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Determination of urinary 2-thiazolidinethione-4-carboxylic acid after exposure to alkylene bisdithiocarbamates using gas chromatography–mass spectrometry

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Abstract

This is a newly developed method which permits the quantitative determination of 2-thiazolidinethione-4-carboxylic acid (TTCA, an established biomarker of exposure to CS_2) as a metabolite of alkylene bisdithiocarbamates (ABDCs) in human urine. After separation of TTCA from the urinary matrix using liquid–liquid extraction the analyte was converted into its diethyl derivative. Separation and quantitative analysis was carried out by capillary gas chromatography and mass selective detection in single ion monitoring mode. 4-(4-Chloro-2-methylphenoxy)butanoic acid (MCPBA) served as internal standard. The detection limit was 0.7 μg/l in urine. The relative standard deviation of the within-series imprecision was 4.3% at a concentration of 13 μg/l. The relative recovery was within the range of 86 to 98%. In order to determine the suitability of TTCA for biological monitoring after exposure to ABDCs, we analysed 87 24-h urine samples from occupationally exposed workers. The results were compared with the levels of TTCA excreted in urine by 50 control persons without known exposure to dithiocarbamates or CS_2. This collective of unexposed persons also provided TTCA reference values for the general population. The urinary TTCA concentrations of the exposed persons were in the range from 0.8 μg/g creatinine to 515 μg/g creatinine. Unexposed persons excreted TTCA in concentrations from below the detection limit to 182 μg/g creatinine. The median concentration found in exposed persons (27 μg/g) was nearly 2.5 times higher than in non-exposed persons (11 μg/g). The difference between the exposed and unexposed collective was highly significant. Assessment of an individual's exposure by determining the level of TTCA in urine nevertheless was not possible. This was due to the relatively wide range of concentrations and because the ranges of both collectives overlapped.

Keywords: 2-Thiazolidinethione-4-carboxylic acid; Alkylene bisdithiocarbamates

1. Introduction

Alkylene bisdithiocarbamates (ABDCs) are important agrochemical fungicides and are widely used. Furthermore, in industry they are used as slimicides in water cooling systems, in the manufacturing of sugar, pulp and paper, and as vulcanisation accelerators and antioxidants in rubber. Due to their chelating properties they are also used as scavengers in waste water treatment [1]. Disulfiram is used in medicine as a therapeutic agent in the treatment of alcohol abuse [2].

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The fungicides used in agriculture are metal salts containing manganese (maneb, mancozeb), iron (ferbam), zinc (zineb, mancozeb, propineb), or sodium (metiram).

Although this class of fungicide is regarded as relatively harmless because of its low mammalian toxicity (e.g. an acute oral LD_{50} in rats of 6750 mg/kg for maneb [1]) the chronic and subchronic toxicity of these substances cannot be ignored. Usually bisdithiocarbamates are classified as eye and skin irritants. Some may induce skin sensitisation in susceptible individuals. The metabolites and degradation products of ABDCs, ethylene thiourea or propylene thiourea (propineb), affect the thyroid [3]. Neurotoxic effects have also been observed [4]. Furthermore, some alkylene bisdithiocarbamates have been mentioned as being teratogenic in high doses [5]. Due to their chelating properties, dithiocarbamates inhibit enzymes containing Fe, Cu, Zn or thiol groups [6].

The metabolism of alkylene bisdithiocarbamates in plants and mammals, especially in rats and mice, has been studied extensively [1,7–9,10–12]. Animal experiments revealed that rats excrete 90% of incorporated ABDCs with the urine during the first 24 h [9]; no accumulation was observed. Both ethylene and propylene bisdithiocarbamates release CS_2 as a product of metabolism (Fig. 1). It is well-known that carbon disulphide is metabolised to TTCA via addition to the cysteinyl-SH group of glutathione and subsequent ring condensation (Fig. 2) [13,14]. About 3% of orally administered carbon disulphide is metabolised in rats to TTCA and excreted in urine [15]. Rats metabolise about 2% of the CS_2 equivalents of ABDCs to TTCA after oral ingestion of dithiocarbamates [16].

Occupational exposure mainly occurs during the manufacturing of ABDCs and their use as fungicides. The general population can be exposed to these compounds and their degradation products as a result of residues in the diet [7,17–19]. Another non-negligible source of urinary TTCA is the consumption of brassica vegetables [20]. These vegetables contain TTCA. The ingestion of raw cabbage, for example, may lead to concentrations higher than 2 mg/l [21]. Furthermore, the pesticide captan may produce TTCA during its metabolism [22].

