Identification of a New Urinary Metabolite of Carbon Disulfide Using an Improved Method for the Determination of 2-Thioxothiazolidine-4-carboxylic Acid

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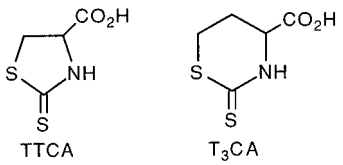
A new method is reported for the analysis of 2-thioxothiazolidine-4-carboxylic acid (TTCA) in urine that is amenable to automation and provides greatly simplified chromatograms. The method comprises the addition of tetrahydro-2-thioxo-2*H*-1,3-thiazine-4-carboxylic acid, which is chemically similar to TTCA, as internal standard, purification on an Oasis HLB solid-phase extraction column, and analysis by HPLC with UV detection. The limit of detection for TTCA was 40 pmol/mL of urine, recovery was 79.3 ± 1.0%, and detection was linear over at least 3 orders of magnitude. In addition, during the analysis of urine samples from workers exposed to CS_2, a novel urinary metabolite of CS_2 was recognized. The new metabolite demonstrated a dose response, was present at approximately 30% the level of TTCA, and was charaterized to be 2-thioxothiazolidin-4-ylcarbonylglycine (TTCG). Administration of TTCG to rats resulted in excretion of TTCA suggesting that TTCG is a likely precursor of TTCA. Although urinary excretion of both TTCA and TTCG resulted from administration of captan, only TTCA was detected following administration of methyl isothiocyanate. The greater selectivity of TTCG suggests that co-analysis of TTCA and TTCG in urine may aid in differentiating exposures to CS_2, captan and isothiocyanates.

Introduction

Identification of 2-thioxothiazolidine-4-carboxylic acid (TTCA,[1] raphanusamic acid) in the urine of workers



exposed to carbon disulfide introduced a biomarker for exposure to this important industrial solvent (*1*, *2*). Although the quantity of TTCA has been correlated with the level of exposure to CS_2, the details of the steps leading to the formation of TTCA from CS_2 are still not completely understood. As one would expect TTCA is also excreted in urine on exposure to those chemicals that release CS_2 in vivo, such as, disulfiram (used in rubber manufacture or administered orally in alcohol aversion therapy) and dithiocarbamates (utilized as pesticides) (*3*). The fungicide Captan (*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) that undergoes hydrolytic cleavage to thiophosgene also generates TTCA in vivo. Excretion of TTCA has been reported following consumption of cruciferous vegetables possibly as a result of preformed TTCA or glucosinolates present in the vegetables (*4*). Even with these limitations, determination of urinary TTCA has become the method of choice for monitoring workers' exposure to CS_2 (*5*, *6*), and a biological exposure index of 5 mg of TTCA/g of creatinine in urine has been established.

Recently, while undertaking to estimate the concentrations of TTCA in more than 200 urine samples from workers of a rayon factory in the Peoples Republic of China, we found that the currently available assays (Table 1) did not satisfy all our requirements of sensitivity, reproducibility, and ease of operation. Therefore, a new HPLC based method was developed that includes a chemically similar internal standard, removes many closely eluting impurities before chromatography, and gives highly reliable and reproducible values. The method can be automated to process multiple samples obtained when assessing occupational exposures. In addition, since the chromatograms obtained using the new method contained only a few well-separated peaks, it was possible to recognize a new peak in addition to that of TTCA that was unique to urine samples obtained from exposed workers. This paper provides evidence to show that the extra peak arose from a novel urinary metabolite of CS_2. Structural characterization of the new metabolite sheds light on the possible mechanism of formation of TTCA

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[1] Abbeviations: cys-gly, cysteinylglycine; DMF, *N*,*N*-dimethylformamide, GSH, glutathione; HPLC, high-performance liquid chromatography; MITC, methyl isothiocyanate; PBS, physiologically buffered saline, TTCA, 2-thioxothiazolidine-4-carboxylic acid; TTCG, 2-thioxothiazolidin-4-ylcarbonylglycine; T_3CA, tetrahydro-2-thioxo-2*H*-1,3-thiazine-4-carboxylic acid; TLC, thin-layer chromatography; UV, ultraviolet.

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Table 1. Comparison of the Methods Available for the Measurement of TTCA in Urine

ref	method	advantages	disadvantages
4	HPLC with column switching	direct injection of urine	column switching is inconvenient; dilution of sample and limited column life
3	GC-MS of a volatile derivative	less interference from other components of urine	requires derivatization with diazomethane or silyl reagents
8	HPLC-UV detection with gradient elution	a detection limit of 1.5 pmol	several closely spaced peaks
9	HPLC-UV detection with isocratic elution		lack of an internal standard requires exhaustive extraction

and provides complimentary information to using TTCA alone as a biomarker for assessing CS_2 exposure.

