and its intermolecular disulfide and tetrasulfide forms is necessary.

HPLC in conjunction with fluorescence detection has been a useful technique for the sensitive and selective determination of biogenic monothiols. Fluorogenic reagents that alkylate thiols, such as *o*-phthalaldehyde, N-substituted maleimides, bromobimane and halogenobenzoxadiazoles, yield unique derivatives that are readily separated by reversed-phase HPLC and at the same time prevent the oxidation of thiols on the column [11—14]. None of these reagents, however, have been applied to the determination of dithiols. We have investigated the use of bromobimane (BB) for the determination of dithiols and have developed a method for the quantitative determination of the therapeutically useful dithiol, DMPS, by precolumn derivatization with BB and reversed-phase HPLC. Bromobimane is a derivative of syn-9,10-dioxabimane (3,4,6,7-tetramethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione).

EXPERIMENTAL

Reagents

Chemicals were obtained from the following sources: DMPS sodium salt and [1,3-^{14}C]DMPS (specific activity 87.7 μCi/mmol) from Heyl (Berlin, F.R.G.); N-(2,3-dimercaptopropyl)phthalamidic acid (DMPA), the internal standard, from Dr. T. Yonaga (Teikyo University School of Medicine, Tokyo, Japan); DMSA from Johnson & Johnson (Skillman, NJ, U.S.A.); sodium sulfite, 2-mercaptoethanesulfonic acid sodium salt, mercaptosuccinic acid and dithiothreitol (DTT) from Sigma (St. Louis, MO, U.S.A.); 5-*sec.*-butyl-5-ethyl-2-thiobarbituric acid sodium salt (Inactin) from Gary Lockwood Assoc. (East Lansing, MI, U.S.A.); BB from Calbiochem-Behring (La Jolla, CA, U.S.A.); tetrabutylammonium bromide (TBAB) from J.T. Baker (Phillipsburg, NJ, U.S.A.); sodium tetrahydridoborate from Alfa Products (Danvers, MA, U.S.A.); dichloromethane, acetonitrile and methanol (HPLC quality) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Double-distilled, deionized water was used to prepare all standard and reagent solutions.

Stock solutions of thiol standards (20 m*M*) were prepared in 0.01 *M* hydrochloric acid (pH 2.0), 0.1 *M* NH_4HCO_3 (pH 8.0) or water. Working standards were prepared by diluting stock solutions (1:10 to 1:1000) with 0.01 *M* hydrochoric acid or water to appropriate concentrations and were stored frozen a

−20°C. These solutions were found to be stable for three months at −20°C. The BB reagent (40 m*M*) was prepared in acetonitrile and stored at −20°C in a bottle wrapped in aluminum foil to protect it from light. Aqueous 0.1 *M* DTT and 0.5 *M* sodium tetrahydridoborate were prepared as reducing reagents just prior to use. The 0.1 *M* NH_4HCO_3 reaction buffer (pH 8.0) was purged with nitrogen for at least 1 h immediately prior to use. The mobile phase solvents consisted of 20 m*M* TBAB in methanol (solvent A) and 20 m*M* TBAB in water (solvent B). These solvents were filtered (0.45-μm Nylon-66 filter, Rainin, Woburn, MA, U.S.A.) prior to use. DMPS solutions (0.67 *M*, pH 7.3) used to inject rabbits were prepared in an aqueous solution of 0.9% sodium chloride—5% $NaHCO_3$ and stored at −20°C until used. No conversion or DMPS to oxidized species was evident upon thawing the solutions as determined by BB derivatization and HPLC.

Apparatus

A Beckman Model 334 gradient liquid chromatograph equipped with two Model 110A pumps was used for separations. Mobile phase composition was adjusted by high-pressure mixing of solvents in a dynamically stirred gradient mixing chamber by varying the speed of the pumps using a Beckman 421 system controller. A Beckman Model 157 fluorescence detector equipped with a 9-μl flow cell and 356-nm (Corning 7-60) excitation and 450 ± 20-nm emission filters was used. A Hewlett-Packard 3390A reporting integrator was used for data acquisition. Injections were made with a Beckman Model 210 injection valve via a 20-μl sample loop. HPLC fractions were collected with an LKB 2112 Redirac fraction collector. Radioactivity was determined using a Beckman LS7800 liquid scintillation counter. Fast atom bombardment (Xenon) negative-ion mass spectra (FAB-NI-MS) were obtained with a Varian MAT 311A double-focusing mass spectrometer equipped with an SS200 data system. Samples were inserted into the source (50°C) on a direct FAB probe containing a glycerol matrix. The FAB gun (Ion Tech) was operated at 7 kV.

