URINARY 2-THIOTHIAZOLIDINE-4-CARBOXYLIC ACID (TTCA) AS THE MAJOR URINARY MARKER OF CARBON DISULFIDE VAPOR EXPOSURE IN RATS

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Male Sprague-Dawley rats (200–250 g; 60 per exposure group) were exposed to carbon disulfide (CS_2) air concentrations of 0, 50, 150, and 500 ppm(v/v) for 6 hr/day, 5 days/week over six months. Following the exposures, nine rats from each exposure group had four sets of cumulated urines collected (between 0–8, 8–16, 16–24, and 24–48 hr). The urinary parameters measured were: 2-thiothiazolidine-4-carboxylic acid (TTCA), total thioethers (TE), and the compounds responsive to the iodine-azide (IA) test. Urinary TTCA elimination obeyed pseudo-first-order, one-compartment model kinetics of half-time ($t_{0.5}$) 5.2 +/– 0.3 hr up to 16 hr of collection. The elimination of TE within 16 hr had a *$t_{0.5}$ of 8.5 +/– 0.6 hr. TTCA, IA, and TE were correlated highly in the first 16 hr. After 16 hr, the $t_{0.5}$ for TE lengthened to 13.1 hr. At CS_2 concentrations of 50, 150, and 500 ppm, the respective $t_{0.5}$ for IA-responsive compounds were 12.6, 6.1, and 4.4 hr. TTCA had the highest correlation coefficient and* p-*value*

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2. Abbreviations: ACGIH, American Conference of Governmental Industrial Hygienists; BEI, ACGIH biological exposure index; CS_2, carbon disulfide; CV, coefficient of variation; IA, iodine azide; k_{el}, rate constant of elimination; r, correlation coefficient; $t_{0.5}$, half-time; TE, total thioethers; TLV-TWA, threshold limit value–time-weighted average; TTCA, 2-thiothiazolidine-4-carboxylic acid.

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relative to CS_2 exposure concentration, and also was the most sensitive, precise, and selective urinary marker.

INTRODUCTION

The Biological Exposure Indices (BEI) Committee of the American Conference of Governmental Industrial Hygienists (ACGIH) has established the BEI for carbon disulfide (CS_2) of 5 mg of 2-thiothiazolidine-4-carboxylic acid (TTCA) per g of creatinine for a urine sample collected at the end of a work shift (ACGIH, 1986). This corresponds to a threshold limit value (TLV®) of 10 ppm as an eight-hour time-weighted average (TWA) with skin exposure not factored in (ACGIH, 1995). The BEI was set on personal exposure data and corresponding urinary TTCA concentrations for viscose rayon workers as reported by van Doorn et al. (1981a) and Campbell et al. (1985).

TTCA is also found in human urine after oral exposure to Antabuse (disulfiram) (van Doorn et al., 1982). Maximum concentrations of 0.48 mg TTCA/g creatinine were detected 12 hours after dosing. DeBaun et al. (1974) showed that TTCA accounted for about 19% of the total metabolites excreted by rats exposed to the pesticide Captan. *Brassica* vegetables such as cabbage are a food source of urinary TTCA (Simon et al., 1994), with reported geometric means of pre- and postingestion samples of 0.09 and 0.77 mg TTCA/g creatinine, respectively.

Many field and laboratory studies have determined the relationship of CS_2 exposure to the production of TTCA in human urine (van Doorn et al., 1981a; Rosier et al., 1982, 1987a,b; Campbell et al., 1985; Ogata and Taguchi, 1989; Meuling et al., 1990; Van Poucke et al., 1990; Riihimaki et al., 1992; Kitamura et al., 1993; Drexler et al., 1994). Cox et al. (1992) derived a proposed BEI of 2.7 mg TTCA/g creatinine for the TLV-TWA, and 1.1 mg TTCA/g creatinine for the 1989 OSHA permissible exposure limit (PEL) of 4 ppm CS_2. Other urinary biological monitoring tests, including the iodine-azide (IA) test and the test for total thioethers (TE), have been used to assess human and rat exposure to air concentrations of carbon disulfide (Vasak et al., 1963; Djuric et al., 1965; Graovac-Leposavic et al., 1967; Jakubowski and Piotrowski, 1967; Herber, 1976; McKenna and DiStefano, 1977; Biollat, 1979; van Doorn et al., 1981b; Rosier et al., 1984; Campbell et al., 1985; van Poucke et al., 1990). No studies have addressed the major aim of the present study, i.e., to observe rat populations exposed to CS_2 in which the interrelationships of urinary TTCA, TE, and IA have been determined.