TTCA is already an established urinary parameter for biological monitoring of exposure to CS_2 [24] (BEI: 5 mg/g creatinine [23], BAT: 4 mg/g creatinine [25]). Biomonitoring studies of exposure to carbon disulphide in man showed that less than 6% of the CS_2 absorbed was excreted as TTCA in urine [13], other studies found 3% was excreted as TTCA after inhalation of CS_2 [26].

The WHO suggested TTCA in urine could be used as a biological index for monitoring exposure to dithiocarbamates [5]. To date, however, there is no data available concerning its suitability [27,28].

The methods already published for analysis of urinary TTCA [11,16,22,29,30–32] do not fulfill the requirements regarding sensitivity when detecting TTCA in the low μg/l range expected after alkylene bisdithiocarbamate exposure. The most sensitive method published so far offers a detection limit of 16 μg/l [31] using high-performance liquid chromatographic separation and UV-detection. The most sensitive procedure using gas chromatography–mass spectrometry after liquid–liquid extraction yields a detection limit of 30 μg TTCA/l urine.

The aim of this study was to develop a practical and sensitive method suitable for investigating urinary TTCA concentrations after occupational exposure to alkylene bisdithiocarbamates. In addition, the method was intended to enable the determination of baseline excretion of TTCA in the general population in order to examine standard excretion values caused by dietary habits, the intake of ubiquituous CS_2 [37], and/or the intake of fungicide residues in food.

2. Experimental

2.1. Chemicals and methods

2-Thiazolidinethione-4-carboxylic acid (TTCA, certified assay: 99%), 4-(4-chloro-2-methylphenoxy)-butanoic acid (MCPBA, certified assay: 98%) and sodium borohydride ($NaBH_4$ pellets; certified assay: 98%) were purchased from Aldrich (Steinheim, Germany); hydrochloric acid 37% p.a., sodium chloride p.a., potassium hydroxide p.a., diethyl ether p.a., toluene Uvasol®, methanol Suprasolv®, and highly purified water were obtained from Merck (Darmstadt, Germany). *N*-nitroso-*N*-ethyl urea was ob-