Derivatization protocol

To a 7-ml glass tube containing 0.1 *M* NH_4HCO_3 solution (pH 8.0) and 50 μl of the 40 m*M* BB reagent were added 0.1 or 0.2 ml of working standard or urine sample. The final volume was 2 ml. After purging the head-space with nitrogen, the reaction tube was capped, contents mixed and shaken at ambient temperature in the dark for 5 min on a mechanical shaker (Eberbach clinical rotator, setting 190). The excess BB reagent was extracted by shaking with 2 ml of dichloromethane for 10 s. Phase separation was accomplished by centrifuging (IEC International clinical centrifuge Model CL) at full speed for 2 min. The aqueous phase was removed, adjusted to pH 7 by adding 15 μl of 6 *M* hydrochloric acid and injected (20 μl) onto the HPLC column. All glass reaction tubes were rinsed with 10% hydrochloric acid to remove residual metals.

Reduction procedures

DMPS was treated with DTT according to a modification of the method of Zahler and Cleland [15] for the reduction of disulfides. To 1.8 ml of 0.2 *M*

Na_2CO_3—$NaHCO_3$ buffer (pH 9.0) and 0.1 ml of 0.1 *M* DTT was added 0.1 ml of a 2 m*M* DMPS standard. The mixture was shaken for 30 min at room temperature. A 0.1-ml aliquot was taken for BB derivatization according to the above derivatization protocol except a ten-fold greater concentration of BB was used since BB also reacts with DTT. In addition, the volume of dichloromethane was doubled for the extraction of excess BB and derivatized DTT. For comparison, a control was prepared without DTT treatment. Urine samples were treated with sodium tetrahydridoborate to convert any oxidized DMPS to reduced DMPS. To a long glass culture tube (15 × 1 cm) containing water purged with nitrogen and 0.1 or 0.2 ml of urine sample or working standard were added 1.4 ml of 0.5 *M* sodium tetrahydridoborate (final volume 2 ml). After purging the head-space with nitrogen, the tube was capped, contents mixed and shaken for 15 min. The tube was immersed into liquid nitrogen and 0.5 ml of cold 2 *M* hydrochloric acid was added to consume excess sodium tetrahydridoborate and to lower the pH to 1—2. After reaching ambient temperature, the contents of the tube were mixed and 0.2 ml was taken for BB derivatization according to the standard protocol. Sodium tetrahydridoborate has been used successsfully for the reduction of urinary disulfides [16].

Sample collection

Male New Zealand white rabbits (1.5–2.0 kg) were sedated with intramuscular injections of Ketamine (45 mg/kg). Urine was collected directly from the bladder using a catheter. The collection was facilitated by attaching a hypodermic syringe to the catheter. Physiological saline was supplied continuously intravenously to replenish body fluids. After emptying the bladder, a single intramuscular injection of DMPS (0.2 mmol/kg) was given. Urine was collected at various time points (0—4 h) and 0.1- or 0.2-ml aliquots immediately were processed according to the standard BB derivatization protocol. Other aliquots were added to glass tubes containing 9 *M* hydrochloric acid (75 μl acid per 1 ml urine) or no additive and frozen (—20° C) to be processed later.

HPLC conditions

Chromatographic separations were accomplished with a 250 × 4.6 mm stainless-steel Ultrasphere IP C_{18} reversed-phase column containing 5-μm packing (Beckman). The analytical column was protected by a Rheodyne Model 7302 inlet filter (Beckman) and by a 45 × 4.6 mm guard column containing 10-μm Ultrasphere IP C_{18} packing. Separations were performed at ambient temperature and at a flow-rate of 1 ml/min. The mobile phase gradient was as follows: isocratic at 45% solvent B for 11 min; 45% to 25% B in 1 min; isocratic at 25% B for 7 min; 25% to 45% B in 1 min; equilibration at 45% B for 15 min. Elution time per injection was 35 min.

Quantitation of DMPS

Calibration curves were constructed from the chromatographic data obtained from standard reaction mixtures containing varying concentrations of DMPS. The working standards were prepared in 0.01 *M* hydrochloric acid or acidified urine (pH 2.5). The suitability of DMPA as an internal standard was assessed

by preparing reaction mixtures containing a constant concentration of DMPA with varying concentrations of DMPS. Linearity was determined from linear regression analyses of plots of BB-DMPS peak area or peak-area ratio (BB-DMPS/BB-DMPA) versus concentration of DMPS. The amount of DMPS in urine samples was calculated from the calibration curves. The purity of the batch of DMPS used in this study was taken into account when calculating the amount of DMPS found in urine samples. As determined by the manufacturer, the batch used contained 90% of the material as DMPS with about 9% representing isomeric acyclic disulfide and isomeric cyclic tetrasulfide polymers of DMPS [17].