Materials and Methods

Chemicals. Carbon disulfide is volatile, flammable, toxic, and a skin irritant, MITC is toxic and easily absorbed through the skin, and Captan (Chem Service, West Chester, PA) is toxic, a suspected carcinogen and an ocular irritant. Gloves and a fume hood should be used when handling these compounds. Carbon disulfide, HPLC-grade solvents, silica for column chromatography, and silica coated aluminum sheets for thin-layer chromatography were obtained from EM Science (Gibbstown, NJ). Sigma Chemical Co. (St. Louis, MO) was the source for cys-gly, corn oil, 1,2-propanediol, and premeasured powder for phosphate buffered saline and Fluka-Aldrich (Milwaukee, WI) for the other chemicals used. The syntheses of TTCA (*7*) and T_3CA (*3*) have been published.

2-Thioxothiazolidin-4-ylcarbonylglycine Ethyl Ester. TTCA (10 mmol) was dissolved in DMF (20 mL) and stirred with dichloromethane (20 mL) and triethylamine (3.5 mL, 25 mmol) before cooling in ice. Isobutyl chloroformate (1.35 mL, 10 mmol) was added and the mixture was stirred for 1 h. Glycine ethyl ester hydrochloride (1.54 g, 11 mmol) was added and the stirring was continued as the ice melted and the reaction mixture warmed to room temperature. After 16 h, solvents were removed and the residue was dissolved in ethyl acetate (30 mL) that was washed with brine containing 1 M $NaHCO_3$ (20 mL), dried, and evaporated. The crude product was purified by flash chromatography (50−0% hexane in ethyl acetate). The fractions containing the pure product (with R_f of 0.5 on TLC in ethyl acetate) were combined and evaporated to a viscous oil; 1.74 g (70%); ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 9.5 Hz, CH_3), 3.79 (dd 1H, J = 6.6 and 15.2 Hz, C^5-H), 3.96 (dd, 1H, J = 12.1 and 15.2, C^5-H), 3.98 (m, 2 H, NCH_2), 4.10 (q, 2H, J = 9.5 Hz, OCH_2), 4.91 (dd, 1H, J = 6.6 and 12.1 Hz, C^4-H); ^{13}C NMR δ 14.5 (CH_3), 37.6 (C-5), 42.1 (NH−CH_2), 62.4 (OCH_2), 65.6 (C-4), 170.0 and 170.2 (C═O), 202.9 (C-2).

2-Thioxothiazolidin-4-ylcarbonylglycine. Ethyl ester of TTCG (0.5 g, 2 mmol) was dissolved in dioxane (5 mL) and stirred with 1 N NaOH (3 mL) for 2 h. The reaction mixture was washed with ethyl acetate (2 × 5 mL), acidified to pH 2 with 2 N HCl, and extracted with ethyl acetate (3 × 10 mL). The crude TTCG obtained from the combined extracts was purified on a column of silica (0−30% methanol in ethyl acetate); 0.33 g (75%) of off-white solid; ^{1}H NMR (D_2O) δ 3.58 (dd 1H, J = 5.5 and 11.8 Hz, C^5-H), 3.89 (dd, 1H, J = 9.4 and 11.8, C^5-H), 3.86 (m, 2 H, NCH_2), 4.90 (dd, 1H, J = 5.5 and 9.4 Hz, C^4-H); ^{13}C NMR δ 37.3 (C-5), 43.4 (NH−CH_2), 66.8 (C-4), 171.5 (CON), 175.9 (CO_2H), 203.8 (C-2) MS (negative ion) m/z 219 (M − 1).

Environmental Monitoring and Human Urine Sample Collection. Monitoring pumps were used to obtain static area sampling of inhaled air by drawing air at a fixed rate through a drying tube into a charcoal trap. The pumps were calibrated before and after the work shift and a tandem charcoal trap used to ensure that saturation of the initial trap did not occur. CS_2 was desorbed from the charcoal and quantified by gas chromatography using a flame photometric detector. Three areas, clerical, packaging, and spinning, were monitored and urine samples were obtained from individuals working in each of the three areas immediately before and after a 12 h work shift.

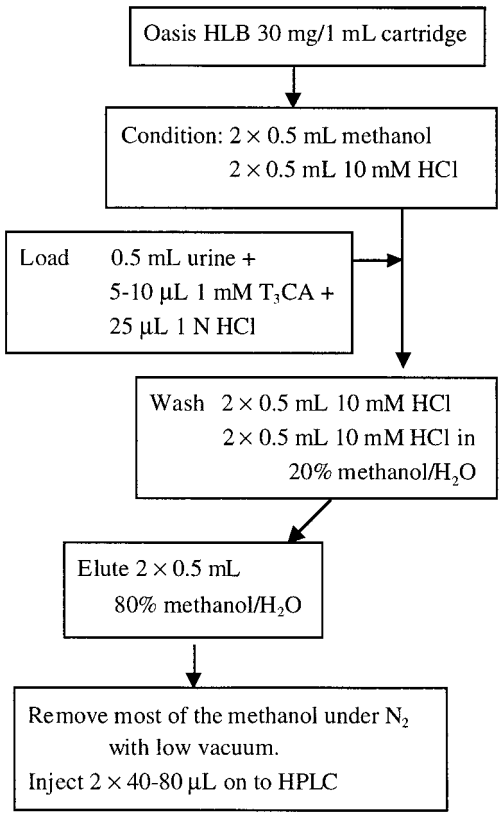


Figure 1. Sequence of steps used to analyze for TTCA and TTCG in urine samples.