MATERIALS AND METHODS

Animals

Researchers from the National Institute for Occupational Safety and Health (NIOSH) conducted a study (Lynch, 1985) using rats as a model to investigate a possible mechanism for the etiopathogenesis of atherosclerotic lesions in workers exposed to CS_2. A hypothyroid

condition was supposed to cause increased serum lipids, a precursor marker. These same animals were used for the present study.

Adult male Sprague-Dawley [Crl: CD® (SD) BR] rats weighing 200–250 g were purchased from Charles River Breeding Laboratories, Incorporated (Wilmington, Massachusetts). The animals were certified murine virus– and *Mycoplasma pulmonis*–free on receipt and immediately before exsanguination. The rats were housed in stainless steel cages inside 4.5 m^3 stainless steel and glass inhalation chambers (Hinners et al., 1968). They were fed NIH 07 pellet diet (Ziegler Brothers, Gardner, Pennsylvania) and CS_2-free water *ad libitum*, except during exposures. Cages were changed and sanitized weekly. Automatic lighting cycles (12 hr on [from 0700 to 1900 hr]/12 hr off) were maintained. Chamber cage locations were rotated weekly. The rats were observed individually once in the morning and again in the afternoon for clinical signs, morbidity, and mortality.

CS_2 Vapor Generation and Exposures

CS_2 (pesticide grade) was purchased from MCB (Norwood, Ohio). Analysis of CS_2 by NIOSH Method 1600 (NIOSH, 1984) demonstrated the compound's stability and purity by gas chromatography/mass spectrometry (GC/MS) prior to, during, and after rat exposure. Purity was >99%. Inhalation exposures were conducted under dynamic flow conditions at a chamber pressure of –0.254 cm of water relative to ambient atmospheric pressure. To minimize ammonia buildup, tangential air feeds were maintained at about 1.1 m^3/min, i.e., 12–15 air changes/hr. CS_2 atmospheres were generated by injecting CS_2 liquid with a fluid metering pump (Fluid Metering, Oyster Bay, New York) at a constant (<10% CV) injection rate into the tangential airfeed manifold airstreams at the inhalation chamber tops. To confirm CS_2 concentration, lack of contamination, and uniform individual chamber CS_2 distribution, chamber atmospheres were sampled and analyzed with a Wilkes-Miran portable infrared analyzer (Foxboro, Norwalk, Connecticut) at least hourly. Temperature and humidity were measured hourly. Target airborne concentrations were achieved by changing the metering rate and extent of dilution with filtered chamber air. Separate groups of 60 rats were exposed to 0, 50, 150, and 500 ppm CS_2 for 6 hr/day, 5 days/week, over six months. Rat body weights were determined before exposure, weekly, and at termination.

Urine Collection, Storage, and Measurements

Nine rats exposed for three consecutive days in each exposure group in the last exposure week were selected randomly for urine collection at the end of the final exposure day. They were housed in Nalgene metabolism cages with food and water available *ad libitum*. Urines were collected in 100-ml polymethylpentane containers for 0–8, 8–16, 16–24, and 24–48 hr for each rat. Creatinine was determined for each fresh urine void by the alkaline picrate Jaffe reaction (Tietz, 1976).

IA assays were conducted on 2 ml of fresh urine by the urinary sodium azide/iodine reaction (first developed by Vasak et al., 1963 and Djuric et al., 1965) utilizing time t in seconds for the iodine color to fade. This was determined by the disappearance of purple iodine-starch

paper color on application of one drop, which is a slight modification of the procedure as set forth by NIOSH (1977). The index, E = (creatinine concentration in mg/L)x $\log_{10}$t, was then calculated. The detection limit was about an E of 18 at a high creatinine of 3 mg/L. Color monitoring occurred over ten days.

Fresh urine volumes of 3 ml and 4 ml were aliquotted for TTCA and TE, respectively, and stored at –20°C. The spectrophotometric measurement of urinary TE was based on the standard method of van Doorn et al. (1979), as modified by Igwe et al. (1988). The detection limit was about 40 nmol-SH/g creatinine using glutathione as substrate in the Ellman (1959) reaction (Igwe et al., 1988).

