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Liquid chromatographic determination of urinary 2-thiothiazolidine-4-carboxylic acid, a biomarker of carbon disulphide exposure

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Abstract

An effective gradient high-performance liquid chromatographic method for baseline separation of urinary 2-thiothiazolidine-4-carboxylic acid (TTCA), with photodiode array detection at 271 nm was described. *o*-Methylhippuric acid was used as an internal standard (I.S.). A 1-ml urine sample was saturated with 300 mg of sodium sulphate, acidified with 100 μl of 6 *M* hydrochloric acid, extracted twice with 2 ml of diethyl ether, and after evaporation, the residue was taken up in 1 ml of 0.1% (v/v) phosphoric acid. The two mobile phases used for gradient elution were: (A) 10 m*M* ammonium dihydrogenphosphate (pH 3.5) and (B) same concentration of buffer but containing 20% (v/v) of methanol (pH 4.8). The flow-rate was set at 1.0 ml/min. TTCA and I.S. were detected at 2.2 and 9.1 min, respectively. The method was validated with urine samples collected from normal subjects and workers occupationally exposed to carbon disulphide. The present method enables the detection of urinary TTCA at a concentration of 0.025 mg/l. Analytical recovery and reproducibility generally exceeded 90%. The proposed method is considered more sensitive, specific and reliable than other existing methods.

1. Introduction

Carbon disulphide (CS_2) is a notorious toxic substance causing central and peripheral neurotoxic effects, increased cardiovascular mortality, injury to the sensory organs, and disturbances in reproductive functions [1–2]. Occupational exposure to CS_2 usually occurs from inhalation and skin absorption in the production of rayon, soil disinfectants and electronic vacuum tubes. Due to the serious toxicity of CS_2, determination of its urinary metabolite 2-thiothiazolidine-4-carboxylic acid (TTCA), is being carried out as biological monitoring to assist in the estimation of occupational exposure and for disease prevention. TTCA is not a natural urinary constituent and its excretion in urine is rapid (2–4 h) after CS_2 exposure [3,4].

So far, only a handful of methods is available for the determination of urinary TTCA, using capillary gas chromatography [5] or high-performance liquid chromatography (HPLC) [6–10]. These methods have been applied for biological monitoring of occupational exposure to CS_2, with or without modifications [11–14]. However, these earlier methods were found to

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be non-selective and insensitive for low-level CS_2 exposure. A more specific and sensitive HPLC method for routine screening of TTCA in urine for risk assessment of exposure to low-level CS_2 is therefore required.

In the present study, *o*-methylhippuric acid was used as an internal standard (I.S.) to reduce the variability of the traditional ether extraction method. The efficiency of the extraction technique was also investigated for maximum recovery. Separations of TTCA and I.S. were free from interferences and peak purity was examined by a computerized photodiode array detection system.

2. Experimental

2.1. Reagent and chemicals

TTCA is not available commercially. The standard material was obtained from Dr. Zhaoyang Ma of the Institution of Occupational Medicine, Chinese Academy of Preventive Medicine (Beijing, China), and was synthesized according to the method described by Kopecky and Smejkal [15]. *o*-Methylhippuric acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals and reagents such as HPLC-grade methanol, phosphoric acid, ammonium dihydrogenphosphate, diethyl ether, hydrochloric acid and sodium sulphate were obtained from Merck (Darmstadt, Germany). All solutions were prepared with distilled deionized water.

2.2. Standard preparation

A stock standard of 10 mg/ml of TTCA was prepared by dissolving 100 mg of TTCA in 10 ml of water. A stock solution of 10 mg/ml of *o*-methylhippuric acid (I.S.) was prepared in methanol. I.S. solution of 1 mg/ml was prepared daily by diluting the stock 10-fold with water. The working standards of TTCA with concentrations ranging from 0.025 to 5 mg/l for every day calibration, were all freshly prepared with 0.1% (v/v) phosphoric acid containing 0.1 mg/ml of I.S. These working standards were found to slowly decompose at room temperature. The degradation of TTCA standards of 10–15% at various concentrations, was observed after 3-week storage at 4°C.