RESULTS AND DISCUSSION

HPLC separation of BB-DMPS derivative and comparison to monothiols

A typical chromatogram of DMPS after derivatization with BB is shown in Fig. 1. The early eluting peaks (3—6 min) represent hydrolysis products of BB [13] as determined by analyzing a reagent blank not containing DMPS. The extraction with dichloromethane greatly reduces the size of these early peaks. The remaining peaks in the chromatogram represent DMPS-derived products as determined by scintillation counting of column fractions collected from the HPLC separation of BB-derivatized [^{14}C] DMPS. Resolution of the major BB-DMPS peak (11.0 min) from the early eluting peaks was accomplished by including the ion-pairing reagent TBAB (20 m*M*) in the mobile phase methanol–water (55:45). Elution of the later cluster of smaller peaks (18—21 min) was accomplished by increasing the mobile phase methanol content to 75%. The relative standard deviation (R.S.D.) of the retention time of the major BB-DMPS peak was 1.17% (n = 6), indicating that conditioning the column with the initial mobile phase composition for 15 min was sufficient for column equilibration. The multiple peaks, however, do not represent multiple reaction products of DMPS with BB.

The structure of the major BB-DMPS derivative (Fig. 2), as determined by FAB-NI-MS (see below), shows that both sulfhydryl groups of DMPS reacted

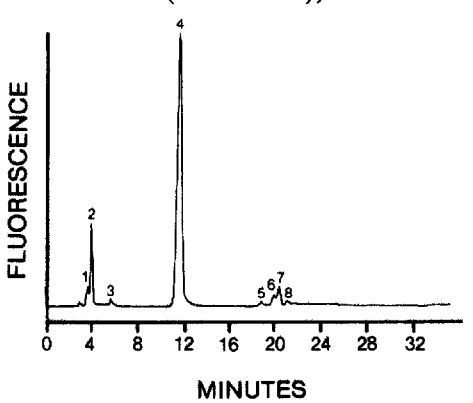


Fig. 1. Chromatogram of 2,3-dimercaptopropane-1-sulfonic acid (75 μM) derivatized with bromobimane (BB). Derivatization and chromatographic conditions as in Experimental. Peaks: 1—3 = hydrolysis products of BB; 4 = BB-DMPS derivative; 5—8 = BB-DMPS isomeric intermolecular disulfide derivatives.

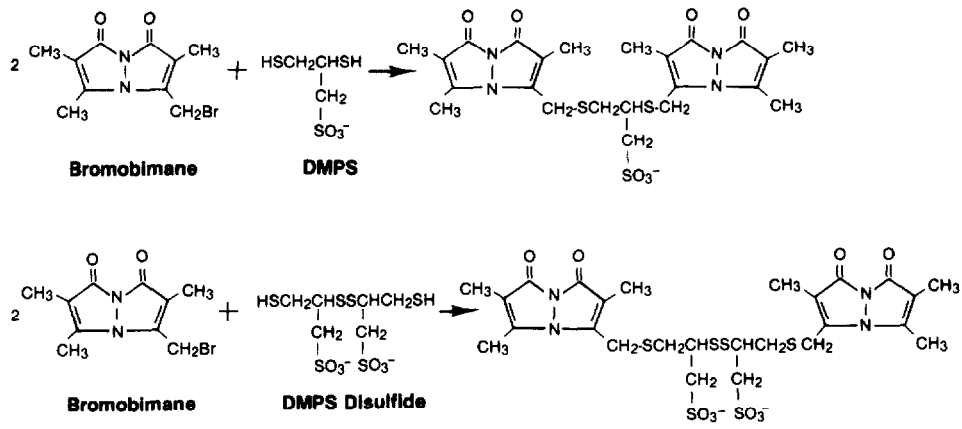


Fig. 2. Proposed reactions of bromobimane with 2,3-dimercaptopropane-1-sulfonic acid (DMPS) and one of its isomeric intermolecular disulfides.

with BB. The cluster of smaller peaks (5—8, Fig. 1) appear to be isomers of the intermolecular disulfide form of DMPS. The structure of one of these disulfides is shown in Fig. 2. The mixture of isomeric disulfides represented 7% of the total fluorescence response as determined by area percentage under the peaks. The presence of these isomeric disulfides in the drug has also been confirmed by the manufacturer; 6.5% as determined by HPLC and NMR analyses [17]. Treatment of DMPS with the reducing agent DTT followed by derivatization with BB resulted in a decrease in the peak areas of the cluster of small peaks with a concomitant increase in the area of the major DMPS peak.