TTCA measurement was based on the ether extraction of acidified thawed urine and subsequent high-performance liquid chromatography (HPLC) using ultraviolet detection. The ether extraction method of van Doorn et al. (1981a) was modified by Cox and Que Hee (1996) to optimize the extraction number (three). The HPLC method of Campbell et al. (1985) was modified because of background problems by using two 250-mm x 4.6-mm I.D. HPLC columns in series after injection of 15 µL of urine concentrate. The columns were a Hibar LiChrospher 100 CH-8 (10 µm; E. Merck, Darmstadt, Germany), followed by a Partisil 5 ODS-3 C_{18} (5 µm; Whatman, Clifton, New Jersey), with detection at 272 nm and flow rate of 1.0 ml/min using a solvent flush/isocratic solvent elution scheme. The flush solvent consisted of 95% methanol, 4% distilled water, and 1% acetic acid; isocratic solvent consisted of 98% (v/v) distilled water, 1% acetonitrile, and 1% acetic acid. The isocratic solvent was used for 4 min after injection, the flush solvent was used for the next 8 min, and then the isocratic solvent was used again for a further 24 min. The TTCA retention time was approximately 13.1 min, and the detection limit was a urine concentration of 100 nM.

To ensure quality control in the IA, TE, and TTCA tests, one reference urine sample of known concentration was analyzed for every ten samples, as was a known concentration of TTCA in water. All samples were analyzed blind and selected randomly.

STATISTICAL METHODS

Whether the data obeyed a log normal or Gaussian distribution was ascertained using the PC-SAS (SAS Institute Incorporated, Cary, North Carolina) univariate procedure. Analysis of variance techniques and Student *t*-tests were employed to define interactions and significance, respectively. Multiple linear regressions were also performed to detect correlations and assess dose-effect relationships.

RESULTS AND DISCUSSION

The results of the TTCA, TE, and IA assays are presented in Tables 1, 2, and 3, respectively, and also Figures 1, 2, and 3/4, respectively, for rats exposed to 50, 150, and 500 ppm of CS_2. Arithmetic means are used since the data were distributed normally. Table 1 and Figure 1

show that the excretion essentially was complete for TTCA within 16 hr, as was excretion for IA-responsive metabolites (Table 3, and Figures 3 and 4), but not for TE (Table 2 and Figure 2). Figure 4 indicates that at 500 ppm CS_2, the elimination of metabolites responding to the IA assay was capacity-limited.

TABLE 1. TTCA (μmol) Excreted after End of CS_2 (ppm) Exposure

Collection time (hr)	50 ppm CS_2	150 ppm CS_2	500 ppm CS_2
0–8	0.75 (0.27)	2.39 (0.53)	5.47 (2.70)
0–16	0.96 (0.41)	2.92 (0.48)	7.18 (2.92)
0–24	0.97 (0.42)	2.93 (0.48)	7.18 (2.93)
0–48	1.00 (0.41)	2.94 (0.49)	7.20 (2.91)

Urinary TTCA as arithmetic mean level (standard deviation) in micromoles excreted over time in rat urines after the end of CS_2 exposures (ppm) over six months at 6 hr/day, 5 days/week. The data have been corrected for the control animals.

TABLE 2. TE (μmol-SH) Excreted after End of CS_2 (ppm) Exposure

Collection time (hr)	50 ppm CS_2	150 ppm CS_2	500 ppm CS_2
0–8	1.00 (0.61)	1.52 (0.86)	4.38 (2.43)
0–16	1.45 (0.70)	2.63 (1.11)	6.74 (2.07)
0–24	1.81 (0.78)	2.97 (1.04)	7.60 (2.07)
0–48	2.15 (0.84)	3.42 (0.70)	8.48 (2.25)

Arithmetic mean urinary TE level (standard deviation) in micromoles of thiol groups (-SH) excreted in rat urines over time after the end of CS_2 exposures (ppm) over six months at 6 hr/day, 5 days/week. The data have been corrected for the control animals.