2.3. Urine sample

Urine samples were collected from 24 workers from a rayon fiber manufacturing plant in Guangzhou, China. Exposure to CS_2 at the workplace was monitored by using a charcoal tube with a personal sampling pump throughout the whole shift of 8 h. The samplers were detached at the end of the workshift and stored at −4°C until analysis. A gas chromatograph with flame ionization detector was used for determination of CS_2. Urine samples (10 ml) were collected in polycarbonate bottles before the workers entered the plant at the beginning of the shift, and also at the end of the workshift. These workers were exposed to CS_2 with time weighted average (TWA) concentrations ranging from 0.2 to 24 ppm. Urine samples of normal subjects with no known history of CS_2 exposure were obtained from 23 and 26 medical staff members from Singapore and China, respectively. Urine samples were preserved with 1% (v/v) 6 *M* hydrochloric acid immediately after collection and stored frozen until analysis.

2.4. Sample preparation

For sample preparation, a modified organic extraction method comprising an internal standard technique was used. A 1-ml urine sample was introduced into a test tube with 100 μl of I.S. solution (1 mg/ml), approximately 300 mg of sodium sulphate and 100 μl of 6 *M* hydrochloric acid. After vortex-mixing for 30 s, 2 ml of diethyl ether was added. Then a multi-purpose shaker (Harvard/Ltd, UK) with the shaking speed set at 200 oscillations per minute, was used to shake a batch of 20 to 40 samples simultaneously for 5 min. This was followed by centrifugation at 2000 *g* for 5 min at 4°C. The upper ether layer was transferred and capped in a test tube. The organic extraction was repeated

with 2 ml of fresh ether. The two organic extracts were then combined and mixed thoroughly. After centrifugation, 2 ml of this extract was then transferred to a 10-ml glass tube and evaporated to dryness at 40°C. After drying, the residue was resuspended with 1 ml of 0.1% (v/v) phosphoric acid and 10 μl was injected onto the column for HPLC analysis. The results were presented as observed (mg/l) or after correction for urinary creatinine (mg/g creatinine).

2.5. Chromatography

A gradient HPLC system comprised of a Hewlett-Packard 1050 series quaternary pumping system (Palo Alto, CA, USA), a Gilson Model 234 autoinjector (Villers-le-Bel, France) and a Waters Model 996 photodiode array (PDA) detector (Milford, MA, USA) was used. UV scans at peak apex showed absorption maxima at 271 nm for TTCA. The analytical column was a replaceable cartridge (ODS Hypersil, 5 μm, 125 × 4 mm I.D.) protected by a guard cartridge system, and were obtained from Hewlett-Packard.

The two buffer solutions used for gradient separation were 10 m*M* ammonium dihydrogenphosphate. The pH of buffer A was adjusted to 3.5 with phosphoric acid and for buffer B the pH was 4.8 to which was added 20% (v/v) methanol. The analysis started with 90% buffer A and reduced to 40% from 4 to 9 min. It was further changed to 90% of buffer B from 10 to 14 min for column cleaning. The column was re-equilibrated with an initial conditioning for 4 min before the next injection. The flow-rate was set at 1.0 ml/min.

3. Results and discussion

3.1. Efficiency of sample preparation

Although several recent studies have reported on the use of urinary TTCA for biological monitoring of carbon disulphide exposure, very few of them have focused on the accuracy and reliability of the analytical method. It is important to note that chromatographic analyses of organic metabolites in urine are always very complicated, in particular for urine samples collected from cigarette smokers or after coffee consumption. They tend to have more organic metabolites and caused serious interferences. Thus, sample pretreatment plays an important role in urinary chromatographic analysis. An automated column-switching procedure with anion-exchange chromatography using urine samples without extraction has been reported recently [10–16]. Although this technique is very convenient and attractive for routine analysis, it has some disadvantages. Firstly, the complex column-switching procedure is rather impractical for inexperienced chromatographers. Secondly, owing to the dilution factor (at least 5-fold), a rather high detection limit of 0.1 mg/l was reported. It is thus not sensitive enough for the determination of the background levels of urinary TTCA. Thirdly, direct injection of the urine sample could easily damage the column. This problem could be overcome by a clean-up procedure using the solid-phase (C_{18} cartridge) extraction technique prior to HPLC analysis [9,10]; however, due to the poor retention of TTCA in the C_{18} column, it was found not very effective in reducing the complexity of void volume interference as compared with the organic extraction method.

In most of the earlier studies, the organic extraction method was used for sample preparation for urinary TTCA analysis [6–8]. However, it was observed that the recovery of TTCA tend to vary with different extraction conditions. The use of ethyl acetate as an extraction solvent gave a poor recovery of less than 50%, whereas, using diethyl ether, a recovery of about 80% was achieved [6,13]. However, the reproducibility was very poor. A variation of more than 20% was observed in our within-day assay. In fact, it was noted that the use of highly volatile ether often leads to significant variation [17–19].

