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Simultaneous detection of trichloroethylene alcohol and acetate in rat urine by gas chromatography–mass spectrometry

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Abstract

In order to better understand the cytotoxic effects of trichloroethylene (TCE) and its metabolites in TCE-induced carcinogenicity, it is necessary to determine the molecular species in biological samples. We have developed an efficient gas chromatography–mass spectrometry assay for the quantitative analysis of trichloroethylene alcohol and acetate. This method utilizes a simple esterification procedure, and a single liquid–liquid extraction with hexane–dichloromethane (1:1) that allows >90% recovery of the metabolites, followed by gas chromatography–mass spectrometry. This protocol allows for the accurate, sensitive, and reproducible analysis of the toxic TCE metabolites. The utility of the assay is demonstrated through the analysis of TCE metabolites in urine from rats administered with TCE. The limit of quantitation (precision and accuracy<20%) was 1.7 ng/ml for TCE alcohol and 2.3 ng/ml for TCE acetate.

Keywords: Trichloroethylene

1. Introduction

Metabolic activation of trichloroethylene (TCE) produces toxic metabolites that subsequently lead to TCE-induced carcinogenicity. A major focus in the study of metabolism and disposition of TCE is to identify metabolites that can be evaluated through the various metabolic pathways of TCE. The major metabolites include trichloroethanol and trichloroacetate (Fig. 1). It is important to identify those metabolites that are associated with toxic sequel. Detection of TCE metabolites can play an important role in assessing the pharmacokinetics and pharmacodynamincs of TCE-induced carcinogenicity in humans. Inter-individual variations in absorption and distribution of TCE may play some role in explaining differences in metabolism and susceptibility to toxicity from TCE exposure. The differences in susceptibility are believed due to differences in activities of the various enzymes that are responsible for its metabolic activation and its subsequent toxicity.

Previous methods used to determine TCE metabo-

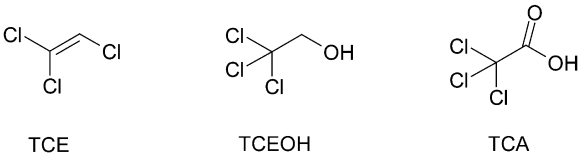

Fig. 1. Molecular structures of the trichlorethylene metabolism: (1) trichloroethanol and (2) TCE acetate.

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lism in biological samples have included indirect chemical analysis protocols and bioassays [1]. A spectrophotometric method for the measurement of the activity of specific enzymes involved in the metabolic activation of TCE was reported. Examples of this type of analysis include determination of cytochrome P450s [2,3]. Another approach provides the basis for potential applications of silica-based materials as adsorbent used for sorptive chromatographic substrates for selective separation of environmental pollutants [4]. The common protocol for the analysis of TCE by high-performance liquid chromatography requires addition of anionic surfactants that are used to enhance the recovery of the compounds from contaminated aquifers prior to extraction [5]. The quantitative limit of the method is 0.05 mg/l for TCE. Both of these approaches suffer from limitations that make their application to the analysis of TCE metabolism in biological samples difficult. Other analytic technique involves mass spectrometry with electron-capture detection for TCE [6–8]. The analysis of organic pollutants such as trichloroethylene is a topic of wide interest. However, the methodologies often require a time-consuming sample pre-treatment. A recent report has described the analysis of TCE metabolism by an EPR/SPIN trapping study [9]. Although this procedure is sensitive, it provides limited information about the individual molecular metabolites of TCE. Other common method for measurement of trichloroethylene and its major metabolites in tissue homogenate in sealed vials by gas chromatography is available [10]. The procedures are cumbersome.

Silymarin is an active component from seeds of the plant *Silybum marianum*. A number of reports have shown that silymarin is an effective anti-inflammatory [11] and anticancer agent [12]. It is also shown to have hepatoprotective functions [13]. However, its biologic mode of actions remains sketchy.

In this study, we report a sensitive and efficient method to detect the toxic metabolites of TCE present in the rat urine using gas chromatography–mass spectrometry (GC–MS) assay. This protocol utilizes a single liquid–liquid extraction step, which allows for high metabolite recovery followed by GC–MS analysis after derivatization of the metabolites.