TABLE 3. Inverse E-Index for Urines Excreted after CS_2 (ppm) Exposure

Collection time (hr)	50 ppm CS_2	150 ppm CS_2	500 ppm CS_2
0–8	0.19 (0.07)	1.16 (0.52)	18.1 (5.2)
0–16	0.46 (0.15)	1.49 (0.62)	19.0 (4.8)
0–24	0.60 (0.19)	1.68 (0.66)	19.1 (5.6)
0–48	0.66 (0.23)	1.79 (0.68)	19.2 (5.6)

Arithmetic mean (standard deviation) urinary inverse E-index values for the IA test excreted in rat urines over time after the end of CS_2 exposures (ppm) over six months at 6 hr/day, 5 days/week. The data have been corrected for the control animals.

Multiple regression analyses (Table 4) of the data in Tables 1–3 produced predictive regression equations relating the assay parameters with urine collection time and CS_2 exposure concentration. All of these equations had p-values of <0.0001. The equations without intercepts had better correlation coefficients (r) and p-values than those with them. The TTCA regression has the highest r and lowest p-value relative to CS_2 concentration.

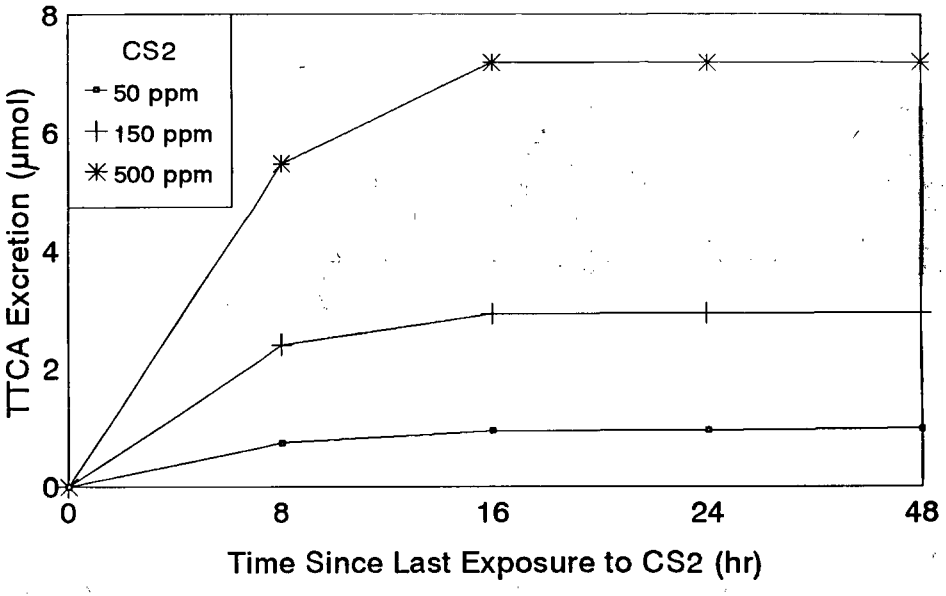

FIGURE 1. Cumulative excretion of urinary TTCA for male Sprague-Dawley rats exposed to 50 ppm, 150 ppm, and 500 ppm CS_2. The data were corrected for unexposed rat controls.

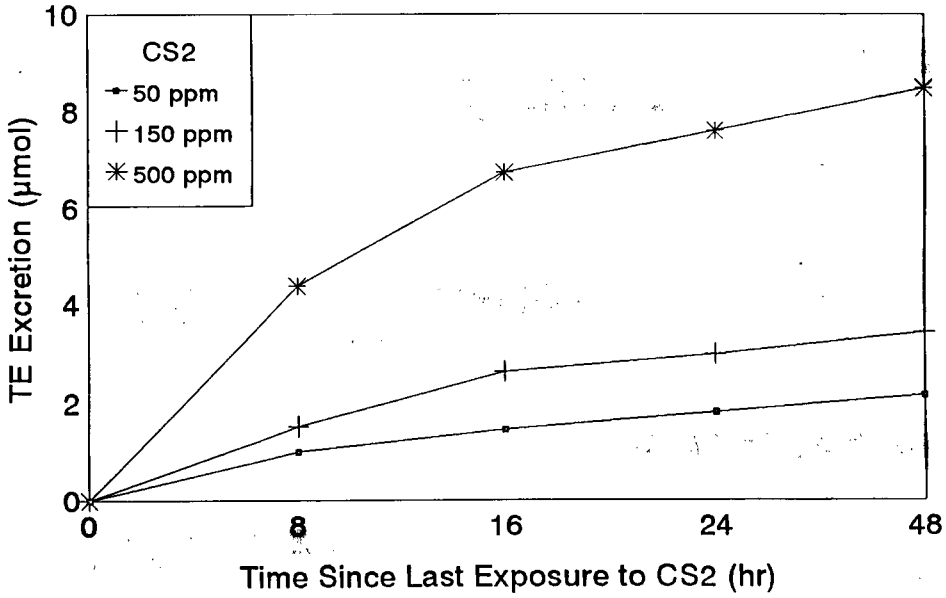

FIGURE 2. Cumulative excretion of urinary thioethers for male Sprague-Dawley rats exposed to 50, 150, and 500 ppm CS_2. The data were corrected for unexposed rat controls.