In order to reduce the variability between sample extractions, the use of an internal standard was found necessary. *o*-Methylhippuric acid is not a natural urinary constituent [20] and due to its capability of full recovery from extraction,

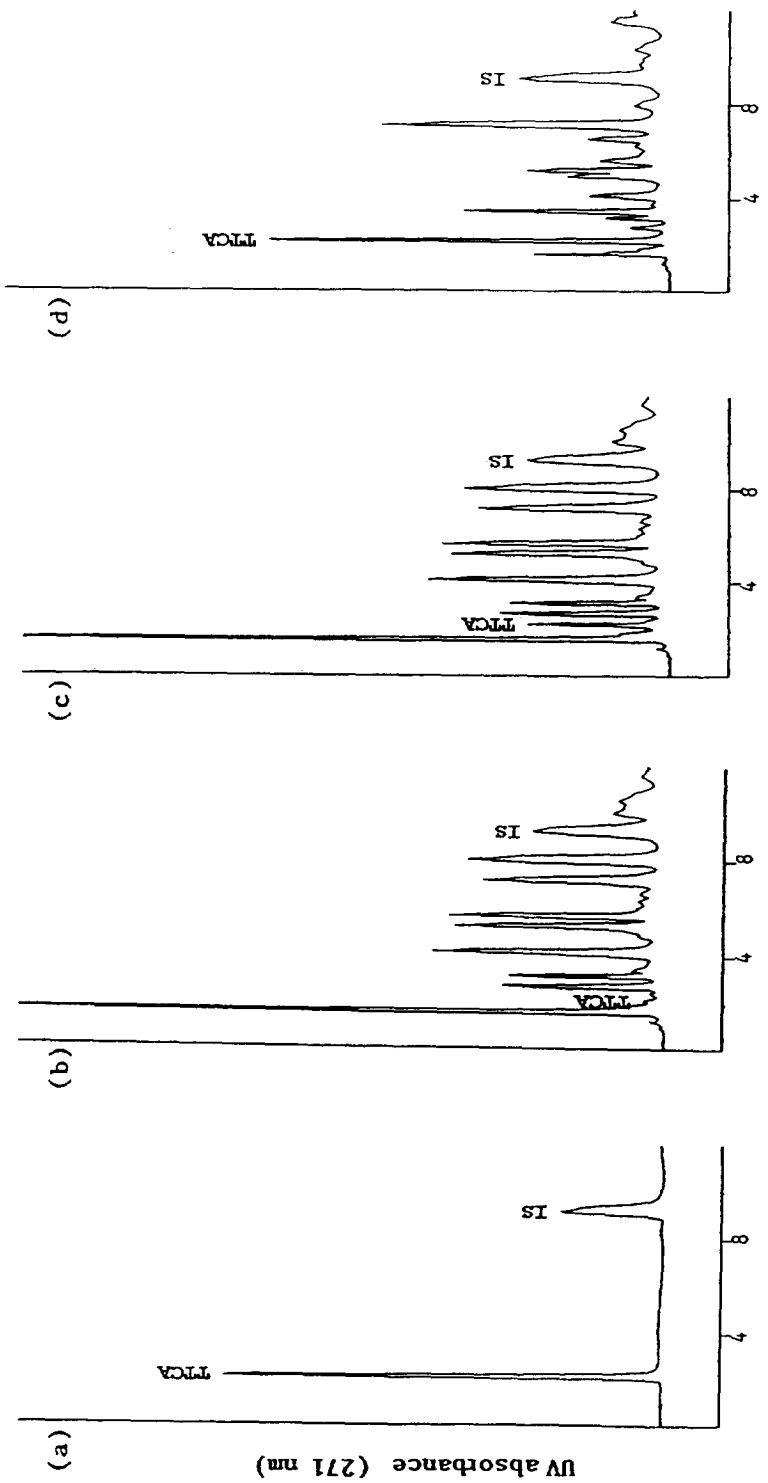

Fig. 1. Chromatograms of (a) an aqueous standard containing 5 mg/l of TTCA and 0.1 mg/l of I.S., (b) a blank urine sample of a normal subject (TTCA, 0.07 mg/l), (c) the same sample spiked with 1 mg/l TTCA and (d) a urine sample of a worker occupationally exposed to CS_2.

it has been successfully used as an internal standard for several other organic acid analyses [18]. The present study has shown that an internal standard was useful for TTCA determination.