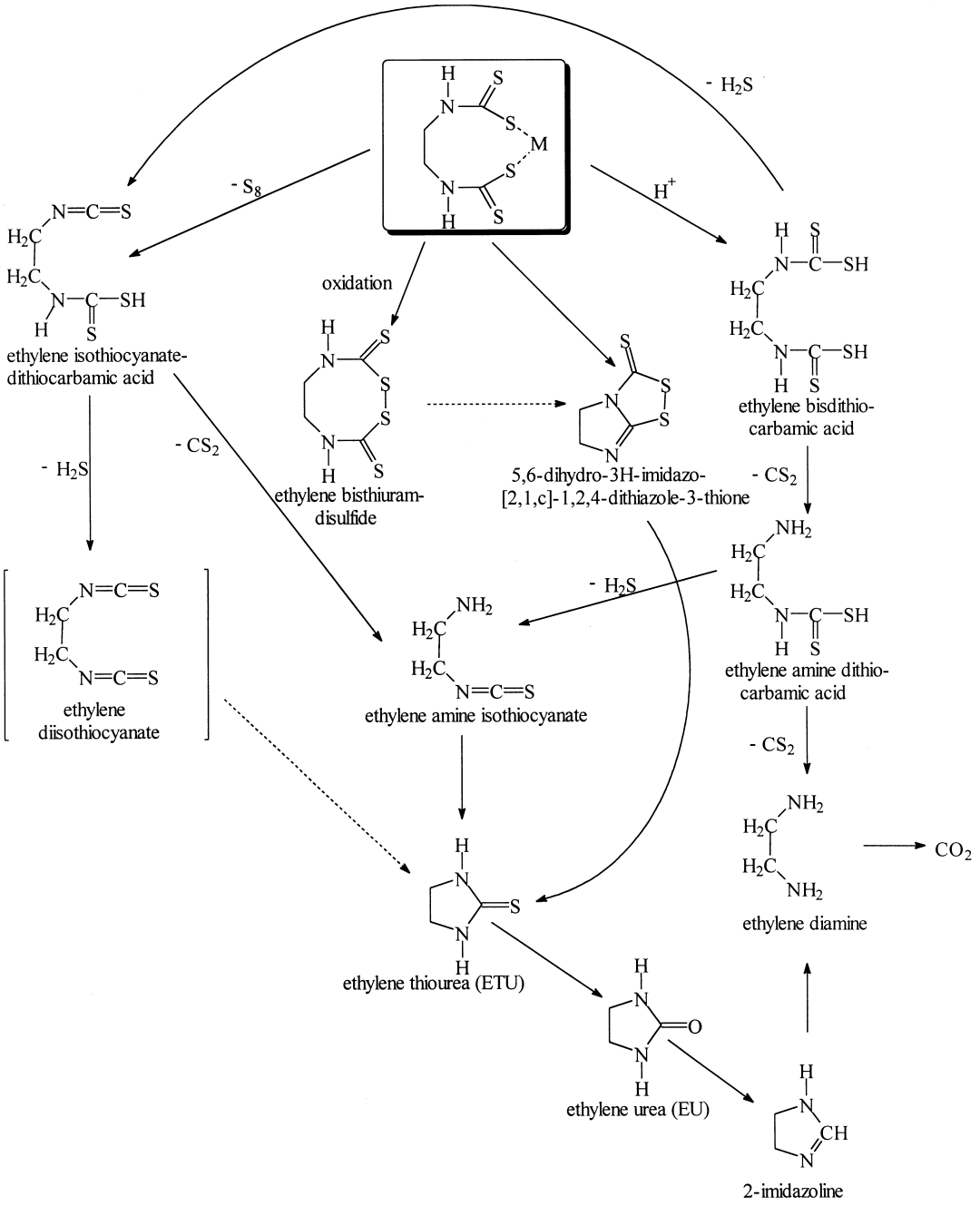

Fig. 1. Simplified metabolic pathway for the decomposition of an ethylene bisdithiocarbamate (dashed line indicates proposed reaction, M=metal) [1,8].

tained from the Institute of Organic Chemistry of the University of Erlangen-Nürnberg. Diazoethane synthesis was carried out by adding 5 g *N*-nitroso-*N*-ethyl urea a little at a time to a 15 ml solution of cooled 4 *M* potassium hydroxide in water layered to 90 ml toluene. The temperature was kept below 5°C.

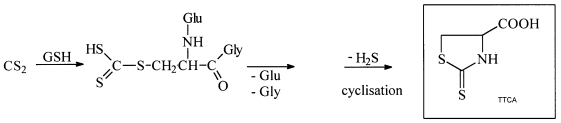

Fig. 2. Metabolic pathway of CS_2 to TTCA in mammals (GSH= glutathione, Gly=glycine, Glu=glutamic acid).

The toluene diazoethane solution was stored in sealable glass bottles at −25°C.

Urinary creatinine concentrations were determined according to Larsen [36].

Statistical analysis was carried out using SPSS 7.5 for Windows®.

2.2. The internal standard solution (I.S.)

The stock solution for the I.S. was prepared by dissolving 25 mg 4-(4-chloro-2-methyl-phenoxy)butanoic acid (MCPBA) in 50 ml methanol (500 mg/l). A volume of 1250 μl of this stock solution was diluted to the mark in a 50-ml glass volumetric flask with highly purified water (12.5 mg/l). The resulting MCPBA standard solution was used for spiking urine samples before sample preparation (see Section 2.3).

2.3. Sample preparation

A 2 g amount of sodium chloride was added to a 5 ml urine sample. Then the mixture was spiked with 100 μl of the I.S. solution. Transformation of the analyte and the I.S. into their uncharged forms was performed by adding 400 μl of 2 *M* hydrochloric acid. This solution was twice extracted with 5 ml diethyl ether (amounts of 100 ml diethyl ether were previously freed from oxidative impurities by shaking with 180 mg sodium borohydride solution in 20 ml water) by shaking in a laboratory shaker for 10 min, and centrifuged for 5 min at 1500 *g*. The combined diethyl ether extracts were evaporated to dryness in a gentle stream of nitrogen. The residue was ethylated with 300 μl of a diazoethane solution in toluene with a reaction time not shorter than 12 h. About 100 μl of this solution was transferred to a microvial for subsequent quantitative GC–MS analysis. An overview of the preparation procedure can be found in Fig. 3.