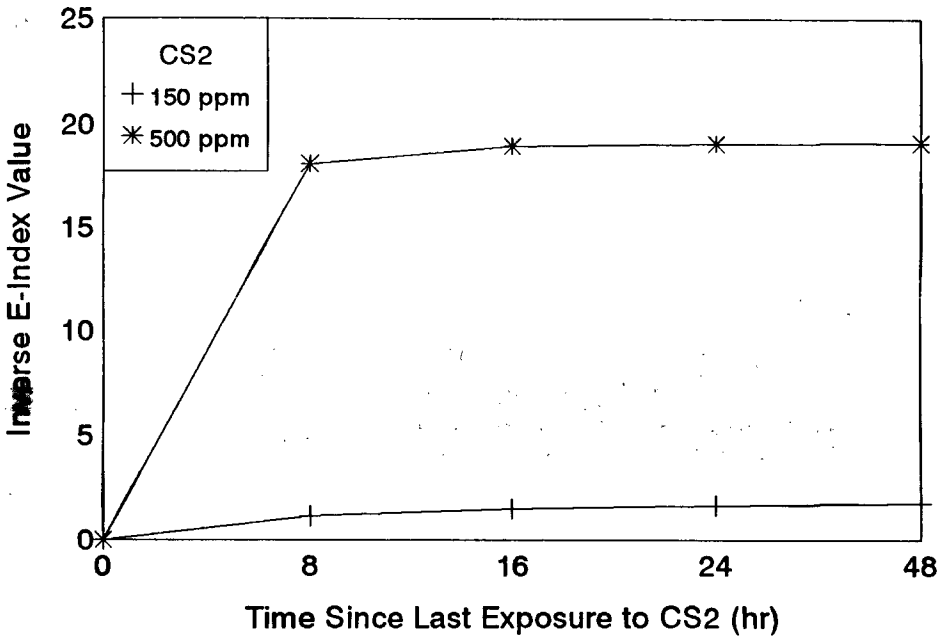

FIGURE 3. Cumulative urinary inverse E-index values for the IA assay for exposure of male Sprague-Dawley rats to 150 and 500 ppm CS_2. The data were corrected for unexposed rat controls.

