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Synthesis of 2-thiothiazolidine-4-carboxylic acid and its chromatography in rat and human urine

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Abstract

2-Thiothiazolidine-4-carboxylic acid (TTCA), the urinary biological monitoring marker for carbon disulfide in humans was synthesized by reacting carbon disulfide with L-cystine. Validation of TTCA purity required elemental as well as mass spectral and chromatographic analyses. Two reversed-phase high-performance liquid chromatographic columns in series were necessary to resolve picomole amounts of TTCA from rat and human urine background peaks on detection at 272 nm even after prior diethyl ether extraction from acidified urines in the presence of saturated sodium chloride. The isolation procedure for TTCA from urine had a recovery of 94.0±8.1% in the linear 1.1–330 μM concentration range (0.0165–4.95 nmol injected mass) with a coefficient of variation of 8.6%. The detection limit was about 100 nM (1.5 pmol injected mass).

Keywords: 2-Thiothiazolidine-4-carboxylic acid; Carbon disulfide

1. Introduction

2-Thiothiazolidine-4-carboxylic acid (TTCA; 2-thioxothiazolidine-4-carboxylic acid; thiazolidine-2-thione-4-carboxylic acid; raphanusamic acid) is a urinary metabolite produced in humans and rats exposed to carbon disulfide used mostly in the viscose rayon industry [1–6]. TTCA is also a urinary metabolite of disulfiram utilized in rubber manufacturing or Antabuse administered orally to induce nausea in drinking alcoholics [7]. Rats exposed to the pesticide Captan excrete TTCA [8]. TTCA reflects absorbed doses of chemicals metabolizing to TTCA [9]. TTCA is the end-of-shift urinary marker (5 mg/g creatinine) for the Biological Exposure Index (BEI) for carbon disulfide (threshold limit value-time weighted average (TLV-TWA) of 10 ppm) of the American Conference of Governmental Industrial Hygienists (ACGIH) [10]. Since the Occupational Safety and Health Administration (OSHA) permissible exposure level (PEL) was lowered to 4 ppm in 1989 (but overturned in 1992), the ACGIH is investigating whether to lower its TLV-TWA [10]. This may cause analytical problems since urinary background assumes greater importance.

The present study reports that elemental analysis is essential to validate TTCA purity before use in analyses. Two different reversed-phase HPLC columns in series (C_8 then C_{18}) are necessary to resolve

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TTCA from urinary background of rats and humans at 272 nm after low-ppm TWA air exposures to carbon disulfide even if diethyl ether extraction is used for urine clean-up.

2. Experimental

2.1. Chemicals

The following chemicals (ACS reagent grade or better) were obtained from Fisher Scientific (Fairlawn, NJ, USA): carbon disulfide (98.96%), L-cystine, ethyl acetate (Optima), anhydrous diethyl ether (peroxide-free by the potassium iodide test [11]), hexane (Optima), concentrated hydrochloric acid, anhydrous magnesium sulfate, potassium carbonate, potassium hydroxide and sodium chloride. Nitrogen and air for evaporation purposes were ultra high purity from Linde Specialty Gases (Danbury, CT, USA). Distilled water was ASTM Type II [12].

2.2. Equipment

The major equipment used was a Kratos Analytical (Ramsey, NJ, USA) MS-80 high-resolution mass spectrometer equipped with DS-55 data system on a NOVA/4 computer, interfaced with a direct-probe mass spectrometer inlet at 150°C and 50 eV electron impact ion voltage or interfaced with a Carlo Erba (Valencia, CA, USA) gas chromatograph equipped with non-polar capillary columns; a Jarrell Ash (Waltham, MA, USA) 9000 simultaneous vacuum inductively coupled plasma (ICP) atomic emission spectrometer with (N+1) scanner for Cl and C used to detect elements as described elsewhere [13]; a Perkin-Elmer (Norwalk, CT, USA) 552 ultraviolet (UV) spectrophotometer with 1-cm Suprasil cells; a Waters (Milford, MA, USA) Model 6000A HPLC solvent delivery system with a Model U6K universal liquid chromatograph injector (volume 25 μl), a Whatman (Clifton, NJ, USA) prefilter, a Merck (Darmstadt, Germany) Hibar LiChrospher 100 C_8 column (250×4.6 mm I.D., 10 μm) followed by a Partisil 5 (Whatman) ODS-3 C_{18} column (250×4.6 mm I.D., 5 μm) before a Kratos Analytical Spectroflow 783 variable-wavelength UV detector at 272 nm; a linear flat-bed chart recorder and a Gulton (Greenwich, RI, USA) Rustrak Ranger data logger.

