2-Thioxothiazolidine-4-carboxylic acid (TTCA)

Application	Determination in urine
Analytical principle	High pressure liquid chromatography (HPLC)
Completed in	August 1990

Summary

The procedure described here permits reliable analytical determination of 2-thioxo-thiazolidine-4-carboxylic acid (TTCA) excreted in the urine of persons exposed to carbon disulfide. For this reason and because of its practicability, this method is suitable for routine monitoring of the existing biological tolerance value for this working material.

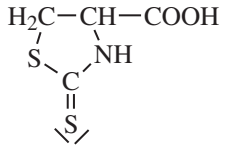
After acidification of and addition of sodium chloride to the urine, TTCA is extracted with diethyl ether. The ether phase is evaporated and the residue is dissolved in methanol. TTCA is analysed by high pressure liquid chromatography.

Detection is achieved with a UV detector at 273 nm.

Calibration is carried out using standard urine solutions containing known TTCA concentrations. The standards are processed in the same way as the assay samples.

Within-series imprecision:	Standard deviation (rel.)	s_w = or 2.6 % or 1.7 %
	Prognostic range	u = 5.9 % or 3.8 %
	At concentrations of 1.0 mg and 5.0 mg TTCA per litre urine and where n = 10 determinations	
Inaccuracy:	Recovery rate	r = 91–95 %
Detection limit:	0.2 mg TTCA per litre urine	

2-Thioxothiazolidine-4-carboxylic acid (TTCA)



is a metabolite of carbon disulfide (CS_2). Carbon disulfide is mainly used in the artificial silk and wood pulp industries. It is still permissible (with restrictions) to use CS_2 against the vine louse (Phylloxera) in vineyards. Furthermore, it serves as a solvent for sulfur, iodine, phosphorus, rubber, guttapercha, wax, paraffin. It is used as a reagent for producing secondary amines, for the vulcanization of rubber, in the manufacture of vulcanizing agents, flotation agents and carbon tetrachloride as well as for the synthesis of sulfurous heterocylic compounds and other organic compounds containing sulfur.

The toxicology of carbon disulfide has been comprehensively described in the documentation of the MAK values [6]. Like all organic solvents it has a depressive effect on the central nervous system. Thus, typical symptoms, such as headache and fatigue, etc. could be observed as a result of the intake of CS_2 at the workplace. Characteristic for CS_2 is that depressive psychoses can occur after long-term occupational exposure. Polyneuritis is also observed. Moreover, intake of CS_2 over many years can alter the blood vessels, as in arteriosclerosis, which can be manifested in various clinical symptoms. Carbon disulfide inhibits a number of enzymes, especially hydrolases or oxidases, which explains certain effects including alcohol intolerance after CS_2 exposure. Chronic exposure causes functional disturbances in the liver, kidneys, pancreas and endocrine glands.

Berufsgenossenschaftlicher Grundsatz G6 (Principle G6 of the Professional Association) on the "Risks of exposure to carbon disulfide" [7] describes the differential diagnostic considerations and the occupational medicinal exclusion criteria of a carbon disulfide intoxication based on organic evidence and the symptoms of disease.

The metabolism and kinetics of carbon disulfide are presented in detail in the documentation of the BAT values (cf. Fig. 1) [1]. According to this publication 2/3 of the inhaled CS_2 is exhaled unchanged. About 30 % of the carbon disulfide is metabolized and reacts mainly with groups bearing lone electron pairs, such as aminosulfhydryl or hydroxy groups. The urine of exposed persons contains metabolic products which are formed when CS_2 reacts with glycine (2-mercapto-2-thiazoline-5-one) as well as cysteine (2-thioxothiazolidine-4-carboxylic acid, TTCA).

Van Doorn et al. [2] found the metabolite TTCA in the urine of people who were exposed to carbon disulfide. Less than 6 % of the CS_2 taken up by the body is metabolized to TTCA [3, 4].

Field studies have shown that TTCA excretion is dependent on exposure to carbon disulfide [1, 5]. Taking these field studies into account the BAT value was set at 8 mg TTCA per litre urine.

No TTCA is excreted by persons who are not occupationally exposed to carbon disulfide.