TTCA Analysis. Urine samples were centrifuged for 5 min at 4000*g* and frozen at −20 °C prior to TTCA analysis. The solid-phase extraction steps prior to the chromatographic analysis (Figure 1) were performed on multiple samples using a LiChrolut sample preparation unit with drying attachment (EM Science). A Waters 2690 LC with 996 diode array detector and Millennium software was used for the chromatographic analysis. Two columns (a Lichrosphere 100RP-8 10 μm 4 × 250 mm column connected to a Whatman Partisil 5 ODS-3 μM 4.6 × 250 mm column) were used. The solvent systems were 3.0% acetonitrile (human samples) or 2.5% acetonitrile (rat samples) with 1% acetic acid (A) and 95:4:1 methanol−water−acetic acid (B). The elution profile consisted of running 100% A isocratically for 18 min, 100% B for 6 min, and then 100% A again for 10 min before the next injection.

Recovery. TTCA, T_3CA, and TTCG (10 nmol each) were added to 2.0 mL of a 40% methanol in water solution or to 0.5 mL of urine. The former was analyzed by HPLC directly, while the latter was processed with Oasis cartridge and the eluate was diluted to 2 mL with water before analysis. The peak areas of the two analytes were compared to arrive at the percentage of recovery which was average of three measurements.

Standard Curves. To generate a standard curve for the determination of TTCA and TTCG in human or rat urine samples, 0.1−1.0 nmol of TTCA and TTCG were added to 0.5 mL aliquots of control urine. These were then processed using the procedure in Figure 1. Peak area ratios of TTCA/T_3CA and

TTCG/T_3CA were plotted against their molar ratios (x) to obtain straight lines given by the equations $0.1301 + 1.026x$ ($R^2 = 0.998$) for TTCA and $0.011 + 1.257x$ ($R^2 = 0.999$) for TTCG. From the peak area ratios of the analyzed samples TTCA and TTCG in nmoles per 0.5 mL of urine were calculated.

Identification of TTCG in Urine of Rats Treated with CS_2. Urine collected from rats treated with CS_2 (see below) was pooled (~25 mL), washed with ethyl acetate (2 × 10 mL), acidified to pH 2, and saturated with salt. It was vigorously stirred with ethyl acetate (20 mL) for 10 min, centrifuged at 8000 rpm for 10 min, and the organic layer removed. The supernatants from four such extractions were combined, dried, and evaporated. The residue was coevaporated with absolute ethanol (3 × 5 mL), dissolved in 2 mL of the same solvent, and heated at 70 °C with BF_3-OEt_2 for 1 h. The residue after the removal of solvent was purified on a column of silica (80−0% hexanes−ethyl acetate). The fractions producing spots on TLC (ethyl acetate) with an R_f of 0.2−0.8 were collected, concentrated, and analyzed by HPLC (column: Xterra MS column 2.1 × 100 mm (3.5 μm particle size) with a 2.1 × 10 mm guard cartridge; flow rate 200 μL/min; isocratic elution 20% acetonitrile in 5 mM formic acid). A peak with an identical retention time and UV spectrum to the ethyl ester of TTCG was analyzed by using a Finnigan TSQ 7000 mass spectrometer (with electrospray ionization) and found to produce the same molecular ion of 249 $(M + H)^+$ and daughter ions of 203 ($M - OC_2H_5$) and 175 (203 − CO) observed for the ethyl ester of TTCG.

Animals. This study was performed in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals, and was approved by the Institutional animal care committee. For animal experiments, male Sprague Dawley rats, 7−9 weeks old and 230−300 g (Harlan, Indianapolis, IN) were used. Rats were housed in a room on a diurnal light cycle, and while in metabolic cages given finely ground rodent chow and water ad libitum.

Carbon Disulfide Exposures. Prior to CS_2 administration, five groups of male rats ($n = 3$/group) were placed into metabolic cages for 24 h. A 24 h urine collection was obtained as control urine and was spun for 5 min at 4000g and frozen at −20 °C prior to TTCA analysis. Next, each group was administered one dose of CS_2 in corn oil by gavage. Groups 1−5 received 0.04, 0.20, 1.0, 3.0, and 5.0 mmol of CS_2/kg, respectively. The rats were placed back in their metabolic cages and a 24 h urine sample was collected the next day.

Captan, MITC, and TTCG Exposures. Male rats ($n = 3$) were placed in metabolic cages and control urine collected for 24 h. A single dose of either MITC (0.1 mmol/kg by i.p. injection using 40 mM MITC in 1,2-propanediol), TTCG (0.4 mmol/kg by i.p. injection using 50 mM TTCG in water), or Captan (0.05 mmol/kg p.o. by gavage using 0.166 M Captan in 1:1 v/v 1,2-propanediol-PBS) was administered. The rats were returned to their metabolic cages and the urine collected for 24 h and processed as described above.