When various monothiols were examined by derivatizaton with BB (Table I) only one HPLC peak was found. Derivatization with BB, separation by HPLC and detection by fluorescence are applicable to the determination of other dithiols besides DMPS. Each of the dithiols, DMSA and DMPA, however, gave two peaks. Like DMPS, the minor eluting peaks may represent the intermolecular disulfide form of the particular drug. Thus, the intermolecular disulfide forms of dithiols can be detected by this method.

pH Dependence of reaction

The optimal pH range for the reaction of DMPS with BB was determined by

TABLE I

HPLC CAPACITY FACTORS OF BB DERIVATIVES OF MONOTHIOLS AND DITHIOLS

Thiol	Capacity factor (k')	
	Major peak	Minor peak(s)
Mercaptosuccinic acid	0.58	—
Sodium sulfite	0.78	—
2-Mercaptoethanesulfonic acid	1.19	—
2,3-Dimercaptosuccinic acid	2.55	6.36
2,3-Dimercaptopropane-1-sulfonic acid	3.05	6.09, 6.23, 6.49, 6.65
N-(2,3-Dimercaptopropyl)phthalamidic acid	4.93	6.84
4-*sec.*-Butyl-5-ethyl-2-thiobarbituric acid	5.49	—

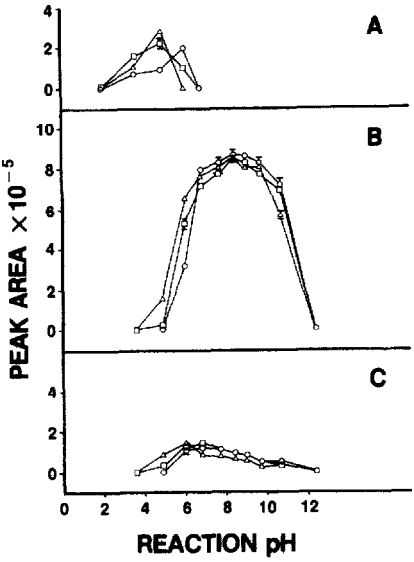


Fig. 3. Influence of pH of the reaction mixture on the reactivity of BB (1.0 m*M*) with DMPS (0.05 m*M*). (A) 9.4-min peak; (B) 11.0-min peak; (C) 18–21 min peaks. Teorell–Stenhagen buffers [18] were 0.05 *M* (pH 2–12). Reaction times were 5 min (○), 25 min (□) and 60 min (△) at room temperature. Reactions were stopped by extraction with dichloromethane. Data represent mean ± mean deviation of duplicate reaction mixtures. Chromatographic conditions as in Experimental.

performing reactions from pH 2.0 to 12.4 (Fig. 3). The reaction mixtures were shaken for 5, 25 or 60 min and then extracted with dichloromethane to stop the reaction by removing unreacted BB. The pH values of the aqueous phases were measured and the aqueous phases analyzed by HPLC. The results indicated that the optimal pH range for maximum formation of the BB-DMPS derivative (11.0-min peak) was 7.7–9.7 (Fig. 3B). An early eluting peak (9.4 min, Fig. 3A) appeared when the pH of the reaction mixture was between 2.0 and 7.0. This peak did not appear when DMPS was omitted from the reaction mixtures. As the reaction time was increased from 5 to 60 min, the fluorescence response represented by the peak at 9.4 min increased between pH 2.0 and 5.0 and its maximum response shifted from pH 6.0 to 4.9. The area of the 9.4-min peak, however, decreased at pH 6.0 with time while the response of the BB-DMPS peak (11.0 min) increased with time. The response of the disulfide peaks (Fig. 3C) increased with time below pH 6.0 but decreased above pH 6.0. These data suggest that below pH 5 only one sulfhydryl group of DMPS is reacting with BB to any extent (represented by the 9.4-min peak) and as the pH is increased above 5, the second sulfhydryl group reacts with BB (represented by the 11.0-min peak). Evidently the anionic sulfonate group of DMPS is lowering the pK_a of the neighboring sulfhydryl group via electrostatic attraction to the sulfhydryl hydrogen.

Reaction time

Reaction mixtures containing 0.1 m*M* DMPS and 1.0 m*M* BB were prepared at ambient temperature in 0.1 *M* NH_4HCO_3 at about the midpoint of the

optimal pH range (pH 8.3, Fig. 3B). The reaction time was varied (0, 5, 10, 15 and 25 min) prior to extraction of the reaction mixture with dichloromethane. After mixing for 5 s, the reaction mixtures were either shaken on a mechanical shaker or allowed to stand. Time of 0 min denotes extraction with dichloromethane immediately after mixing for 5 s. The reaction appeared to be almost instantaneous with no significant change in the fluorescent response with time. The R.S.D. in mean peak area (n = 2) of the five reaction times was 1.38% for shaking and 2.39% for standing. The difference between shaking and standing was not significant ($p > 0.05$). Shaking, however, is included in the derivatization protocol since the reproducibility of four replicate 5 μM DMPS reaction mixtures was improved from 13.4 to 8.8% (R.S.D. in peak area, 10 min reaction time). The 5 min reaction time was chosen because of the gradual hydrolysis of BB at longer reaction times (data not shown). The peak-area ratio of disulfide to DMPS was constant with time indicating that the oxidation of DMPS or disulfide at the reaction pH of 8.3 was negligible.

Concentration of BB

The optimal concentration range for BB is 0.4—2 mM (Table II). Based on these results, at least a ten-fold excess of BB is recommended for the quantitative analysis of DMPS in the micro- to millimolar concentration range. Multiple peaks were obtained when the BB-to-DMPS molar ratio was less than 2.