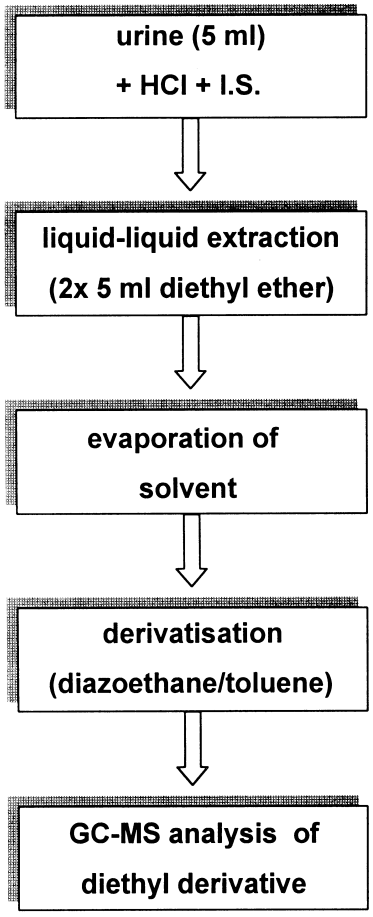

Fig. 3. Sample processing.

2.4. Calibration procedure

A TTCA starting solution, containing 500 mg/l, was prepared by dissolving 25 mg TTCA in 50 ml highly purified water. From this starting solution two standard stock solutions were prepared.

Stock solution A: 1000 μl of the starting solution was diluted to the mark with highly purified water in a 100-ml glass volumetric flask (5 mg/l).

Stock solution B: 2000 μl of the starting solution was diluted to the mark with highly purified water in a 50-ml glass volumetric flask (20 mg/l).

Seven calibration standards, both aqueous and urinary, with spiked concentrations in the range from 5 to 500 μg/l were prepared from these standard stock solutions by diluting with pooled urine or

water. The calibration standards were stable at −25°C for at least 6 months. Linear calibration curves were obtained by plotting the quotients of the peak areas of TTCA and the I.S. as a function of the concentrations used. Both water and pooled urine standards produced the same linear slope of regression. These graphs were used to ascertain the unknown concentrations of TTCA in urine samples.

2.5. Gas chromatography

Analysis was carried out on a Hewlett-Packard HP 5890 gas chromatograph fitted with a Hewlett Packard HP 7673A autosampler and a split/splitless injector operating in splitless mode. The operating temperature of the injector was 260°C. Chromatographic separation was performed on a (35% phenyl)methylpolysiloxane capillary column (60 m× 0.22 mm I.D., 0.25-μm film thickness) purchased from SGE (Weiterstadt, Germany). Helium 5.0 was used as carrier gas with constant flow (1.27 ml/min). The initial column temperature was 100°C; it was then raised at a rate of 7°C/min to 240°C. Afterwards the temperature was finally raised at a rate of