2. Materials and methods

2.1. Chemicals

Trichloroethylene, trichloroacetic acid, trichloroethanol, *p*-nitrophenol (*p*-NP), β-nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH), 2,6-dichlorophenol-indophenol (DCPIP), uridine 5′-diphosphoglucuronic acid trisodium salt (98%, UDPGA), were purchased from Sigma (St. Louis, MO, USA). Silymarin was provided by the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Other chemicals and solvents were of analytical-reagent grade.

2.2. Animals, dosing and sample collection

Male Sprague–Dawley rats (150–180 g) were obtained from the Laboratory Animal Service Center of the Chinese University of Hong Kong. Rats were housed under controlled conditions (12 h light–dark cycle, 22 °C, 60% humidity) and randomly placed three in each group. They were fed a standard rodent chow and had water ad libitum. Rats were administered orally with silymarin (0.5 mg/kg) for 3 days, followed by TCE (2.4 g/kg) for 2 days. Urinary samples were collected at 24 and 48 h after TCE administration. On the sixth day, rats were sacrificed. The rat urine was collected for analysis.

2.3. Analysis of the metabolites of TCE in rat urine

GC–MS analysis was conducted to determine the metabolites of TCE in rat urine. The GC–MS system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP6890 gas chromatograph, a 5973 series mass-selective detector, a HP 6890 auto-injector, a G1512A auto sampler controller and a Vectra XM series 4 5/166 computer using HP G1701AA MS ChemStation software (Version A.01.00). The capillary column was a HP-5 5% phenylmethyl siloxane (30 m×0.25 mm I.D.) with 0.50 μm film thickness. The carrier gas was set at a flow-rate of 1.0 ml/min and a head pressure of 13.8 p.s.i. on the column