TABLE II

EFFECT OF BB CONCENTRATION ON THE DERIVATIZATION OF DMPS

DMPS (0.1 mM) and BB were reacted at room temperature for 5 min in 0.1 M NH_4HCO_3 (pH 8.3). After extraction with dichloromethane, 15 μl of 6 M hydrochloric acid were added to the aqueous phase (final pH 7). The acidified aqueous phases were analyzed by HPLC. Reaction mixtures were prepared in duplicate.

Concentration of BB (mM)	Peak area (± mean deviation) (mV · s)
0.2	242.7 ± 7.8
0.4	312.4 ± 2.6
1.0	324.4 ± 19.8
2.0	295.5 ± 10.4

Stability of BB-DMPS derivative

The derivative was prepared by reaction at pH 8.3 and was isolated by HPLC. Solutions of BB-DMPS (0.1 mM) were prepared in buffers of various pH values. The solutions were stored at 5°C for 0, 1 and 7 days and analyzed by HPLC. The peak areas indicated that the BB-DMPS derivative was stable between pH 2.0 and 9.0 (Fig. 4). It is recommended, however, that the pH of the aqueous phase be adjusted to 2.5—7 to avoid forming pin hole voids at the head of the column caused by injection of solutions at pH > 7.5.

Identification of BB-DMPS derivative

Volatilization of the BB-DMPS derivative, isolated by HPLC, was accomplished by FAB. FAB ionizes non-volatile or ionic substances that are

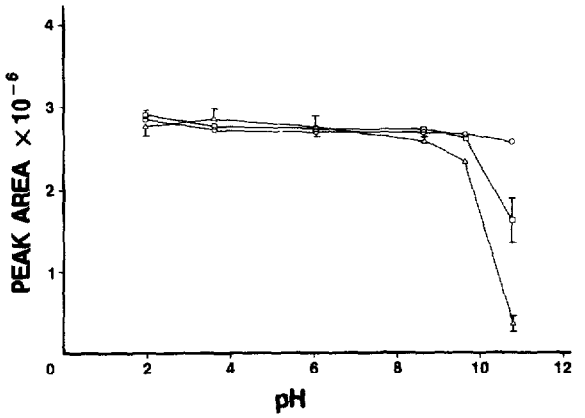


Fig. 4. The stability of the BB-DMPS derivative at different pH values. Solutions of BB-DMPS (0.1 m*M*) were prepared in 0.05 *M* Teorell–Stenhagen buffers (pH 2–11) and stored at 5°C for 0 day (○), 1 day (□) and 7 days (△). Chromatographic conditions as in Experimental. Data represent the mean ± mean deviation of duplicate reaction mixtures.

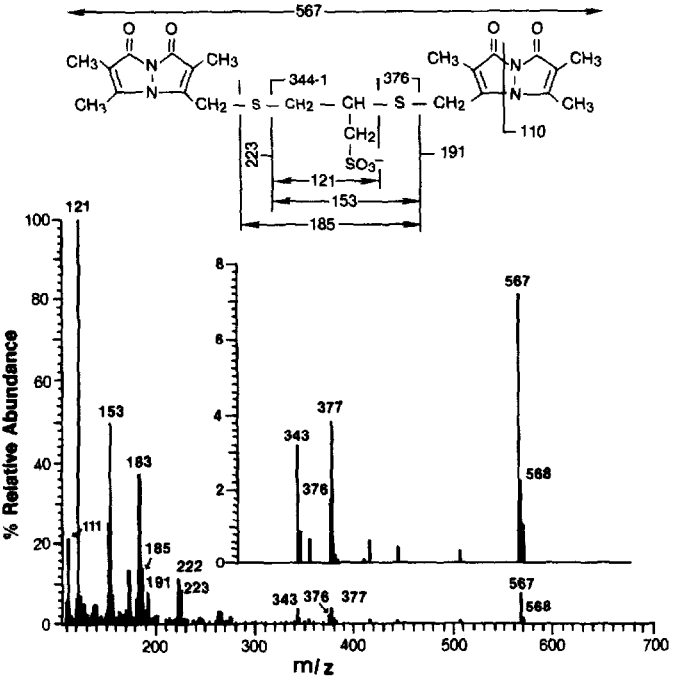


Fig. 5. Fast atom bombardment negative-ion mass spectrum and proposed structure of the BB-DMPS derivative isolated by HPLC.