Other equipment utilized in sample preparation for HPLC injection included a Power Designs (Westbury, NY, USA) Model 4010 precision power source (0-40 VDC, 0-1 A), a Fisher Scientific Industries (Bohemia, NY, USA) Vortex-Genie Model K-550-G vortex mixer, an Adams (West Warwick, RI, USA) analytical centrifuge (Cat. No. 0151), an Organomation Associates (Berlin, MA, USA) Meyer N-EVAP Model No. 112 analytical evaporator and constant-temperature bath, a Lab-Line Instruments (Melrose Park, IL, USA) magnestir, a Beckman (Fullerton, CA, USA) Model DU-50 spectrophotometer, a Hamilton 802 microliter syringe (0–25 μl) and an Eppendorf digital pipet (100–1000 μl) with disposable tops, analytical balance, graduated cylinders, reagent bottles, polyethylene disposable test tubes, glass PTFE-lined screw-capped test tubes and a mechanical shaker.

2.3. TTCA synthesis

When this research was started, TTCA was not available commercially. It is now available from Aldrich (Milwaukee, WI, USA) and Nihon Kankoshikiso (Okayama, Japan).

The synthetic method was one modified from Kopecky and Smejkal [14]. L-Cystine (48 g) was added to potassium carbonate (56 g) aqueous solution (300 ml) and stirred until homogeneous. Carbon disulfide (68 g) was added and the solution stirred mechanically for 13 h at room temperature. The pH was adjusted to 1.0 (with HCl) and the solution passed through a 0.45-μm PTFE filter to remove colloidal sulfur. The filtrate was extracted with hexane (3×100 ml) to remove non-polar impurities. The water was evaporated under a stream of pure air in a fume hood for one week. The first precipitate was discarded. After an additional two weeks of evaporation, the second precipitate was recrystallized from water and the white crystalline residue, after being ground in a porcelain mortar and pestle, was dried to constant weight in a vacuum desiccator containing Drierite.

The product was validated as pure by direct-probe MS (high/low voltage), by isotopic composition of the MS molecular ion, by UV spectrophotometry of

aqueous solutions, by HPLC–UV, by direct-probe MS analysis of the collected suspected TTCA peak on HPLC and by ICP elemental analysis specifically for C, Cl and S in aqueous solutions of known concentration.

2.4. Optimized TTCA assay

The method was one modified from van Doorn et al. [1]. TTCA spiking studies, at a concentration equivalent to the BEI in human urine of a human not exposed to CS_2, were used to optimize the van Doorn et al. [1] clean-up method before HPLC injection. The volumes of HCl and diethyl ether were varied so that all the diethyl ether extractions could be placed in the one 16×100 mm tube. This resulted in solid NaCl being used to facilitate salting out rather than a NaCl solution. The diethyl ether volume was reduced from 8 to 5 ml with three extractions rather than just one.

For the optimized method, creatinine concentration and urine volume were determined for each fresh urine collected in 500-ml polyethylene containers for humans and 100-ml polymethylpentene containers for rats before storing individual 3-ml aliquots at −20°C for TTCA analysis. On thawing to room temperature (25°C), a 2-ml aliquot was acidified with 0.150 ml 5 *M* HCl in a 13×100 mm glass, PTFE-lined, screw-capped test tube and solid NaCl was added until the solution was just saturated. A 5-ml volume of peroxide-free diethyl ether (no color in ether layer after shaking 10 ml of ether with 1 ml of 10% KI [11]) was added, the tube capped, the solution shaken vigorously and then centrifuged at 500 *g* for 2 min. The upper ether layer was transferred by Pasteur pipet into another 13×100 mm glass test tube where amalgamation of extracts occurred on two more extractions. The diethyl ether was evaporated in a stream of nitrogen at 37°C. The residue was then reconstituted in 0.500 ml of distilled water for HPLC.

A 15-μl aliquot was injected onto the HPLC column at room temperature (25°C). The flow-rate was 1.0 ml/min. The mobile phase consisted of 98% (v/v) distilled water, 1% acetonitrile and 1% acetic acid. The flush solvent was 95% methanol, 4% distilled water, and 1% acetic acid. At the time of injection, the mobile phase was utilized followed by the flush solvent 4 min after injection and then the mobile phase again 12 min after injection. The next sample could be injected 36 min after injection of the previous sample. One urine sample of known TTCA concentration was analyzed every ten samples as well as a known concentration of TTCA in water. Quantitation was by interpolation on an external standards curve of at least five concentrations of TTCA in water over the linear concentration range, here 1.1–330 μ*M*.