Determination of Creatinine. The determination of creatinine in the urine samples was performed using the Sigma Diagnostics creatinine kit 555-A (Sigma, St. Louis, MO). This kit uses a modified Jaffe reaction and was scaled down for use as a microplate assay on the SpectraMax 250 Plate Reader (Molecular Devices). A standard curve for linearity and a known 3 mg/dL creatinine standard were run with each sample plate. From the nmoles of TTCA or TTCG in 0.5 mL of urine samples and the creatinine values, TTCA and TTCG were expressed as nmoles per milligram of creatinine in urine.

In Vitro Studies. Cysteine or cys-gly (1 mM) was treated with either MITC or thiophosgene (2 mM) in phosphate buffer (pH 8.0) at 37 °C for 18 h and analyzed by HPLC using the conditions given above.

Results and Discussion

Assay Method. The first step in most methods of analysis (Table 1) is extraction of TTCA from urine. When using UV detection, it is particularly important to separate TTCA from several other UV absorbing components of urine. Solvent extraction of TTCA, a carboxylic acid, from acidified urine is commonly used. Recovery with ethyl acetate was reported to be less than 50% (*8*), although the yield can be increased by using multiple extractions with ether (*9*).

The sensitivity and ease of chromatography of the last two methods in Table 1 are usable but both involve time-consuming and relatively labor-intensive solvent extraction. Replacing solvent extraction with solid-phase extraction (SPE) to allow the handling of multiple samples simultaneously using a manifold would expedite sample preparation. As noted previously (*8*), a reversed-phase C-18 silica cartridge did not adsorb TTCA preferentially to effect purification. Oasis HLB sorbent (from Waters) exhibiting both hydrophilic and hydrophobic properties seemed to be a good candidate for isolating TTCA. Because TTCA is a polar carboxylic acid with a lipophilic −S−C(=S)− group, it seemed reasonable that through changing the pH the ability of TTCA to bind to the Oasis cartridge could be controlled. Preliminary experiments indicated that nonionized TTCA was readily adsorbed to Oasis under acidic conditions and that it could be eluted using an 80% methanol−water mixture, suitable for concentration.

To account for small variations during extraction and concentration, an internal standard was added. In previous studies tetrahydro-2-thioxo-2*H*-1,3-thiazine-4-carboxylic acid (T_3CA), a six membered analogue of TTCA, was used (*3*). Also for the present analysis T_3CA was chosen, rather than 2-methylhippuric acid (*8*) as internal standard, because of its chemical similarity to TTCA. Further, under the present chromatographic conditions, it was well separated from TTCA and the UV maxima of 272 and 282 nm for TTCA and TT_3CA, respectively, allow for single wavelength monitoring at 278 nm. On the basis of preliminary experiments we arrived at the sample preparation protocol outlined in Figure 1. The recovery of TTCA was 79.3 ± 1.0% while that of T_3CA was 78.5 ± 0.3%.

Results of Analysis. The urine samples from workers of a rayon factory were analyzed by the new method and Figure 2 compares the chromatograms of three samples of subjects exposed to differing ambient levels of CS_2. The chromatograms contain very few peaks other than those for TTCA and the internal standard T_3CA, and the peak areas could be measured accurately at quantities less than 100 pmol/0.5 mL of urine. Since the determination of TTCA concentration was based on standard curves using T_3CA as internal standard, the values were highly reproducible. Day to day variation was 2.66% and same day variation was 0.29%. The sensitivity of determination was 20 pmol of TTCA in 0.5 mL of urine (or 40 nmol/L) making it applicable to even low-level exposures (*10*). A comparison of the chromatograms points to the established relationship between TTCA levels and CS_2 exposure levels.

One outcome of the new method that was not anticipated during method development was the recognition of an additional peak (appearing ~1.6 min after TTCA) in the samples from workers exposed to CS_2 (Figure 2). This peak was not present in the urine samples obtained from control subjects. In addition, the area of the peak appeared to be dependent on the exposure level. To establish that the new peak originated from CS_2, rats