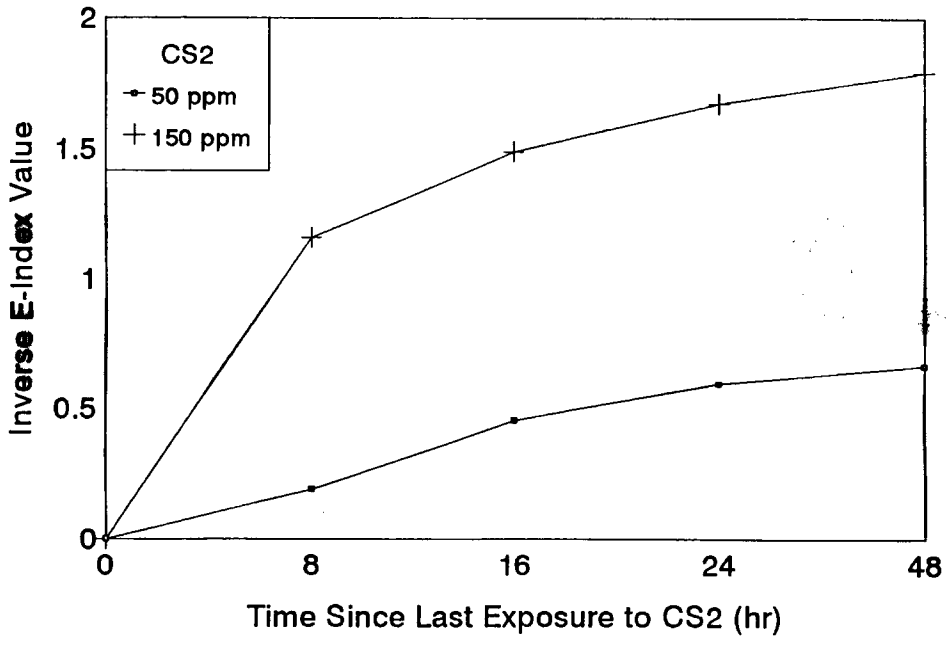

FIGURE 4. Cumulative urinary inverse E-index values for the IA assay for exposure of male Sprague-Dawley rats to 50 and 150 ppm CS_2. The data were corrected for unexposed rat controls.

TABLE 4. Dose-Effect Relationships for the Rat Urinary Assays for Time in Hr (t) after the End of Exposure to a Carbon Disulfide Concentration (ppm) at 6 Hr/Day, 5 Days/Week for Six Months, and Related Correlation Coefficient (r), and *P*-Value (p) in Terms of Regression Equations with and without Intercept

Assay	Regression equation	r/p
TTCA	TTCA = 0.0141t + 0.0178 ppm – 0.2360	0.96/0.0001
	TTCA = 0.0079t + 0.0175 ppm	0.97/0.0001
TE	TE = 0.0229t + 0.0137 ppm – 0.1744	0.90/0.0001
	TE = 0.0183t + 0.0135 ppm	0.94/0.0001
IA	IA = 0.0221t + 0.0381 ppm – 1.7096	0.94/0.0001
	IA = 0.0223t + 0.0363 ppm	0.95/0.0001

The data have been corrected for the control animals.
IA, iodine-azide-sensitive metabolites in inverse E-index units; TE, thioether arithmetic mean in μmol-SH; TTCA, 2-thiothiazolidine-4-carboxylic acid arithmetic mean in μmol.

Subjecting the data in Tables 1–3 to multiple regression analysis produced the interrelationships among the urinary parameters (Table 5), all with p-values <0.0001. TTCA is a five-atom heterocyclic compound, which is a dithiocarbamate (R-S- (C=S) -NH-R′). The IA assay is supposed to reflect the presence of dithiocarbamates, thiocarbamates, and thioureas (Rosier et al., 1984). Therefore, IA should correlate well with TTCA if the latter dominates, as was observed in this study (Table 5). Other major IA-responsive metabolites also are present, including: dithiocarbamates like 2-mercapto-thiothiazolidine-2-one-5 and those formed through amino acid conjugation of CS_2; the thiocarbamate, 2-oxothiazolidine-4-carboxylic acid; and the thiourea, thiocarbamide (Rosier et al., 1984). In the first 16 hr, the number of moles of TTCA excreted was statistically no different from those for TE for the same exposure concentration of CS_2. TTCA may also act as a thioether (R-S-R′), which should be proven directly.

TABLE 5. Correlation Analysis for the Rat Urinary Assays after Exposure Ended for Time in Hr (t) after End of Carbon Disulfide Exposure (ppm) at 6 Hr/Day, 5 Days/Week for Six Months, and Related Correlation Coefficient (r) and *P*-Value (p)

Regression equation with and without intercept	r/p
TTCA = 0.910TE + 0.248	0.86/0.0001
TTCA = 0.951TE	0.92/0.0001
TTCA = 0.405IA + 0.974	0.89/0.0001
TTCA = 0.459IA	0.91/0.0001
TE = 0.343IA + 0.849	0.91/0.0001
TE = 0.390IA	0.92/0.0001

The data have been corrected for the control animals.
IA, iodine-azide-sensitive metabolites in inverse E-index units; TE, thioether urinary amount in μmol-SH; TTCA, 2-thiothiazolidine-4-carboxylic acid urinary amount in μmol.