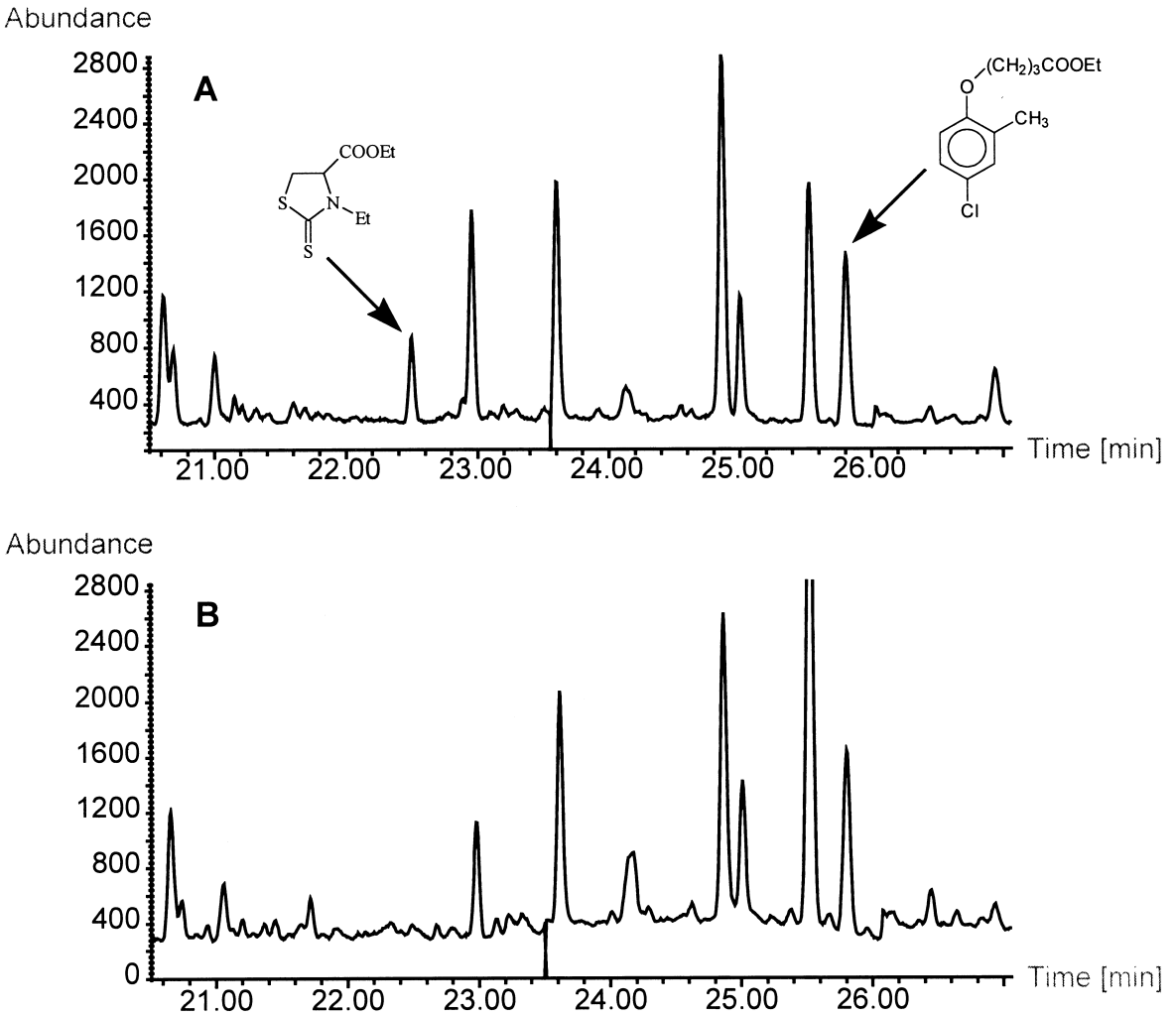

Fig. 4. SIM chromatograms of processed urine samples of (A, 23.7 μg TTCA/l) exposed and (B, TTCA <detection limit) non-exposed persons. The quantifier ions registered were m/z=146, 142. (Et=ethyl).

20°C/min to 250°C and this temperature was held for 21 min. The injection volume was 1 μl.

The retention times for the analyte and I.S. observed under the described conditions were 22.6 min (TTCA) and 25.9 min (I.S.), respectively.

2.6. Mass spectrometry

A Hewlett-Packard HP MSD 5972 mass spectrometer fitted with a quadrupole mass filter was used in electron impact (EI) mode. EI mass spectra were obtained at an ionisation energy level of 70 eV and the electron multiplier voltage was 1900 V. The MSD transfer line temperature was maintained at 300°C. For quantitative analysis of TTCA selected ion monitoring was used and ions with masses m/z= 191 (qualifier; M^+-CH_2CH_3), 146 (quantifier; M^+-$COOCH_2CH_3$) for TTCA, and 211 (qualifier; M^+-OCH_2CH_3), 142 (quantifier; M^+-$C_3H_5COOCH_2CH_3$) for the I.S. MCPBA spectra were recorded. Fig. 5. shows a mass spectrum for ethylated TTCA.

2.7. Collectives and specimen collection

In this study we investigated 87 urine specimens from workers employed in the application of fungicides indoors and outdoors. The workers were occupationally exposed to various alkylene bisdithiocarbamate fungicides during their application in greenhouses, in agriculture/farming, vegetable farming, and orchards. The workers used pesticide formulations containing the ethylene bisdithiocarbamates maneb, zineb, mancozeb, and metiram, and the propylene bisdithiocarbamate propineb. None of the formulations applied contained captan. The creatinine values of these urine specimens were in the range from 0.4 to 2.5 g/l with a median value of 1.2 g/l. The duration of the exposure was in the range from 1.5 to 9 h.