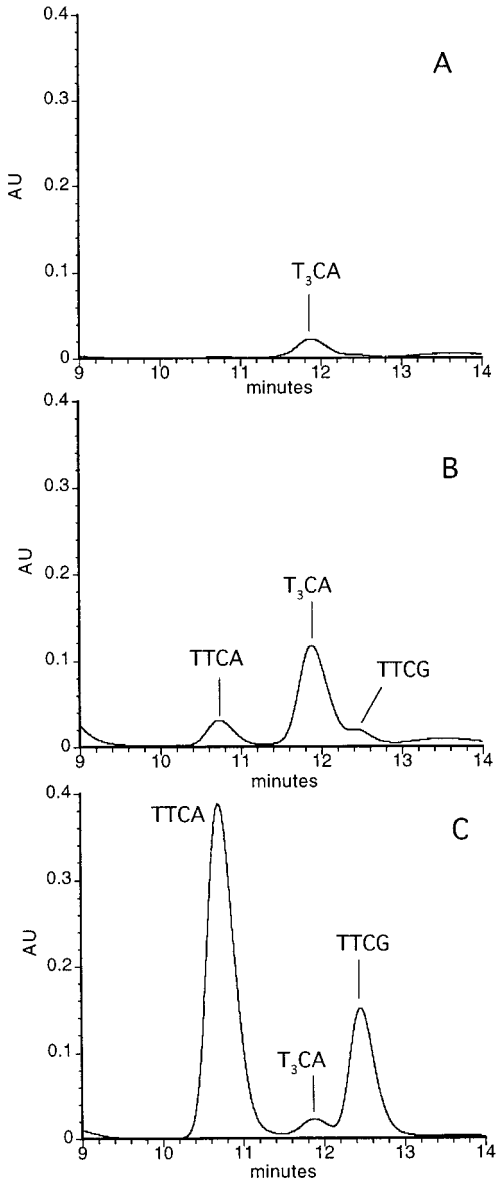


Figure 2. Chromatograms of human urine samples processed as described in Figure 1 and analyzed by HPLC. Separations were performed using a Lichrosphere 100RP-8 10 μm 4 × 250 mm column connected to a Whatman Partisil 5 ODS-3 μM 4.6 × 250 mm column, 3% acetonitile, with 1% acetic acid and monitoring at 278 nm. The range of exposure levels to carbon disulfide (A) less than 0.03−0.4 ppm (clerical area); (B) 0.2−2.0 ppm (packaging area); (C) 8.6−43 ppm (spinning area). The peak at 11.9 min representing added T_3CA is present in all the chromatograms. The level of TTCA (eluting at 10.7 min) is undetectable in panel A, 19.2 nmol/mg creatinine in B, and 48.6 nmol/mg creatinine in panel C. An additional peak with a retention time of 12.5 min could be seen as a shoulder in panel B and as a distinct peak in panel C.

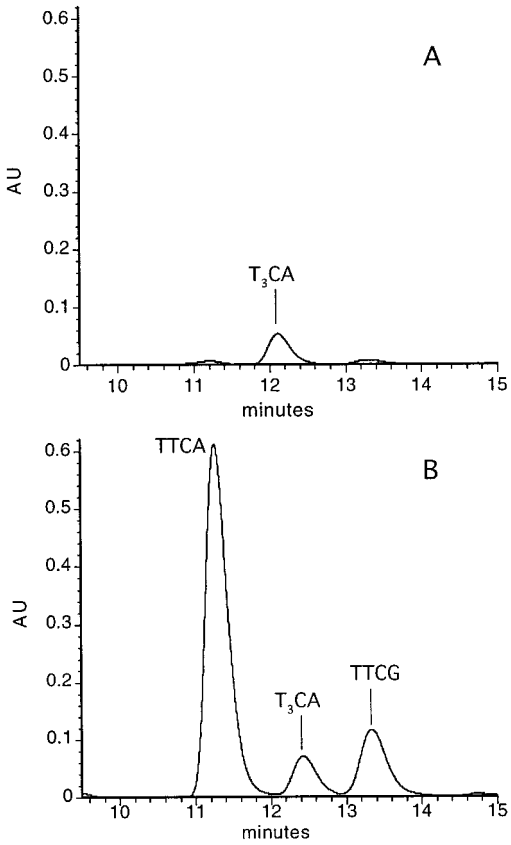
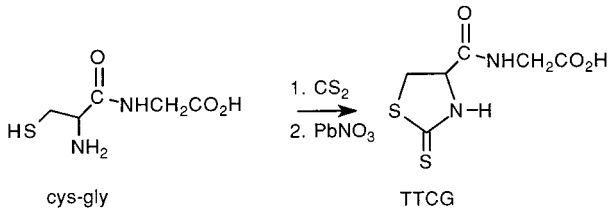


Figure 3. Chromatograms of urine samples obtained from a control (A) and CS_2 exposed (B) rat. The HPLC conditions were the same as specified in Figure 2 except a lower (2.5%) concentration of acetonitrile in the eluent was found to give a better separation of T_3CA and TTCG. A peak for TTCA (11.3 min) as well as a peak for TTCG (13.3 min) were present in urine of rats treated with 5 mmol/kg of CS_2 but not controls. Although two small peaks were observed in control urine samples eluting at 11.2 and 13.3 min, their UV maxima 280 and 260 nm, respectively, and elution times relative to the internal standard were different from TTCA (272 nm) and TTCG (274 nm). The peak at 12.1 (A) and 12.4 (B) min represents added T_3CA.

were exposed to CS_2, urine samples collected and analyzed, and the results compared to those obtained for control rats (Figure 3). Similar to the human samples, the extra peak appearing after the internal standard along with the peak for TTCA was observed only in the urine of exposed rats.