TTCA mass recoveries from spiked urines were calculated relative to the mass recovered from spiking the same volume and TTCA concentration into the same volume of water as urine with the spiked water analyzed directly without clean-up. All experiments were done at least in triplicate. Rat urines were from a study to compare the urinary elimination half-times of TTCA, total thioethers and compounds detected in the iodine-azide test [15]. The human urines came from nineteen exposed rubber production workers in Tennessee and six exposed viscose rayon workers in Virginia in a study to correlate urinary TTCA with personal TWA CS_2 concentrations in air [16].

3. Results and discussion

3.1. TTCA synthesis

Direct-probe MS of the final product showed *m/z* 163 (M^+), *m/z* 118 (M^+−COOH), *m/z* 99 (M^+−2S), *m/z* 86 (118−S), *m/z* 72 (99-C=NH) and *m/z* 59 (86−C=NH) in agreement with DeBaun et al. [8]. Isotope abundances above the molecular ion showed the molecular formula was $C_4H_5NO_2S_2$. Direct-probe MS at 11 eV indicated only one molecular ion and no S_8 (M^+ 256 with mass spectrum consisting of consecutive *m/z* 32 loss from each parent ion). The MS results suggested >99% purity. The maximum UV absorption wavelength was 272 nm with molar absorptivity of 14 815±314 $M^{-1}\,cm^{-1}$ in water. The latter did not differ at $p<0.05$ from the 14 200±500 $M^{-1}\,cm^{-1}$ value of Van Doorn et al. [1]. Only one HPLC peak was observed. Direct-probe MS of the collected peak after HPLC was the same as for unchromatographed TTCA. ICP analysis for C, Cl and S in aqueous

solution [13] showed that the molar C/S ratio was 2.0 with undetectable Cl. Direct-probe MS detects only free acid for a free acid–hydrochloride salt mixture. ICP detects both since differential volatilization is not involved. The ICP purity was >99%. There was no sulfur odor. The yield was 0.8%, sufficient for HPLC standards and to measure validating spectra.

The TTCA characteristics fit those reported by DeBaun et al. [8] rather than those found by Kopecky and Smejkal [14]. HCl recrystallization as recommended by the latter resulted in a mixture of free TTCA and its corresponding hydrochloride salt of m.p. 181–182.5°C (a higher m.p. is usually a sign of increased purity but not here) rather than pure acid (DeBaun et al. [8] reported 179–180°C), a UV maximum red shift at 279 nm relative to DeBaun et al. [8] of 270 nm and the incorrect C/S ratios on ICP elemental analysis as well as the detection of Cl.

What appears pure by one technique is not necessarily pure by another. The validation methods of Kopecky and Smejkal [14] were not adequate since UV and ^{1}H NMR spectroscopies do not discriminate free acid and hydrochloride salt forms. Direct-probe MS may allow only the free acid to be detected or the acid part of the TTCA hydrochloride salt. HPLC–UV detection selects responsive compounds retained and eluted from the HPLC column. Elemental analysis that does not involve differential volatilization is essential. We recommend validation of the purity of commercially available TTCA be performed as above before use as an essential part of TTCA quality control and assurance.

3.2. TTCA HPLC analysis

TTCA spiked into urine and TTCA in water gave non-significantly different slopes (at $p<0.05$) in the same linear range (C.V. <10%) of 1.1 to 330 μM relative to concentration (0.0165–4.95 nmol relative to injected mass). The TTCA detection limits (S/N ratio of 2) were near a TTCA concentration of 100 nM or an injected mass of 1.5 pmol. The TTCA retention time was about 13.3 min. The column void volume elution time was about 2.5 min. The mean TTCA recovery was 94±8.1% in the linear (C.V.< 10%) concentration range 1.1–330 μM ($n=45$ for five concentrations in three sets of triplicates) with an average C.V. of 8.6% with the largest C.V.s being near the lower linear limit. Intra-run C.V. ($n=3$) was 1.7–3.1% at the concentration corresponding to the BEI. During the intra-runs, spiked references were also analyzed; the C.V. ($n=9$) was 4.0% at a concentration corresponding to about 50% of the BEI.