Authors: *A. Eben, F.P. Freudlsperger*
Examiner: *J. Angerer*

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Application	Determination in urine
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Completed in	August 1990

Contents

1 General principles

After acidification and addition of sodium chloride to the urine, 2-thioxothiazolidine-4-carboxylic acid is extracted with diethyl ether. The ether phase is evaporated and the residue is dissolved in methanol. TTCA is analysed by high pressure liquid chromatography.

Detection is achieved with a UV detector at 273 nm.

Calibration is carried out using standard urine solutions containing known TTCA concentrations. The standards are processed in the same way as the assay samples.

2 Equipment, chemicals and solutions

2.1 Equipment

High pressure liquid chromatograph with a UV detector capable of measuring at 273 nm, preferably with autosampler and column thermostat

Integrator

Steel column: length: 25 cm; inner diameter: 4.6 mm

Column packing: Hypersil ODS 5 µm (supplied e.g. by Bischoff, Böblingerstr. 23, D-71229 Leonberg)

LiChrospher 5 µm (e.g. from Merck)

20 µL Syringes for HPLC

15 mL Test tubes (graduated) with ground glass stoppers

Vortex mixer (e.g. Vortex from Cenco, the Netherlands)

10, 20, 100 and 1000 mL Volumetric flasks

10 mL Flasks with tapered necks

Rotary evaporator

Centrifuge

Eppendorf or Pasteur pipettes to remove the organic phase

0.1, 0.2, 0.3, 0.5, 0.8, and 1 mL Pipettes

Universal indicator (e.g. from Merck)

2.2 Chemicals

2-Thioxothiazolidine-4-carboxylic acid (TTCA) (e.g. from Aldrich)

Sodium chloride, p.a.

Hydrochloric acid, 30 %, p.a.

Diethyl ether p.a.

HPLC-grade methanol (e.g. from Baker or LiChrosolv from Merck)

Acetic acid 100%, p.a.

Acetonitrile (e.g. from Baker or LiChrosolv from Merck)

Ultrapure water (ASTM type 1) or double-distilled water

Urine from persons who have not been exposed to carbon disulfide

2.3 Solutions

Mobile phase A:
50% Acetonitrile/50% ultrapure water

Mobile phase B:
0.1 M acetic acid, pH 3.0
About 500 mL ultrapure water are placed in a 1000 mL volumetric flask, 6.0 mL glacial acetic acid are added and the flask is filled to the mark with ultrapure water. The pH value is checked with universal indicator.

2.4 Calibration standards

Starting solution:
The calibration standards are prepared using pooled urine of persons who have not been exposed to carbon disulfide. 20 mg TTCA are weighed exactly and placed in a 100 mL volumetric flask. After filling the flask to the mark with urine it is shaken to ensure that the TTCA dissolves and is homogeneously dispersed (200 mg/L). The solution can be stored in the refrigerator at 4 °C for about 2 months.

Calibration standards with TTCA concentrations up to 20 mg/L urine (cf. Table 1) are prepared by diluting the starting solution with urine from unexposed persons.

Table 1: Pipetting scheme for the preparation of the calibration standards.

Volume of the starting solution mL	Final volume of calibration standard mL	Concentration of calibration standard mg/L
0	10	0 (blank value)
0.1	20	1.0
0.1	10	2.0
0.2	10	4.0
0.3	10	6.0
0.5	10	10.0
0.8	10	16.0
1.0	10	20.0

The calibration standards are freshly prepared every day and processed immediately.

3 Specimen collection and sample preparation

Spontaneous urine specimens are collected in plastic bottles at the end of a working shift. They are acidified with glacial acetic acid (1 mL glacial acetic acid to 100 mL urine). If the specimens are not processed immediately they should be deep frozen. They should not be stored for more than 4 weeks in this state.

5 mL Urine are pipetted into a 15 mL test tube with a ground glass stopper. After saturation with sodium chloride, 0.1 mL of 30 % hydrochloric acid are added. When the sample has been mixed for 1 min on the shaker, 3.5 mL diethyl ether are added and it is mixed again for 1 min. The mixture is subsequently centrifuged at 3000 *g* for 5 min. The ether phase is taken up as completely as possible using an automatic pipette or a Pasteur pipette and transferred to a 10 mL flask with a tapered neck. The solution is evaporated to dryness in a rotary evaporator (at 30 °C). The residue is dissolved in 0.5 mL methanol. This solution is used for the high pressure liquid chromatographic analysis.