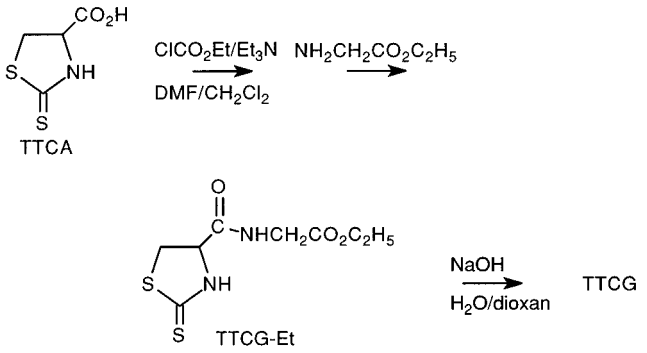
The UV spectrum obtained for the late eluting peak using the diode array detector had a maximum of 274 nm and it was similar to that of TTCA. The presence of a carboxyl group was suggested by the fact that the compound was extracted by ethyl acetate or retained by Oasis sorbent only under acidic conditions. Because GSH has been implicated in the formation of TTCA (*2*) and the new metabolite may have been a precursor of TTCA, we treated GSH or cysteinylglycine (cys-gly) with CS_2 followed by oxidation with lead nitrate, the conditions that produce TTCA from cysteine. When the products



were analyzed by HPLC, only cys-gly subjected to the above conditions gave a peak with the retention time similar to that of the new metabolite suggesting a structure related to both TTCA and cys-gly (2-thioxothiazolidin-4-ylcarbonylglycine, TTCG), for the unidentified metabolite.

To gain further insight into the structure of the metabolite, an independent synthesis of TTCG was undertaken. The formation of the dipeptide bond in

TTCG using the mixed anhydride method (*11*) starting with TTCA and glycine gave a mixture of products from which the isolation of pure TTCG was very difficult. However, when the ethyl ester of glycine was used, the resulting ester of TTCG could be easily purified and subsequently hydrolyzed under basic conditions to generate TTCG whose structure was verified by NMR and negative ion mass spectroscopy [*m/z* 219 (M − 1)]. Its



retention time was also identical to that of the sample obtained from cys-gly (data not shown).

Confirmation for the structure of the new urinary metabolite was achieved utilizing LC/MS/MS. Urine from rats treated with CS_2 was pooled, acidified, extracted with ethyl acetate, and concentrated. The crude mixture was purified by column chromatography and by HPLC for analysis by mass spectrometry, but obtaining a positive molecular ion for TTCG proved difficult. To circumvent this problem the extract was treated with BF_3-OEt_2 in ethanol to obtain the ester of the carboxylic acid which was purified and found to produce the identical parent ion and daughter ions to those obtained for synthetic TTCG ethyl ester (Figure 4).

The potential use of TTCG as a biomarker was explored by treating rats with a single dose of CS_2 (0.04−5.0 mmol/kg) and collecting urine during the following 24 h period. The amounts of TTCA and TTCG present in the urine were determined using standard curves established with T_3CA as the internal standard. When expressed per mg of creatinine, both TTCA and TTCG showed linear increases with the dose of CS_2 (Figure 5), although the concentration of the latter was ∼30% of that of the former. The recovery of TTCG when using the sample preparation of Figure 1 was 80.8 ± 0.9% and the sensitivity of determination was 20 pmol. The day to day variation was 3.7% while the determinations in a single day were within 2.7%.

Mechanism of TTCA Formation by CS_2. Identification of TTCG in urine raises two questions: (1) is TTCG a precursor of TTCA and (2) does TTCG provide additional exposure information to that derived from TTCA alone? The proposed mechanism for the generation of TTCA from GSH and CS_2 is presented in the top of Scheme 1. The first step is the formation of trithiocarbonate from GSH and this reaction has been demonstrated in vitro (*12*). Next, removal of γ-glutamate followed by cyclization leads to TTCG and the final step is hydrolysis of the peptide bond to yield TTCA. To determine if TTCG could be metabolized to TTCA in vivo, rats were given a single dose of TTCG and the urine collected during the next 24 h was analyzed. TTCA was detected in the urine and from the relative peak areas

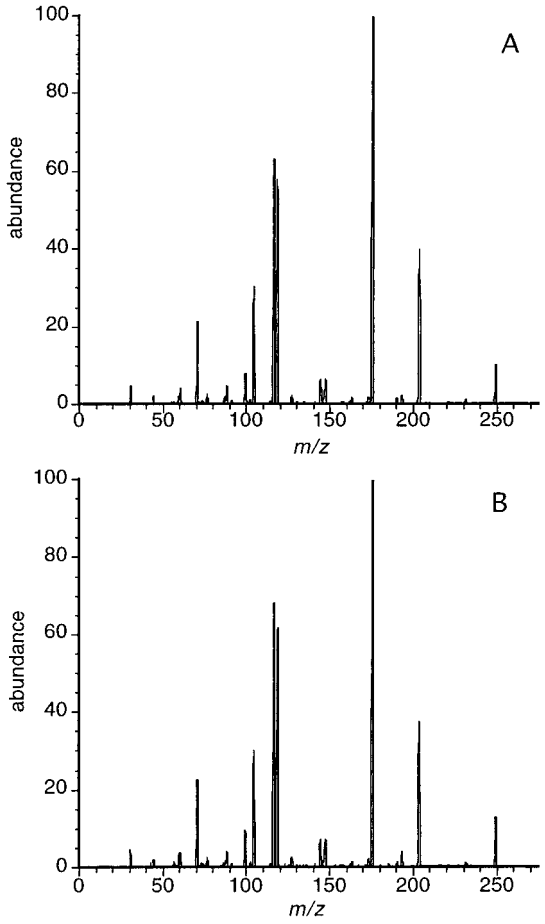


Figure 4. Tandem mass spectra of the [M + 1] parent (*m/z* 249) of TTCG ethyl ester synthetic standard (A) and urine extract (B). Many fragment ions including those at 203 ($M-OC_2H_5$) and 175 (203-CO) were observed in both samples. The spectrum was obtained on a Finnigan TSQ 7000 triple-quadrupole mass spectrometer.