In order to determine the baseline excretion of TTCA in the general population, a collective of 50 persons not occupationally exposed to alkylene bisdithiocarbamates, captan, or CS_2 was also investigated. In this collective the creatinine concentrations were in the range from 0.4 to 2.5 g/l with a median value of 1.5.

Twenty-four hour urine specimens were collected from exposed persons during the period of application in summer. Urine samples from unexposed persons were collected as spot samples. All samples were stored in sealable plastic bottles at −25°C until processing.

3. Results and discussion

Because of differences in absorption of the various alkylene bisdithiocarbamates, the best way to reflect

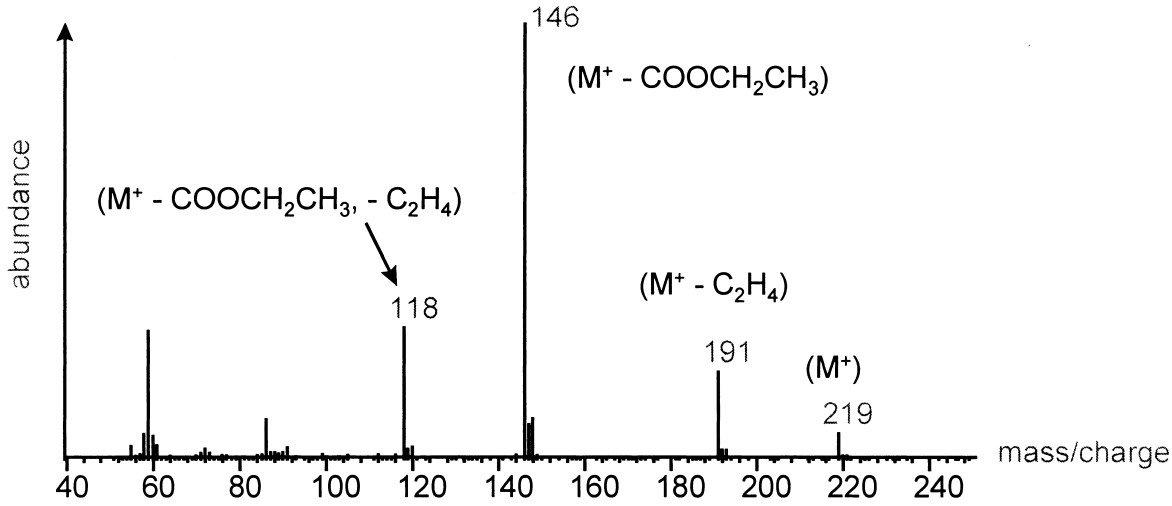

Fig. 5. Mass spectrum of TTCA diethyl derivative.

all routes of absorption (dermal, oral, inhalative) is by determining the unchanged substance or one of its metabolites [33] in body fluids using biological monitoring. Ethylene thiourea is only a metabolite of ethylene bisdithiocarbamates and propylene thiourea only of propylene bisdithiocarbamates [26]; the advantage of TTCA is that it is a common metabolite of all alkylene bisdithiocarbamates.

The gas chromatographic conditions used allowed the separation of TTCA from coextracted substances after a clean-up procedure and using a (35% phenyl)-methylpolysiloxane column. As a mass selective detector was used, the method proved to be much more specific than methods described in the literature using a nitrogen phosphorous detector or UV-detector after high-performance liquid chromatographic separation. Two gas chromatograms recorded in SIM mode (m/z=146, 142) are shown in Fig. 4. A corresponding full scan mass spectrum of TTCA is presented in Fig. 5.

3.1. Calibration graphs

The calibration graphs were linear for the range investigated. Aqueous calibration standards (5–500 μg/l) yielded correlation coefficients higher than 0.98. No pooled urine free of TTCA was obtainable, but the urine used was found to have a blank TTCA concentration of 13 μg/l. Thus, the urinary calibration standards resulted in calibration curves in the range from 13 to 513 μg/l. The calibration curves intersected in the positive range. The associated correlation coefficients were higher than 0.998. Calibration curves generated from both urinary and aqueous standard solutions produced the same slope of regression. Due to better linear correlation, especially in the lower calibration range, urinary calibration curves were used for quantification.

3.1.1. Precision

In order to assess the within-series imprecision, pooled urine of persons not exposed to pesticides with a concentration of 13 μg TTCA per litre urine was analysed ten times. The relative standard deviation was 4.3% (absolute 0.5 μg/l).