refractory to conventional ionization methods. The successful use of FAB for the MS analysis of sulfonates has recently been reported [19]. Counter-ion peaks in the mass spectrum of the sample that were due to TBAB were eliminated by computer subtraction of the corresponding HPLC fraction from a control reaction that did not contain DMPS. The negative-ion mass spectrum showed useful fragmentation as well as the presence of the parent molecular ion (Fig. 5). Several of the ions in the spectrum suggested the proposed

structure shown in Fig. 5. The mass spectrum clearly indicated the presence of the parent anion (BB-DMPS$^-$) at *m/z* 567. The fragment ions at *m/z* 185, 153 and 121 (base peak) were indicative of the sequential loss of sulfur with a concomitant increase in relative abundance. In addition, these fragment ions were the most diagnostic since they represent the skeletal backbone of the original DMPS molecule and were in high abundance. The peak at *m/z* 183 was most likely a cluster ion of glycerol. The fragment ions at *m/z* 377 and 111 may have been formed by abstraction of a hydrogen atom from the surface of the matrix. Likewise, transfer of a hydrogen atom to constituents in the matrix probably resulted in the formation of the fragment ions at *m/z* 343 and 222. The presence of the TBA cation in the sample (ten-fold molar excess) appeared to enhance rather than suppress ionization. This apparent enhancement in ionization may be due to increased volatilization via ion-pairing [20].

Linearity, precision and detection limits

Table III lists calibration curve data obtained from the derivatization of DMPS using the derivatization protocol. DMPS standards were prepared in different media as indicated. The curves showed excellent linearity with correlation coefficients ranging from 0.9959 to 0.9999. Comparison of the slopes and intercepts of the linear regressions [21] showed no significant difference ($p > 0.05$) between standards prepared in 0.01 *M* hydrochloric acid and in acidified urine. Upon storage at −20°C, however, degradation of standards prepared in acidified urine was noted. An acidified urine standard

TABLE III

STANDARD CALIBRATION CURVE DATA

DMPS reaction concentration (μM)	Peak area (mV · s)			Peak-area ratio (DMPS/DMPA)	
	0.01 *M* Hydrochloric acid*	0.01 *M* Hydrochloric acid**	Acidified urine**	0.01 *M* Hydrochloric acid**	Acidified urine**
1	1.1635	1.4011	1.0442	0.182	0.175
2	3.7553	3.2549	2.3864	0.381	0.347
3	9.7589	9.8874	7.4778	0.964	0.890
10	18.508	22.795	19.450	2.128	2.041
20	36.263	53.525	47.420	4.665	4.733
100	192.69	—	—	—	—
400	807.42	—	—	—	—
r	0.9999	0.9976	0.9959	0.9989	0.9973
y-Intercept	−2.4908	−2.7883	−3.1919	−0.1356	−0.1959
Slope	2.0204	2.7580	2.4668	0.2368	0.2412
x-Intercept	1.2328	1.0110	1.2940	0.5725	0.8120

*Working standards were prepared in 0.01 *M* hydrochloric acid and 50 μl were added to reaction tubes containing 100 μl of urine, 50 μl of 40 m*M* BB and 1.80 ml of 0.1 *M* NH_4HCO_3 (pH 8.3).

**Working standards were prepared in 0.01 *M* hydrochloric acid or acidified urine (pH 2.5) and 100 μl were added to reaction tubes containing 50 μl of 40 m*M* BB and 1.85 ml of 0.1 *M* NH_4HCO_3 (pH 8.3).

containing 2 m*M* DMPS was frozen for 0, 1, 3 and 20 days, thawed, and was used in reaction mixtures adjusted to contain 50 μM DMPS. The results of the analysis showed that the concentration of DMPS was 98.5, 81.0, 73.2 and 63.8%, respectively, of theory as determined by calibration curves prepared from working standards in 0.01 *M* hydrochloric acid. Alternatively, standard calibration curves were obtained using reaction mixtures prepared by adding 50 μl of DMPS standards in 0.01 *M* hydrochloric acid and adding 100 or 200 μl of blank urine. A typical curve that encompasses the range of concentration of DMPS found in urine from DMPS-treated rabbits showed excellent linearity (Table III). When DMPA was used as an internal standard the linearity and x-intercept of the standard calibration curve improved (Table III). The feasibility of the method for the determination of other dithiols was demonstrated by performing standard calibration curves of DMPA and DMSA (reaction concentrations from 2 to 20 μM). Linear regression analysis gave correlation coefficients of 0.9987 for both compounds and x-intercepts of 2.0619 and −1.2190, respectively.

Comparison of the slopes and intercepts of linear regressions obtained from reaction mixtures (5 to 100 μM) with or without extraction with dichloromethane showed no significant difference ($p > 0.05$). This indicated that BB-DMPS was not extracted significantly by dichloromethane (data not shown). In addition, HPLC analysis of the dichloromethane phase of duplicate 100 μM reactions showed that only 0.2% (range 0.009%) of the derivative was extracted. Extraction with dichloromethane removes BB hydrolysis products and urine constituents that would otherwise interfere with the column resolution of BB-DMPS when the DMPS reaction concentration is less than 5 μM. Deproteinization or filtration of urine samples or reaction mixtures was found to be unnecessary since extraction with dichloromethane followed by centrifugation removed particulates and precipitates from the aqueous phases of reaction mixtures.