4 Operational parameters for HPLC

Column:	Material:	Steel
	Length:	25 cm
	Inner diameter:	4.6 mm
Column packing:	Hypersil ODS; 5 µm	
Separation mode:	Reversed phase	
Detector:	UV detector which is capable of measurement at 273 nm	
Column temperature:	column thermostat 38 °C	
Mobile phase:	A: 50% acetonitrile/50% ultrapure water	
	B: 0.1 M acetic acid, pH 3	
	The eluents must be filtered	
Flow rate:	1.0 mL/min	
Sample volume:	20 µL	

Gradient program:

Time (min)	A(%)	B (%)
0	2	98
8.0	70	30
18.0	70	30
24.0	2	98
37.0	2	98

Figure 2 shows the high pressure liquid chromatograms for a urine sample of a person exposed to carbon disulfide, a spiked urine sample as well as two calibration standards. The retention time for TTCA is approx. 5.3 min.

5 Analytical determination

The operational parameters are set and 20 µL of the methanolic extracts are each injected into the high pressure liquid chromatograph. If the analytical results do not lie within the range of the calibration curve the urine samples should be diluted and reprocessed.

6 Calibration

The calibration standards as well as the unspiked urine used for their preparation (cf. Section 2.4) are processed and analysed as described (cf. Section 2.3). A calibration curve is obtained by plotting the resulting peak areas as a function of the TTCA concentrations added. Under the working conditions described here the calibration curve is linear up to 20 mg/L.

The urine mixture used to prepare the calibration standards does not normally cause a peak in the chromatogram at the retention time of TTCA (cf. Fig. 3). If, however, a peak occurs another pooled urine sample must be used for calibration.

It is not necessary to plot a calibration curve for every analytical series. The validity of the calibration curve can be checked by analysing an aqueous standard solution of known TTCA concentration. This solution functions as an external standard.

7 Calculation of the analytical result

When the peak areas are obtained for the assay samples their TTCA concentration in mg per litre can be read directly from the calibration curve.

8 Standardization and quality control

As described in Section 6, an aqueous solution of known TTCA concentration, e.g. 10 mg/L, is included in each analytical series. The concentrations obtained from the calibration curve can be arithmetically related to this external standard. Control urine containing a constant TTCA concentration is included in each series to test the imprecision for the purpose of internal quality control. This control material is prepared in the laboratory, divided into aliquots and stored in a deep freezer. It is advisable to adjust the TTCA concentration to correspond to the BAT value (8 mg/L; 1993).

9 Reliability of the method

9.1 Precision

To determine the within-series imprecision, pooled urine was divided into two aliquots and spiked with different amounts of TTCA. The urine samples, which contained 1.0 or 5.0 mg/L TTCA per litre urine, were each analysed ten times. The resulting relative standard deviations were 2.6 or 1.7% which correspond to prognostic ranges of 5.9 or 3.8% respectively.

9.2 Accuracy

The accuracy of the method was determined with recovery experiments and checked by means of intercomparison programmes. To find the recovery rate the urine samples were processed and analysed according to the instructions given in Section 9.1. The results were evaluated using calibration standards added to a different pool of urine than the spiked urine samples used in the recovery experiments. The recovery rate was 95 % for a concentration of 1 mg/L and 91 % for a concentration of 5 mg/L. In an intercomparison programm 10 urine samples from exposed persons were analysed in two different laboratories. The results are presented in Table 2.

Table 2: Comparative investigations of urine samples from exposed persons.

Sample	TTCA concentration Laboratory 1 mg/L	 Laboratory 2 mg/L
1	2.90	3.2
2	4.33	7.9
3	3.50	4.2
4	0.98	2.0
5	3.11	3.8
6	3.95	3.3
7	2.54	3.8
8	1.82	3.3
9	< DL	< DL
10	8.47	11.3
Mean value (n = 9) Standard deviation (n = 9)	3.51 2.13	4.76 2.94

DL = detection limit

The values obtained by both laboratories using this method show significant linear correlation ($y = 1.27\ x + 0.28$, $r = 0.932$).