In order to obtain the between-day imprecision, spiked samples of pooled urine with five different TTCA concentrations were processed and analysed on seven different days. The concentrations ranged between 13 and 213 μg/l. The relative standard deviations were determined as being in the range from 3.3 to 7.8%. The precision data are also presented in Table 1.

3.1.2. Recovery of spiked concentrations

Accuracy was examined by carrying out recovery experiments. In order to determine the recovery, urine from persons not exposed to alkylene bisdithiocarbamates was pooled. Spiking this urine (blank TTCA concentration 79 μg/l) with two different amounts of TTCA (15 and 120 μg/l) resulted in two solutions containing 94 and 199 μg TTCA per litre urine. Aliquots were analysed eight times and the results were calculated from urinary calibration curves. Relative mean recovery was found to be 95% (spiked concentration: 15 μg/l) and 96% (spiked concentration: 120 μg/l), respectively.

In order to investigate the influence of individual urine specimens, six individual samples with different creatinine contents in the range from 0.91 to 1.81

Table 1
Precision data (SD=standard deviation)

Determined concentration (average) (μg/l)	Spiked concentration (μg/l)	Imprecision		*N*
		Percentage	SD (μg/l)	
13	0	4.3% Within-series	0.5	10
13	0	5.4% Between-day	0.7	7
64	50	7.0% Between-day	4.5	7
213	200	3.3% Between-day	7.0	7
89	15	7.8% Between-day	7.0	7
192	120	7.2% Between-day	12.2	7

g/l and TTCA concentrations in the range from 3.6 to 168 μg/l, were additionally investigated. Subsequently, these samples were spiked with 50 μg/l and analysed once again. Relative recovery was determined as being 92.4±6% (mean recovery±standard deviation). No correlation to individual creatinine levels was observed. These experiments and the results from Section 3.1 confirm that the slope of regression was not affected by interference from different urinary matrices in our method.

3.1.3. Detection limit

TTCA could not be detected in blank extracts prepared from purified water. The detection limits were calculated from a signal-to-noise ratio of 3:1. The resulting limit of detection was 0.7 μg/l in urine (determined in an individual urine sample in which TTCA could not be detected) and 0.5 μg/l in water using a single ion fragment ($m/z=146$) as quantifier in both cases. The detection limit of the less intensive qualifier ion fragment ($m/z=191$) was 5 μg/l in urine and 1.5 μg/l in water, respectively.

3.1.4. Sources of error

The diazoethane/toluene solution is unstable. After a storage period of 6 weeks at −25°C the solution should be discarded.

The use of diethyl ether without previous removal of oxidants (Section 2.3) may lead to irreproducible results.

We were not able to obtain any pooled urine free of TTCA. Due to the influence of dietary habits on urinary TTCA concentrations, donors should not have ingested food containing TTCA, such as brassica vegetables, within 3 days before specimen collection.

3.2. Results of biological monitoring and discussion

The results of the analysis of TTCA in the urine of 87 exposed and 50 unexposed persons are presented in Table 2. Twenty-four hour urine samples were collected only in exposed persons but not in controls. To compare 24-h urine and spot urine samples, TTCA concentrations were correlated to individual creatinine concentrations. This correction was intended to standardise the results, as it is recommended that TTCA be related to creatinine [34,35]. However, it cannot be excluded that TTCA values (related to individual creatinine levels) in spot urine samples may significantly differ from the corresponding values in 24-h urine. This problem must be further investigated.

TTCA was found in all samples from exposed workers. The concentrations were in the range from 0.8 to 515 μg/g creatinine. The corresponding values in the control specimens investigated were found to be below the detection limit to 182 μg TTCA/g creatinine. TTCA was not detected in two of the controls. The median value in the exposed persons (27 μg/g) was nearly 2.5 times higher than in non-exposed persons (11 μg/g).