The precision of the instrument was determined by six injections of a 5 μM DMPS reaction mixture. The relative standard deviation (R.S.D.) in peak area was 0.9%. The precision of the derivatization reaction was determined by injecting six replicates each of 5 and 50 μM DMPS reaction mixtures. The R.S.D. in peak area was 7.4 and 3.2%, respectively. Purging the NH_4HCO_3 reaction buffer and the head-space of reaction mixtures with nitrogen improved the reproducibility at low DMPS concentrations from 24.7 to 7.4% (R.S.D. in peak area of six replicate 5 μM reaction mixtures). When DMPA was used as an internal standard, precision improved from 7.4 to 1.6% (R.S.D. in peak area, six replicate 5 μM reaction mixtures). The accuracy of the method was tested by standard addition of DMPS to a rabbit urine sample collected 0.5 h after DMPS treatment. Of the DMPS spike 98% was recovered as determined by a standard calibration curve.

The detection limit of the method for DMPS in urine was 10 pmol per 20-μl injection (signal-to-noise ratio of 3). This corresponded to a 0.5 μM DMPS reaction mixture containing 100 μl of 10 μM DMPS in urine.

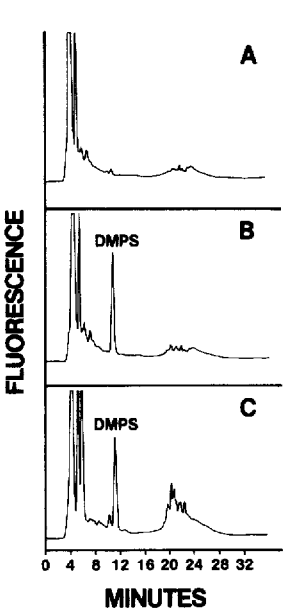
Applications

This method can be used to determine the amount of DMPS excreted in the

urine of subjects given DMPS. Examples of chromatograms of reaction mixtures containing urine, urine spiked with DMPS or urine from a DMPS-treated rabbit are shown in Fig. 6. The BB-DMPS peak is well resolved from peaks representing other urinary constituents (Fig. 6B and C). The range of DMPS concentrations in urines that were analyzed varied from 0.015 to 3.3 m*M* (corresponding to DMPS concentrations in the reaction mixture of 0.75—330 μM).

Analysis of urines, collected via a catheter, from DMPS-treated rabbits (Fig. 7) indicated that 4 h after injection of DMPS only 3.2% of the administered dose was excreted in the urine as unchanged DMPS. The maximum amount of unchanged DMPS was detected 0.5 h after injection. It should be emphasized that urine upon collection must be processed immediately with BB to avoid post-oxidation of the sulfhydryl groups of DMPS. For example, urine samples collected 0.5 h after DMPS administration, frozen (—20°C) for 24 h and then analyzed according to the standard derivatization protocol contained only 9.4% of the DMPS found in the same urine that had been processed immediately with BB. Analysis of urine samples (0.5 h) that were acifidied with hydrochloric acid and then frozen for 24 h also resulted in less DMPS; 69% compared to the same urine that was immediately processed with BB.

When urine samples were treated with sodium tetrahydridoborate followed



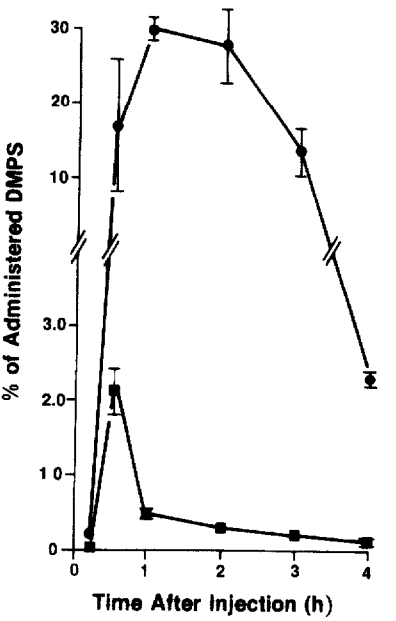


Fig. 6. Representative chromatograms of reaction mixtures from (A) urine of untreated rabbit, (B) urine of untreated rabbit spiked with DMPS (10 μM DMPS in reaction mixture) and (C) urine collected from a rabbit 2 h after intramuscular administration of 0.2 mmol/kg DMPS. Derivatization and chromatographic conditions as in Experimental.