To determine the losses due to sample preparation TTCA solutions prepared in methanol are analysed without further processing. Comparison of the results with those obtained for the processed standard solutions in urine show that only 50 % of the added TTCA is detected after a single extraction. When extraction is carried out three times 85% of the added TTCA is detected. However, a single extraction is preferable for practical reasons. This is justifiable because the calibration standards are processed and analysed in the same manner as the investigated urine samples.

9.3 Detection limit

Under the analytical conditions described here the detection limit is 0.2 mg TTCA per litre urine.

9.4 Sources of error

As shown in the chromatogram in Figure 3, peaks do not generally occur at the characteristic retention time for TTCA when extracts of urine samples from unexposed persons are analysed.

Using acetic acid as an eluent in HPLC can present problems. Long-term use can cause corrosion at the pumps. Therefore, capillaries and pumps must be thoroughly rinsed with ultrapure water after the analysis has been completed. Exhaustive investigations have shown that it is essential to use acetic acid if good separation is to be achieved.

Contamination caused by reagents or glass vessels has not been observed.

10 Discussion of the method

The procedure described here is essentially based on a method developed by *Van Doorn* [2, 8]. At present no other methods for the determination of TTCA are available.

The method is diagnostically specific. No TTCA is found in the urine of persons who have not been exposed to carbon disulfide. Under the conditions for HPLC recommended here TTCA is separated from all the other urinary components, i.e. determination of this metabolite is also analytically specific. Separation is achieved by the eluent program selected in this case; the TTCA retention time is approximately 5 minutes, while most of the components in urine are eluted in 10 to 25 minutes. The sensitivity of this method as well as its detection limit permit the surveillance of the BAT value for TTCA which is 8 mg/L at present (1993). Furthermore, the method is so

practicable that it is suitable for routine biological monitoring investigations. The within-series precision is excellent (1.7–2.6%). Unfortunately no suitable internal standard has been found which can guarantee precision over a longer period. Therefore, it is advisable to include an aqueous control solution of known TTCA concentration in every analytical series instead of an internal standard. Then the results can be appropriately corrected if necessary. The accuracy of the method was checked by means of intercomparison programmes.

Instruments used:
High pressure liquid chromatograph 1090 with diodenarray detector and DPU from Hewlett-Packard
Steel column Hypersil ODS 5 µm
Steel column Nucleosil 120 120-5 C18 from Macherey and Nagel
Precolumn Lichrosorb RP-C18, 5µm from Merck

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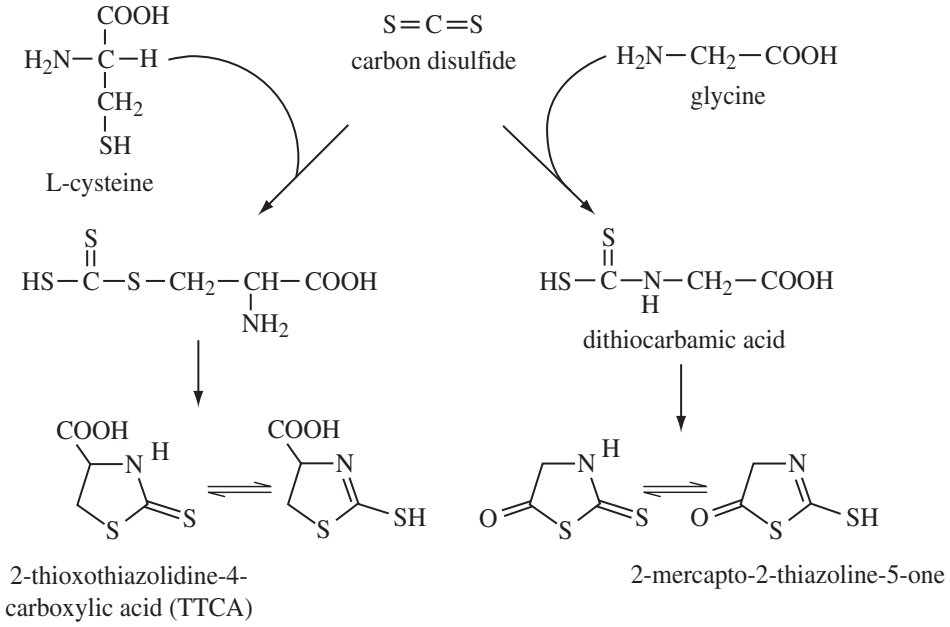

Fig. 1: Metabolism of carbon disulfide.