A Mann–Whitney–Wilcoxon test revealed a highly significant difference between both groups. In Fig. 6 this difference is illustrated in a cumulative frequency chart. For both collectives the results of biological monitoring yielded relatively wide ranges for urinary TTCA concentrations. These ranges overlapped, the standard deviations were higher than the median values of the two groups. A box-and-whiskers-plot presented in Fig. 7 illustrates this overlapping of both collectives. The wide ranges observed may be caused by different occupational

Table 2
Results of biological monitoring of urinary TTCA (SD=standard deviation, D.L.=detection limit)

	Exposed persons ($N=87$)		Control person ($N=50$)	
	Concentration (μg/l)	Concentration (μg/g creatinine)	Concentration (μg/l)	Concentration (μg/g creatinine)
Average	65	54	35	24
SD	124	86	44	32
Median	33	27	16	11
Range	1.4–860	0.8–515	<D.L. –170	<D.L.–182
95th percentile	218	160	123	69

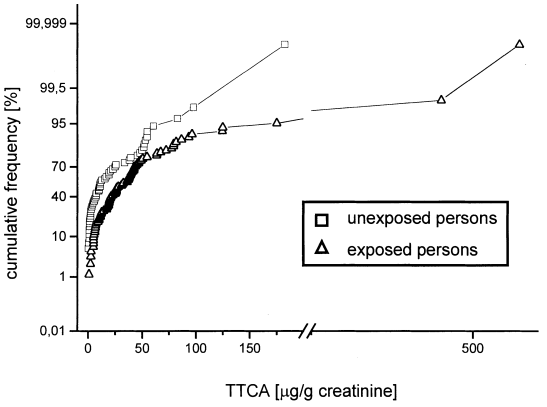

Fig. 6. Cumulative frequency of both unexposed and exposed collectives.

exposure and by differences in individual dietary habits. Whether the TTCA concentrations of non-exposed persons are due to residues of alkylene bisdithiocarbamates in food, incorporation of ubiquitous CS_2 [37] or are exclusively caused by a diet containing TTCA, remains unclear. However, the overlapping TTCA concentrations of our collectives made it impossible to asses the individual body burden caused by ABDCs.

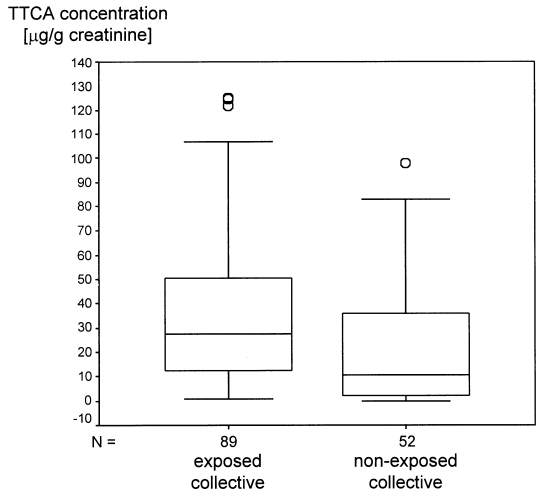

Fig. 7. Box-and-whiskers-plot for comparison of exposed and non-exposed collective. (The lower and upper box edges represent the 25th and 75th percentile, the line inside the box the median value. Fifty percent of cases have values within the box. Cases with values that are more than 1.5 box-lengths (represented by the stamps) from the upper or lower edge of the box are defined as outliers).

Comparing our results with others cited in the literature revealed that the average TTCA concentrations we determined in non-exposed persons (24 µg/g) and in exposed (54 µg/g) persons were lower than those in unexposed persons (90 µg/g, $n=122$) observed by Simon et al. [29]. We therefore conclude that the ABDC exposure of the workers investigated by us was relatively low, provided that overestimation due to the less specific HPLC–UV method (detection limit=0.05 mg/l) used by Simon et al. can be excluded. The varying dietary intake of ABDCs or TTCA itself might explain the individual differences in the baseline excretion of TTCA in the general population.

4. Conclusions

Presented here is a precise, accurate, specific and sensitive method for the quantitative detection of the alkylene bisdithiocarbamate metabolite TTCA in human urine. The reliability criteria, such as within-series imprecision, between-day imprecision and recovery can be described as good. The method proved to be practicable in routine analysis. The excellent detection limit of 0.7 µg TTCA per litre urine enabled the biological monitoring of occupationally and environmentally exposed persons.

Using biological monitoring we found that persons occupationally exposed to ABDCs excrete higher levels of TTCA in urine than the general population. We found a highly significant difference between the exposed and unexposed collective. The ranges of the TTCA concentrations of both groups of persons overlapped (Fig.7). For this reason we were not able to use TTCA as a biological monitoring parameter for individual risk assessment after occupational exposure to alkylene bisdithiocarbamates. Nevertheless, differentiation between occupationally exposed persons and non-exposed persons on a collective basis was possible.

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