The HPLC method was initially based on one by Campbell et al. [2]. The C_{18} column alone produced a TTCA retention time of about 4.9 min for an analysis solvent flow-rate of 1.5 ml/min. Unfortunately, urines of CS_2 unexposed people had a peak at the same retention time that was equivalent to about 500 pmol TTCA. Direct probe MS of the HPLC peak revealed it was not TTCA. Decreasing the flow-rate to 1.0 ml/min improved separation of spiked TTCA and the native urine peak but TTCA was now a small shoulder. Varying the composition of constituents in the mobile phase and changing solvents in place of methanol (acetonitrile, ethanol, isopropanol, nitromethane, butyl chloride, p-dioxane, 2-ethoxyethanol and 2-methoxyethanol) also did not resolve the peaks. When the C_8 column was placed before the C_{18} column, incomplete resolution from the major interferences occurred. Baseline separation was achieved when the flow-rate was lowered further to 1.0 ml/min. TTCA was the major peak but it now had a small shoulder of approximately 17–19 peak height units at a full scale absorbance setting of 0.01. Since the unoptimized chromatogram had TTCA superimposed on a peak equivalent to 53 peak height units at full scale absorbance of 0.01, a third of the interference remained. The interfering peak was nullified by subtracting control peak areas. The interfering peak was about 30% smaller for unexposed humans than for unexposed rats. Chromatograms for rat urines after exposure to 150 ppm CS_2 by inhalation [15] and for unexposed rats are shown in Fig. 1. Similar chromatograms for workers [16] are shown in Fig. 2.

Most other investigators [1–5] have found that one C_{18} HPLC column was adequate for HPLC estimations but this did not suffice for the present human study probably because of the much lower air exposures to CS_2. Van Doorn et al. [1,5] showed that diethyl ether extraction was essential for TTCA resolution purposes from endogenous compounds in human urines and to enhance sensitivity. The effect of diethyl ether peroxides on TTCA analysis has

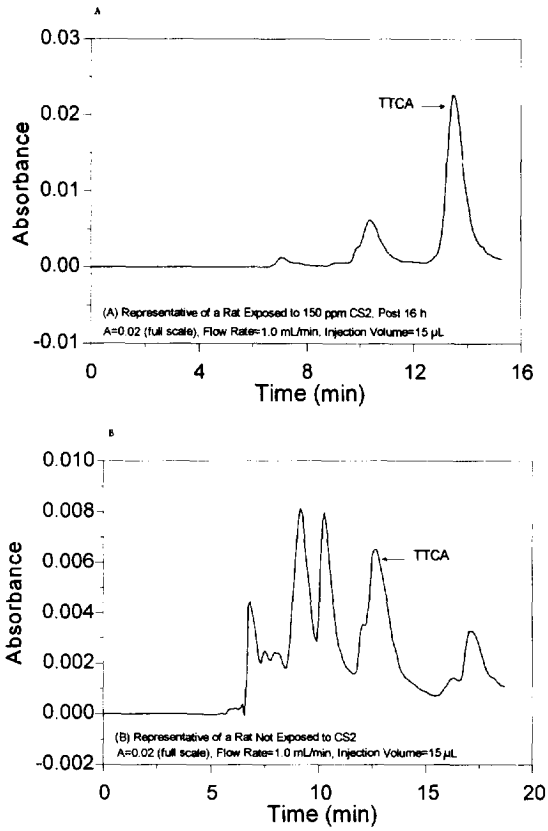

Fig. 1. (A) HPLC at 272 nm wavelength for a post 16 h urine of a Sprague–Dawley male rat exposed to 150 ppm of CS_2 by inhalation over the previous six months at 6 h per day and five days per week with exposures in the final week being for three consecutive days. (B) HPLC for an unexposed rat. The mobile phase sequence at 1.0 ml/min at 25°C was: (1) 0–3.99 min, 98% water, 1% acetonitrile, 1% acetic acid; (2) 4.0–11.99 min, 95% methanol, 4% water, 1% acetic acid; (3) 12.0 min onwards, Step 1. The figure shows only the chromatograms from the optimized method. The HPLC columns were 250×4 mm I.D. with the Hibar LiChrospher 100 C_8 column (10 μm) preceding the Partisil ODS-3 C_{18} (5 μm).