It was also found that the efficiency of ether extraction could be affected by other conditions. It was observed that samples acidified with 10% (v/v) 6 *M* hydrochloric acid showed better recovery of TTCA than those treated with a lower concentration (data not shown). The use of sodium sulphate instead of sodium chloride also enhances the recovery. On the other hand, it was noted that TTCA prepared in methanol showed a broader and smaller peak in chromatograms as compared with those prepared in water or diluted acid. The reason for the difference in chromatographic performance of TTCA prepared in different media is not known. Nevertheless, reconstitution of samples in methanol as suggested in earlier reports [6,14] should be avoided.

3.2. Efficiency of chromatographic analysis

The combination of acetic acid and methanol is a common mobile phase used in liquid chromatography [6–8]. However, it was observed that using the above mobile phase, TTCA was not retained in the reversed-phase C_{18} column. This problem was overcome by eluting with ammonium dihydrogenphosphate buffer. However, the pH of the buffer plays a critical role in the separation. The best separation of TTCA from the adjacent peaks was achieved with a pH of 3.4–3.7. Using the above conditions, TTCA in the standard solution (5 mg/l) was detected at 2.2 min and I.S. (0.1 mg/ml) at 9.1 min, as shown in Fig. 1a. The chromatograms of a urine sample collected from a non-exposed subject and the same sample supplemented with 1 mg/l of TTCA are shown in Figs. 1b and 1c, respectively. A urine sample of a worker exposed to 7.9 ppm of CS_2, with TTCA determined at 3.9 mg/l, is shown in Fig. 1d. The reliability of the separation was verified by the PDA spectrum index and purity test, and the results appeared promising. The retention time of the analyte was reproducible with a variation coefficient of less than 5% for day-to-day analysis. The lowest detection limit at a signal-to-noise ratio of 3 was 250 pg of TTCA, and accordingly, a urinary TTCA level as low as 0.025 mg/l could be detected.

3.3. Calibration, recovery and reproducibility

Using the present method, the relationship between peak height and concentration was linear from 0.025 to 5 mg/l of TTCA. The typical regression equation and correlation coefficient (r) were $y = -0.018 + 1.243x$ ($r = 0.99$), where y is the peak height ratio of TTCA vs. I.S. and x is the concentration of TTCA (mg/l). The variation of the linearity and slope of the calibration graphs for between-day analyses were 0.07 and 7.9%, respectively ($n = 3$). Over 90% analytical recovery of TTCA of aqueous standards with various concentrations and spiked samples were obtained (Table 1) and the results were reproducible. The data shown in Table 1 indicate that the variation coefficients of within-day and between-day assays were less than 3% and 7%, respectively ($n = 3$).

3.4. Urinary concentrations of TTCA

A total of 73 urine samples collected from normal subjects and from workers exposed to low concentrations of CS_2 were analyzed by the proposed method. The results for 24 workers, whose urine samples were collected before and after CS_2 exposure, are presented in Fig. 2. The histogram shows a significant increase of TTCA excretion at the end of the workshift. A correlation between increased concentrations of urinary TTCA and CS_2 with $r = 0.606$ ($p < 0.05$) was obtained (Fig. 3). A mean value of 0.75 (range, 0.18–5.16) mg/l of urine or 0.56 (range, 0.06–2.76) mg/g creatine of TTCA was obtained from these workers exposed to an average of about 6.2 ppm of CS_2 during their workshift. TTCA was not detected in 13 of the normal subjects. A mean concentration of 0.08 (range, 0.03–0.18) mg/l of urine or 0.05 (range, 0.005–0.15) mg/g

Table 1
Precision and recovery of the analysis ($n = 3$)

Sample	Mean (mg/l)	Mean recovery (%)	Coefficient of variation (%)	
			Within-day	Between-day
Aqueous standards				
0.5 mg/l	0.45	90	1.4	3.6
1.0 mg/l	0.88	88	1.1	5.2
5.0 mg/l	4.73	95	0.3	3.6
Spiked samples				
Blank	0.07	–	7.9	6.9
+1 mg/l	1.06	99	0.8	2.4
+5 mg/l	4.77	94	2.6	1.4
Mean		93	2.3	3.9

creatinine was obtained from the remaining 36 subjects. The TTCA detected in the urine of non-exposed subjects could be due to dietary influence [11,16].