Table 6 shows the appearance $t_{0.5}$ of TTCA, TE, and IA as determined from Figures 1–4 at each CS_2 concentration. The range of TTCA $t_{0.5}$ is 5.0–5.5 hr, with an arithmetic mean of 5.2 hr with CV 4.8%; excretion is completed within 16 hr. Similarly, the $t_{0.5}$ for TE was 8.5 hr with CV 7.2%, reasonably close to the data for TTCA. TE, however, were still detectable after 48 hr, unlike TTCA, and after 16 hr the $t_{0.5}$ for TE lengthened to 13.1 hr. Before 16 hr, therefore, both TTCA and TE obeyed pseudo-first-order kinetics, with $t_{0.5}$ being independent of CS_2 initial concentration. In contrast, the $t_{0.5}$ values for IA were 12.6, 6.1, and 4.4 hr for 50, 150, and 500 ppm CS_2, respectively; the arithmetic mean was 7.7 hr and CV 57%, indicative of concentration-dependent processes. The $t_{0.5}$ at the two highest concentrations approximated those for TTCA. The IA-responsive compounds at 50 ppm CS_2 were excreted with a low capacity having little to do with TTCA.

TABLE 6. Comparison of Urinary Excretion Half-Times ($t_{0.5}$) and Elimination Constants (k_{el}) for the TTCA, TE, and Metabolites Sensitive to the IA Test by CS_2 Exposure Concentration (ppm)

	Urinary parameter					
	TTCA		TE		IA	
CS_2	$t_{0.5}$ (hr)	k_{el} (hr^{-1})	$t_{0.5}$ (hr)	k_{el} (hr^{-1})	$t_{0.5}$ (hr)	k_{el} (hr^{-1})
50	5.2	0.133	9.0	0.077	12.6	0.055
150	5.0	0.139	8.6	0.081	6.1	0.114
500	5.5	0.126	7.8	0.089	4.4	0.158
Mean	5.2	0.133	8.5	0.082	7.7	0.109
SD	0.3	0.006	0.6	0.006	4.3	0.062

The standard deviation for individual $t_{0.5}$ is 0.2 hr.

This study is the first to interrelate TTCA, TE, and IA for a rat animal model, and to determine the rat $t_{0.5}$ for the urinary excretion of TTCA and TE. Rosier et al. (1987b) measured a human TTCA $t_{0.5}$ of 2.0 +/– 0.3 hr at 50-watt physical activity. Since the duration of that study was only 200 min for CS_2 concentrations of 3, 10, and 20 ppm, any slow elimination phase would not be detected. The slow elimination phase must be present for workers documented to show TTCA in preshift samples after exposure the previous work day to high CS_2 concentrations. In 1986, Gagnaire et al. conducted a study of the effect of CS_2 on nerve conduction velocity in male Sprague-Dawley rats. The rats were exposed by inhalation to 481 +/– 44 ppm CS_2, 5 days/week, 6 hr/day, for 25 weeks. The results showed that the TTCA arithmetic mean and standard deviation was 4.0 +/– 0.5 μmol TTCA/24-hr urine at the end of two and four days of exposure, following no exposure over the two weekend days in weeks 19 and 23. This is to be compared with 7.2 +/– 2.9 μmol TTCA/24-hr urine excreted after exposure to 500 ppm CS_2 under the conditions of the present study. The causes of the differences in urinary TTCA are not known. Kivisto et al. (1995), while investigating the effects of CS_2 vapor in short-term exposures on cytochrome P-450 enzyme induction and glutathione depletion in male Wistar rats showed that TTCA excretion ranged from 1.4–2.0 μmol/24-hr at 50 ppm, and from 4.7–5.4 μmol/24-hr at 500 ppm. The excretion at 500 ppm

was not statistically different from that observed at 500 ppm for the present study; at 50 ppm, however, it was higher than the 0.97 +/– 0.42 μmol/24-hr in the present study. Different exposure regimens and rat strains are the probable contributing factors.

The IA $t_{0.5}$ of 4.4 hr at 500 ppm agrees with the 3.5-hr value measured for male Sprague-Dawley rats exposed to 667 ppm CS_2 (McKenna and DiStefano, 1977). There are multiple metabolites that have different $t_{0.5}$, and there is a capacity-limited process at 50 ppm that does not involve TTCA. Rosier et al. (1984) have reported a strong correlation between TTCA and IA in humans ($r = 0.92$, $p = 0.05$), but not a correlation equation or $t_{0.5}$. The r for the correlation of IA index to CS_2 in the present study was 0.95, with a much better *p*-value.

CONCLUSIONS

Urinary TTCA is better correlated to CS_2 air concentration than IA and TE, and is as sensitive and selective in male Sprague-Dawley rats as it is in humans. The IA assay has the poorest selectivity at low CS_2 concentrations. The TE assay selectivity is intermediate at low concentrations of CS_2.

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