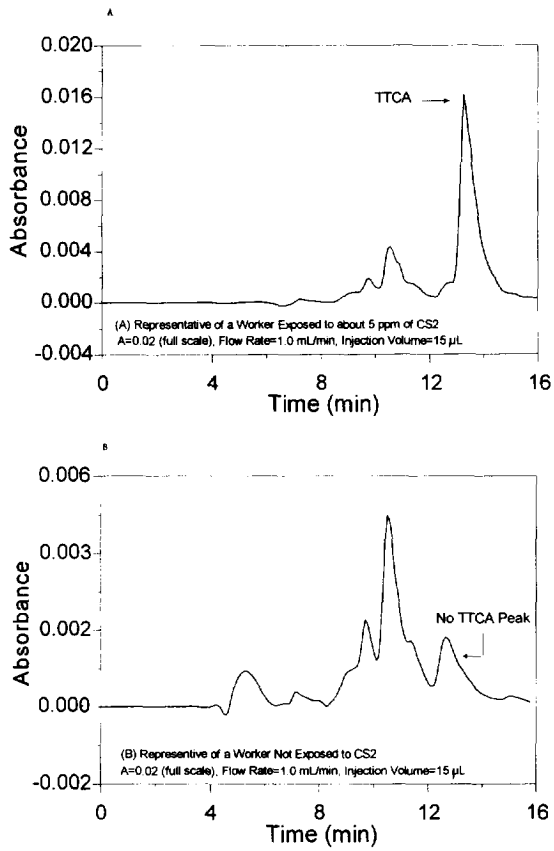

Fig. 2. (A) HPLC at 272 nm wavelength for an end-of-shift spot urine representative of a worker exposed to 5 ppm TWA CS_2 over the previous workshift compared with (B), the HPLC for an unexposed worker. The mobile phase sequence was as in Fig. 1.

been observed by others [17]. The published extraction efficiency for TTCA in the van Doorn et al. method [1,5] was 52±3% for urine TTCA concentrations of 2 to 300 μM. The detection limit was 0.5 μM. Storage of TTCA in urine for 40 h at −20°C or room temperature did not decrease urinary TTCA but storage at 37°C did, by 26% [5]. Campbell et al. [2] showed that his modified van Doorn et al. method using one diethyl ether extraction permitted TTCA recoveries of about 85% (no C.V. stated); there was also no TTCA degradation on storage of urine samples for three days. The HPLC linear concentration range was up to 120 μM TTCA. In contrast, recoveries in the modified method of the present study are >90% because of the multiple extractions and the HPLC method is more sensitive due to better resolution.

4. Conclusions

The purity of TTCA must be validated by elemental analysis as well as by chromatographic and spectroscopic methods. The isolation of TTCA from urine requires multiple diethyl ether extractions to obtain recoveries >90% and nM sensitivities. The

resolution of TTCA from other native urine compounds at low concentrations may necessitate two HPLC columns (C_8 and C_{18}) in series rather than just one C_{18} column.

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References

[1] R. van Doorn, L.P.C. Delbressine, C.P.M.J.M. Leijdekkers, P.G. Vertin and P.T. Henderson, Arch. Toxicol., 47 (1981) 51.
[2] L. Campbell, A.H. Jones and H.K. Wilson, Am. J. Ind. Med., 8 (1985) 143.
[3] J. Rosier, M. Vanhoorne, R. Grosjean, E. Van de Walle, G. Billemont and C. Van Peteghem, Int. Arch. Occup. Environ. Health, 51 (1982) 159.
[4] W.J.A. Meuling, P.C. Bragt and C.L.J. Braun, Am. J. Ind. Med., 17 (1990) 247.
[5] H. Kivisto, E. Elovaara and V. Riihimaki, Arch. Toxicol., 69 (1995) 195.
[6] V. Riihimaki, H. Kivisto, K. Peltonen, E. Helpio and A. Aitio, Am. J. Ind. Med., 22 (1992) 85.
[7] R. van Doorn, C.P.M.J.M. Leijdekkers, S.M. Nossent and P.T. Henderson, Toxicol. Lett., 12 (1982) 59.
[8] J.R. DeBaun, J.B. Miaullis, J. Knarr, A. Milhailovski and J.J. Menn, Xenobiotica, 4 (1974) 101.
[9] S.S. Que Hee, Biological Monitoring: An Introduction, Van Nostrand Reinhold, New York, NY, 1993.
[10] American Conference Governmental Industrial Hygienists, Documentation of the Biological Exposure Indices, ACGIH, Cincinnati, OH, 1991, BEI-57.
[11] N.V. Steere, J. Chem. Educ., 46 (1969) A427.
[12] American Society for Testing Materials, 1989 Annual Book of ASTM Standards: Water and Environmental Technology, Vol. 11.01, ASTM, Philadelphia, PA, 1989, pp. 45-47.
[13] S.S. Que Hee and J.R. Boyle, Anal. Chem., 60 (1988) 1033.
[14] J. Kopecky and J. Smejkal, Bull. Soc. Chim. Belg., 93 (1984) 231.
[15] C. Cox, S.S. Que Hee and D.M. Lynch, Toxicol. Ind. Health, (1996) submitted.
[16] C. Cox and S.S. Que Hee, Am. J. Ind. Med., in preparation.
[17] M. Ogata and T. Taguchi, Ind. Health, 27 (1989) 31.