3.5. Conclusion

There is a growing interest in the use of urinary metabolites of toxicants in environmental

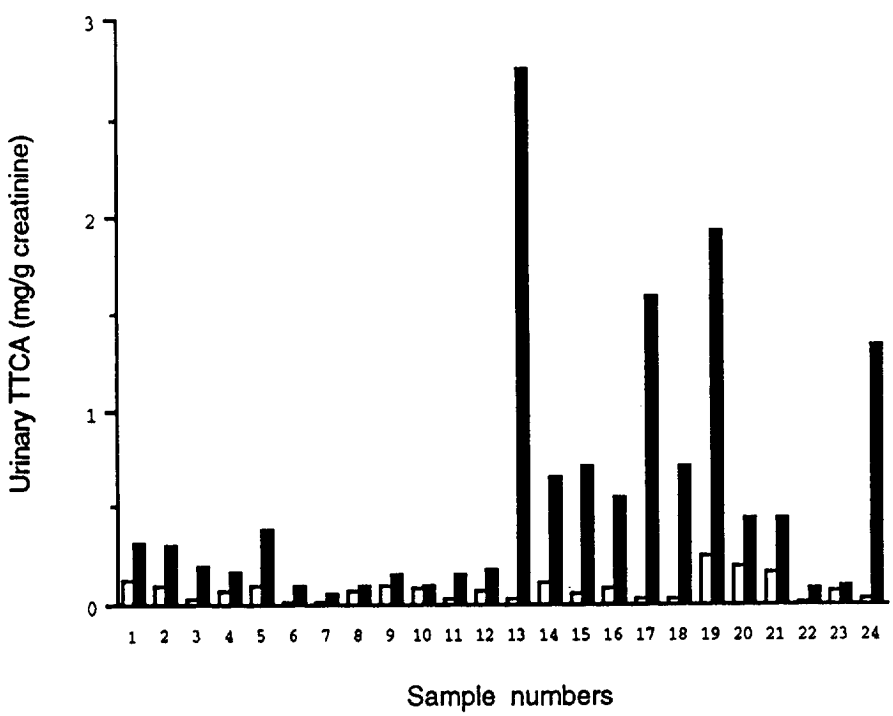

Fig. 2. Urinary TTCA concentration in 24 workers exposed to CS_2. □ = before exposure; ■ = after exposure.

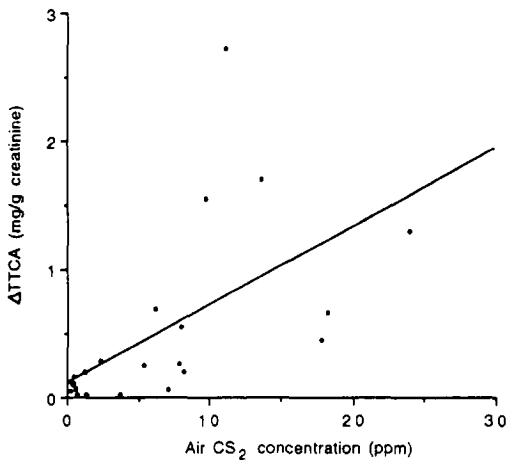

Fig. 3. Correlation between the increase in urinary TTCA concentrations (Δ = difference between after and before exposure) and the CS_2 concentrations in the air. $y = 0.11 + 0.061x$, $r = 0.61$.

and occupational medicine. However, for the assessment of health risk it is always necessary to identify a sensitive and reliable analytical method, for the specific determination of a toxic substance, before they can be used as a biological indicator. Due to the matrix interferences, non-selective chromatographic urinary analyses usually prompt to provide misleading results. The method presented here overcame most of the analytical problems for TTCA analysis. The use of the PDA system confirmed and satisfied our confidence of a clear separation of TTCA. The organic extraction procedure, although not new, was modified for best recovery of TTCA. The application of an I.S. eliminates the variations associated with sample preparation. The whole chromatographic run was only 18 min per injection, including column cleaning and re-equilibration. Using the present method, about 25 samples could be analyzed per day. About 200 samples could be analyzed continuously for 72 h without significant changes in the chromatographic efficiency. Using the proposed method, among the 49 non-exposed subjects 13 were found to have TTCA below the detection limit of 0.025 mg/l. The other 36 samples were detected with a geometric mean value of 0.05 mg/g creatinine. The geometric means of urinary TTCA for 24 workers before and after exposure to CS_2 of a time weighted average concentration (TWA) of 6.2 ppm, were 0.08 and 0.56 mg/g creatinine, respectively. Thus, the proposed HPLC method can be considered as a reliable, sensitive and specific technique for the determination of urinary TTCA.

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