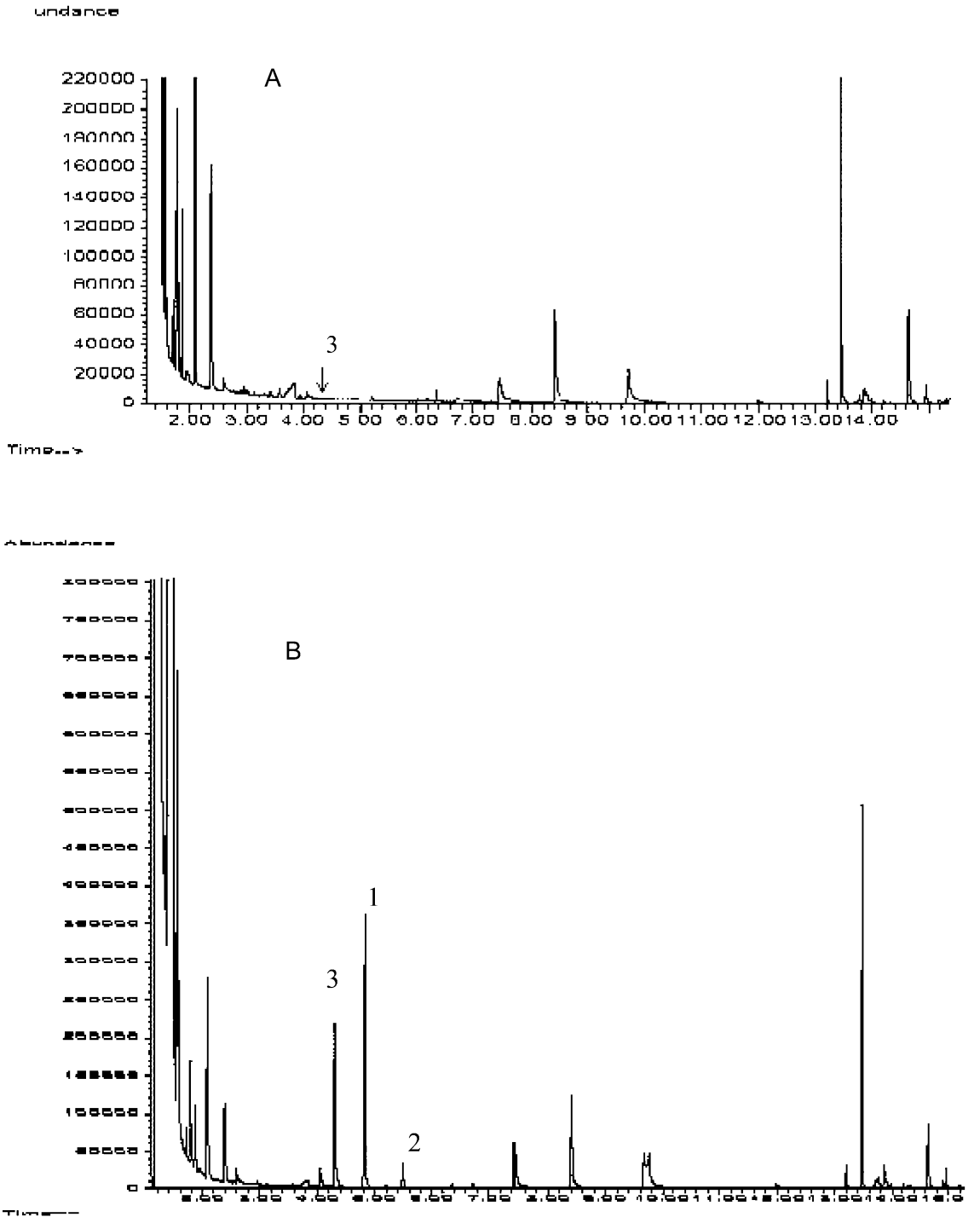

Fig. 2. Chromatogram of TCE metabolites in the rat urine: (A) control, (B) rat after TCE treatment. (1) TCEOH, (2) TCA, (3) dichloroacetic acid as an internal standard.

(1 p.s.i.=6894.76 Pa). A 1-μl volume of the sample in hexane–dichloromethane (1:1) was injected using the splitless mode. The temperatures of the injection port and mass selective detector interface were set at 150 and 280 °C, respectively. The temperature gradient of the GC oven was programmed to start at 60 °C for 4 min, raised to 200 °C at 50 °C/min and held at the final temperature for 2 min. An electron ionization (EI) mode with 70 eV was used. Perfluorotributylamine was used for the calibration of the MS detector.

The derivatization reaction was done according to the published method with modifications [10]. Urine sample (200 μl) and internal standard (dichloroacetic acid, 200 m*M* in methanol, 5 μl) were mixed with 500 μl of water–0.1 *M* sulfuric acid–methanol (6:5:1) in tightly-capped glass tube. The mixture was heated at 70 °C for 10 min. After cooling to room temperature, 500 μl of hexane–dichloromethane (1:1) was added to the mixture and vortexed for 10 min before centrifugation at 600 *g* for 5 min. The organic layer was used for GC–MS analysis. Chemical structures of the metabolites of TCE were identified using a full mass spectra scan mode in comparison with the authentic compounds.

3. Results

Of the various biological matrices, urine was chosen as the investigative medium as most metabolites are present in the urine. A chromatogram showing the profile of the bio-transformation products produced from the parent compound is shown in Fig. 2. Rats were administered TCE, and the rat urines were collected for analysis.

The GC chromatogram (Fig. 2) revealed two major metabolites (1 and 2) and the internal standard (3). The two major components 1 and 2 constituted 80% of the parent compounds in urine. The detection limits of the metabolites in rat urine collected from different experiments are shown in Table 1.

Identification of metabolites was made from GC–MS analyses of the authentic standards of the metabolites and the mass spectra (Fig. 3). Retention time of metabolites was compared with that of the standards. Control (rats with no drug treatment) urine samples collected from rats were also subjected to the same workup protocols and GC–MS analysis. The clue to the presence of these metabolites 1 and 2 came from the application of the protocol described in this study. The technique is capable of analyzing hydroxy and acid products of TCE. Following this method, the rat urine sample was first subjected to liquid–liquid extraction at ambient temperature, during which the major metabolites of TCE present in urine underwent phase transfer, yielding two major metabolites and the internal standard. The recovery of these metabolites was shown in Table 1. The two metabolites were the major products from TCE metabolism. Treatment of rats with silymarin, as an application study, changed the metabolite profile (Fig. 4). Other metabolites of TCE were not detected. Both TCEOH and TCA were observed in the GC and confirmed by MS. To distinguish the two metabolites and eliminate matrix related variation in retention times, co-elution experiments were carried out. No other metabolite derivatives co-eluted with the major metabolites. This result clearly established the presence of the metabolites 1 and 2. The GC profile did not carry compounds that bear similar mass fragmentation patterns. This suggests that there are no other detectable metabolites or structural isomers. The authentic standards were added to evaluate the concentration of the metabolites and facilitate the recovery study. The metabolites from the rat urine matched both in retention time and mass spectral characteristics to the authentic metabolites. The sensitivity of the GC–MS method is reflected in the limit of detection (LOD) values for the two metabolites (Table 1). Over the concentration range of interest from 3 to 40 μ*M*, the calibration curve