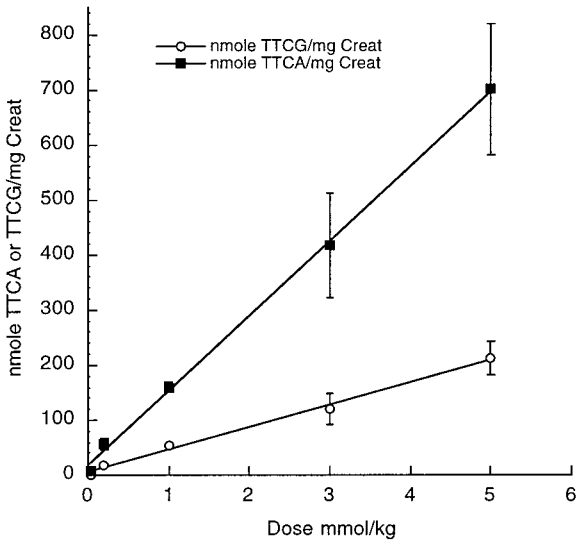
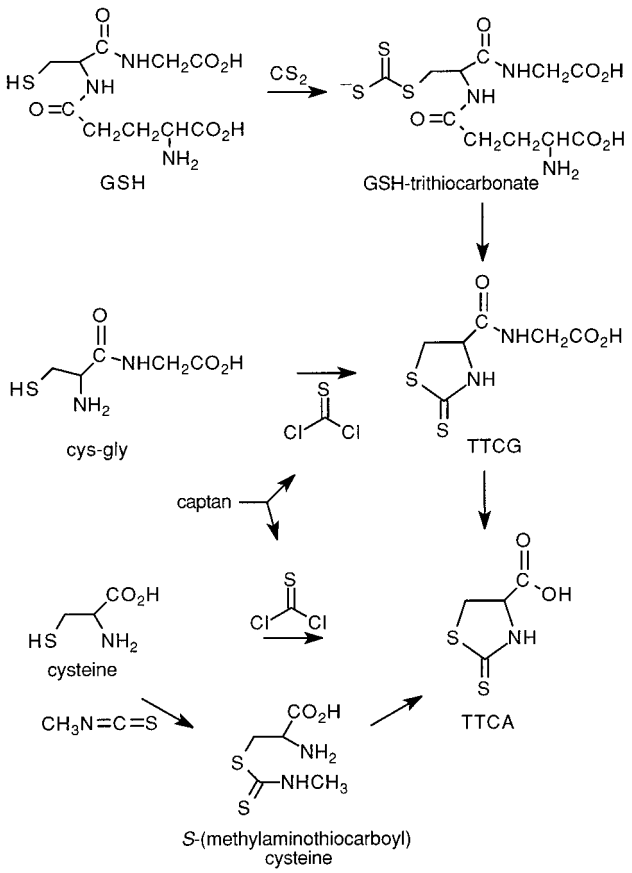


Figure 5. Dose response of TTCA and TTCG in the urine of rats administerd CS_2 per os. Urinary excretion of both TTCA (slope = 17.2, $R^2 = 0.998$)) and TTCA (slope = 5.2, $R^2 = 0.994$) demonstrated a linear response over the exposure range of 0.04−5.0 mmol/kg of CS_2.

of TTCA and TTCG the extent of conversion was calculated to be 88%. Although these results suggest that TTCG is likely to be an immediate precursor to TTCA,

Scheme 1



the pathway leading to TTCG itself still remains to be established. Both addition of CS_2 to GSH followed by cleavage of γ-glutamate or addition of CS_2 to cys-gly could result in production of TTCG.

In addition to direct exposure to CS_2 urinary excretion of TTCA has been reported following intake of dithiocarbamates that release CS_2 in vivo, exposure to the non-CS_2-generating compound captan, and consumption of cruciferous vegetables (*13*). The possible generation of TTCG following exposure to captan was explored. If generation of TTCA from captan occurs exclusively through addition of a captan derived thiophosgene metabolite to cysteine, no TTCG excretion would be expected. Thus, the observation of TTCG following administration of captan in the present study (Figure 6B) suggests that captan derived thiophosgene can react with cys-gly to form TTCG or with GSH to form a relatively stable product that can undergo cyclization with the removal of glutamic acid. When cysteine and cys-gly were treated with thiophosgene at pH 8.0, the formation of TTCA and TTCG, respectively, were observed (data not shown).