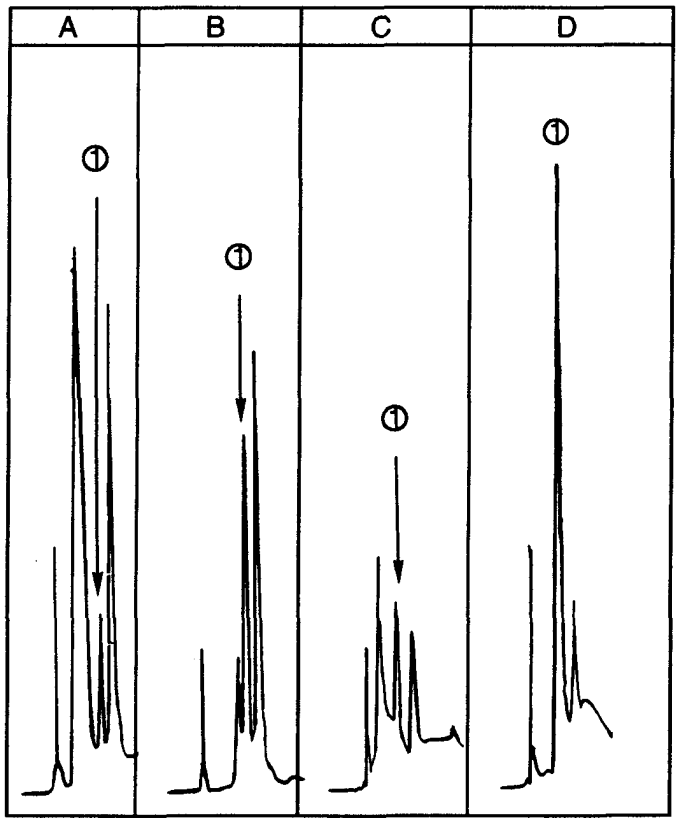

Fig. 2: High pressure liquid chromatograms of:
A Urine extract of a person exposed to CS_2. TTCA 6.1 mg/L urine
B Control urine extract – spiked with 3 mg TTCA
C Calibration standard. 3.4 mg/L urine
D Calibration standard. 14.0 mg/L urine

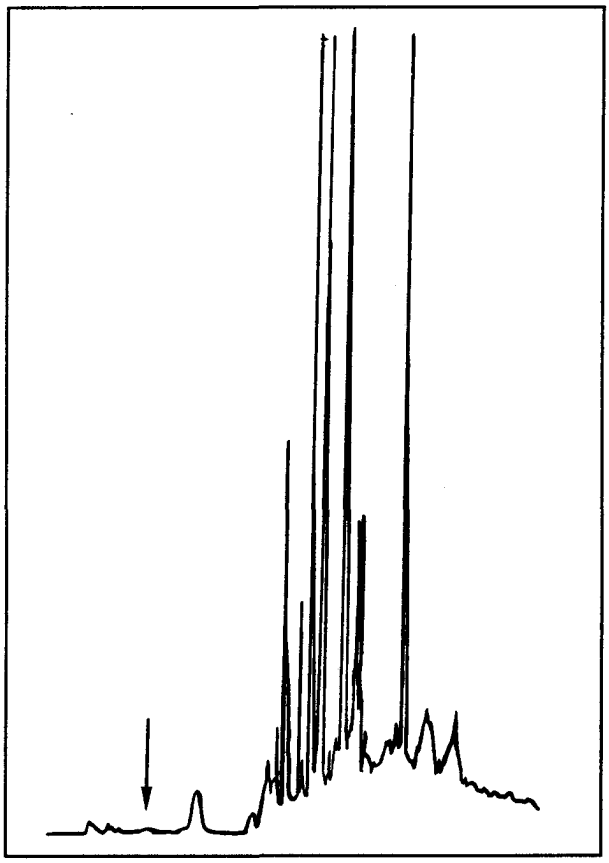

Fig. 3: High pressure liquid chromatograms of urine extract of a person who was not exposed to CS_2.