Fig. 7. DMPS excretion in urine of rabbits treated intramuscularly with 0.2 mmol/kg DMPS. Urine was collected at indicated times using a catheter and analyzed by the BB derivatization protocol and HPLC. Lower curve: unaltered DMPS found in urine; upper curve: DMPS found after sodium tetrahydridoborate treatment of urine. Data represent the mean ± mean deviation of two rabbits.

by BB derivatization, 91.2% of the administered dose of DMPS was found 4 h after injection (Fig. 7). The maximum amount was detected between 1 and 2 h after injection. The results indicate that DMPS is rapidly oxidized or biotransformed in the rabbit and the majority is excreted in urine within 4 h. Gabard [22] injected [^{14}C]DMPS and 6 h later found 89 ± 2% of the administered radioactivity in the urine of rats. In addition, analysis of the urine by Gabard and Walser [23] using thin-layer chromatography suggested that DMPS is not involved in important metabolic reactions.

Treatment of urine by reduction with sodium tetrahydridoborate converted the altered form of DMPS back to its original form as determined by BB derivatization and HPLC with fluorescence detection. The identity, however, of the structure of altered DMPS is unknown at present. DMPS, in vitro, is oxidized readily to the tetrasulfide form in alkaline medium (the tetrasulfide form does not react with BB). DMPS chelates endogenous zinc [24] and increases the excretion of endogenous copper presumably via chelation [25]. Possibly it may form mixed disulfides with cysteine or glutathione or any other endogenous thiol. The chemical structure of the altered DMPS is being investigated in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by NIEHS Grant No. 1 RO1 ES 03356-01. R.M.M. was the recipient of a Heyl Postdoctoral Fellowship. The assistance of P.F. Baker with the MS analysis is appreciated. We wish to express our gratitude and appreciation to D.H. McKelvie and S.E. Wilson for assistance in catheterizing and managing the anesthetized rabbits and to D.E. Carter and K.H. Schram for their critical review of the manuscript.

REFERENCES

1 H.V. Aposhian, Adv. Enzyme Regul., 20 (1982) 301.
2 H.V. Aposhian, Ann. Rev. Pharmacol. Toxicol., 23 (1983) 193.
3 J. Aaseth, Human Toxicol., 2 (1983) 257.
4 B. Gabard, Arch. Toxicol., 35 (1976) 15.
5 H.V. Aposhian, D.E. Carter, T.D. Hoover, C. Hsu, R.M. Maiorino and E. Stine, Fundam. Appl. Toxicol., 4 (1984) S58.
6 E. Friedheim, J.H. Graziano, D. Popovac, D. Dragovic and B. Kaul, Lancet, ii (1978) 1234.
7 J.J. Chisolm and D. Thomas, The John F. Kennedy Institute for Handicapped Children, Baltimore, MD, 1985, personal communication.
8 J.H. Graziano, E.S. Siris, N. Lolacono, S.J. Silverberg and L. Turgeon, Clin. Pharmacol. Ther., 37 (1985) in press.
9 T.W. Clarkson, L. Magos, C. Cox, M.R. Greenwood and L. Amin-Zaki, J. Pharmacol. Exp. Ther., 218 (1981) 74.
10 P.J. Kostyniak, J. Pharmacol. Exp. Ther., 221 (1982) 63.
11 K. Mopper and D. Delmas, Anal. Chem., 56 (1984) 2557.
12 B. Jarrott, A. Anderson, R. Hooper and W.J. Louis, J. Pharm. Sci., 70 (1981) 665.
13 G.L. Newton, R. Dorian and R.C. Fahey, Anal. Biochem., 114 (1981) 383.
14 T. Toyo'oka and K. Imai, Anal. Chem., 56 (1984) 2461.
15 W.L. Zahler and W.W. Cleland, J. Biol. Chem., 243 (1968) 716.
16 K. Bir, J.C. Crawhall and D. Mauldin, Clin. Chim. Acta, 30 (1970) 183.

17 E. Bayer, Heyl & Co., Berlin, F.R.G., 1981, unpublished results.
18 H.A. Sober (Editor), Handbook of Biochemistry, Selected Data for Molecular Biology, CRC, Cleveland, OH, 1st ed., 1968, Section J, p. 195.
19 P.A. Lyon, W.L. Stebbings, F.W. Crow, K.B. Tomer, D.L. Lippstreu and M.L. Gross, Anal. Chem., 56 (1984) 8.
20 D.P. Kirby, P. Vouros, B.L. Karger, B. Hidy and B. Petersen, J. Chromatogr., 203 (1981) 139.
21 J.H. Zar, Biostatistical Analysis, Prentice-Hall, NJ, 1st ed., 1974, Ch. 17.
22 B. Gabard, Arch. Toxicol., 39 (1978) 289.
23 B. Gabard and R. Walser, J. Toxicol. Environ. Health, 5 (1979) 759.
24 V.E. Petrunkin, Tiolovye Soedinen. Med. Ukrain. Nauch-Issledovatel. Sanit.-Khim. Inst., Trudy Nauch. Konf. Kiev, 1957, p. 7.
25 F. Planas-Bohne, Toxicol. Appl. Pharmacol., 50 (1979) 337.