To explore the contribution of isothiocynates derived from dietary glucosinolate and from industrial sources to TTCA formation, rats were administered methyl isothiocyanate and the urinary metabolites analyzed. In contrast to captan, methyl isothiocyanate generated TTCA and an unidentified metabolite of MITC but not TTCG (Figure 6C). The absence of TTCG implies that cyclization proceeds following derivatization of free cysteine but not after derivatization of cys-gly. Consistent with this interpretation in vitro experiments indicated that while cysteine readily cyclized to TTCA on treatment

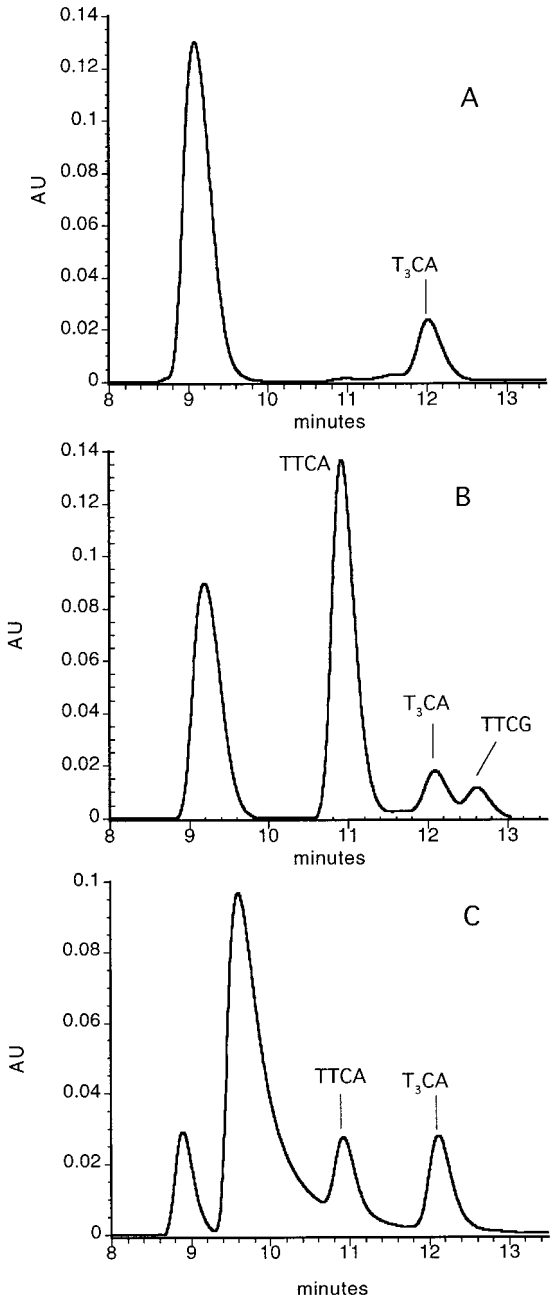


Figure 6. Chromatograms obtained after processing urine samples collected from control (A), captan exposed (0.05 mmol/Kg per os) (B), or methylisothiocyanate (0.1 mmol/kg (C) rats. T_3CA internal standard eluting at 12 min and an unidentified peak at 9 min is present in all three samples. The HPLC conditions were the same as in Figure 3. Whereas TTCA can be identified at 11 min following exposure to either captan (56.2 ± 5.0 nmol/mg of creatinine) or methisothiocyanate (27 ± 10 nmol/mg of creatinine) only captan exposure resulted in measurable amounts of TTCG (4.9 ± 2.3 nmol/mg of ceatinine) eluting at 12.4 m. A large peak eluting at 10 min in the urine of methylisothiocyanate exposed rats was observed but not characterized. Low levels of absorbance were detected in samples prepared from controls at 11.0 min but exhibited a different UV maxima (280 nm) than TTCA (272 nm).

with MITC, the formation of TTCG was very slow with cys-gly under similar conditions. These results suggest that urinary measurement of both TTCA and TTCG will be a better indication of exposure to CS_2 than the determination of TTCA alone. The HPLC method of analysis presented in this paper can accomplish this in a single chromatogram.

Conclusion

An improved method for the determination of TTCA in urine suitable for monitoring exposure to CS_2 is presented. The method is amenable to automation, uses tetrahydro-2-thioxo-2*H*-1,3-thiazine-4-carboxylic acid (T_3-CA) as an internal standard, Oasis HLB cartridge for sample preparation, and HPLC with UV detection for measurement. The sample preparation provided a high recovery and resulted in chromatograms with very few extra peaks thereby improving the accuracy of measurement of TTCA. Use of an internal standard increased the reproducibility while the diode array detector aided in verifying the identity of metabolites. A novel urinary metabolite was identified, 2-thioxothiazolidin-4-ylcarbonylglycine (TTCG), for which the output was found to be dependent on the exposure level of CS_2. TTCG appears to be a precursor to TTCA and presents a potential biomarker to be used in conjunction with TTCA when the presence of TTCA in urine may result from exposure to sources other than CS_2.

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