Table 1
Chromatographic characteristics of the GC–MS assay

Compound	t_R (min)±RSD (%)	LOD[a] (pg)	LOQ[b] (ng/ml)
TCEOH	4.67±0.2	3.4	1.7
TCA	5.34±0.5	4.6	2.3

Data represent the mean±RSD from replicate experiments (n=3).

[a] LOD, Limit of detection at a signal-to-noise ratio of 3.

[b] LOQ, Limit of quantitation at a precision and accuracy of <20%.

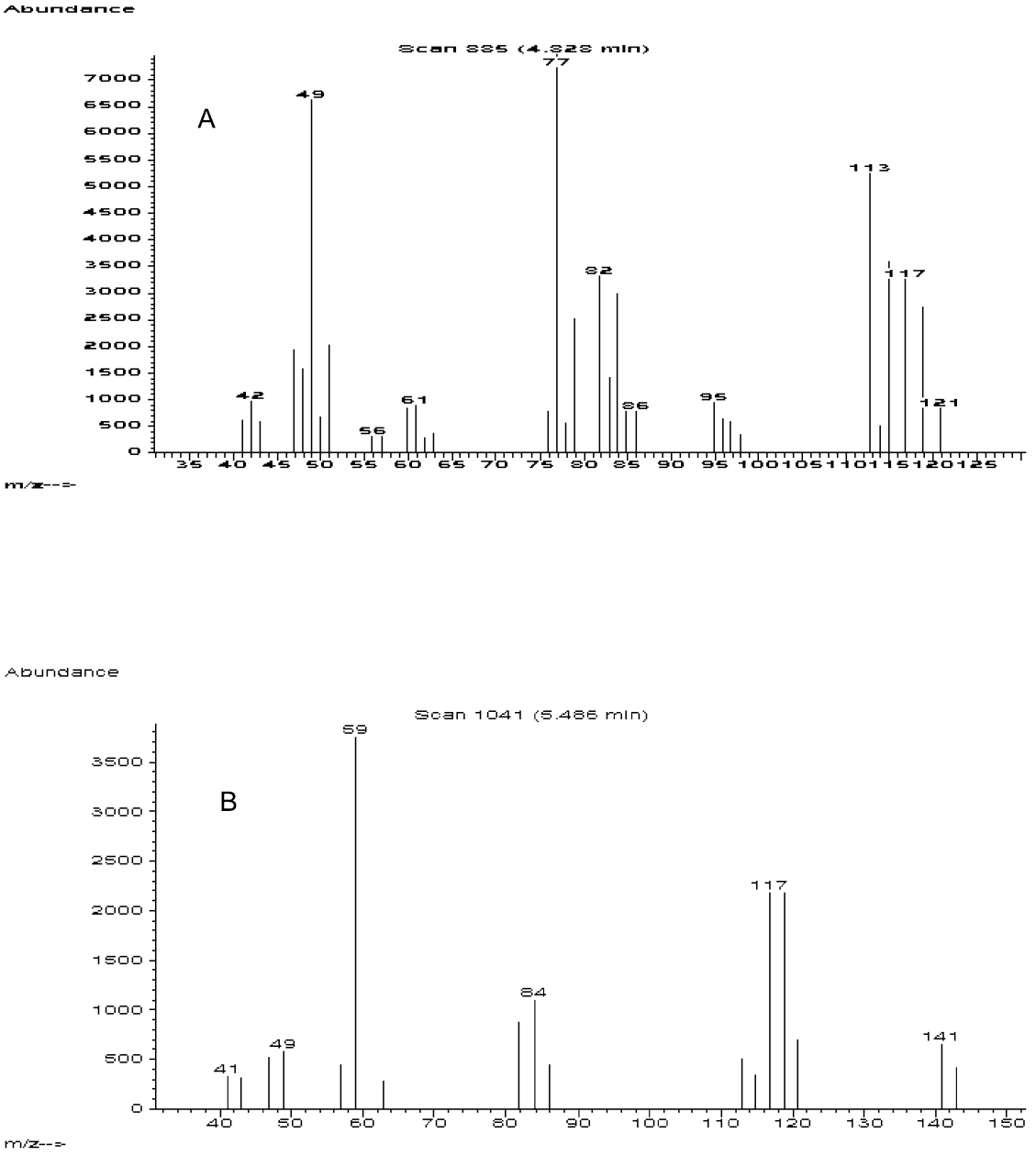

Fig. 3. The mass spectra of (1) TCEOH and (2) TCA identified in the rat urine from Fig. 2.

were linear with coefficient of linearity close to unity (0.99) for the two metabolites. The recovery study showed no significant loss of the metabolites occurred (Table 2).

4. Discussion

TCE is known to produce metabolites through different enzymatic pathways; thus, the types of

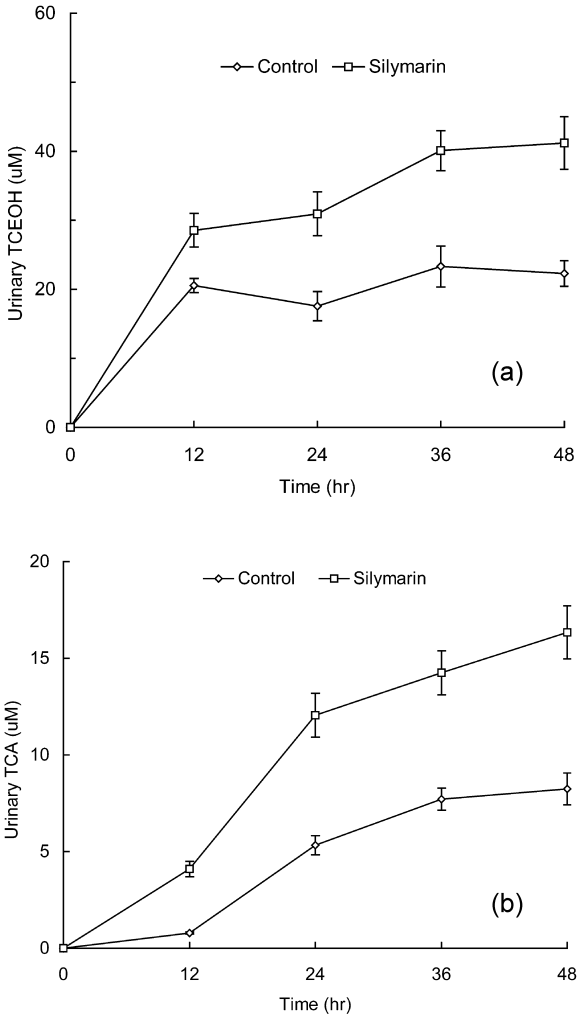

Fig. 4. Time–course study of (A) TCEOH and (B) TCA in the rat after treatment with silymarin. At various different time points the urinary TCEOH and TCA were quantitated by GC–MS as described in Materials and methods. The control urine was obtained from rats with TCE treatment. The data represent the mean of replicates (n=3).

metabolites suggest what enzymes could be involved in the metabolic activation of TCE. There is no report on the analysis of TCE metabolism in urine. The procedures normally used in identification of metabolites in biological matrices may not be sensitive enough for urine sample due to significant differences in chemical properties between the two. A milder workup procedure can be used for urine sample. Concentrated acid was avoided to minimize

Table 2
Recovery of trichloroethanol and trichloroacetate from rat urine after liquid–liquid extraction

Concentration (ng/ml)	Between day[a]	
	TCEOH	TCA
1.25	1.29±0.4	1.46±0.04
7.5	1.33±0.06	1.47±0.07
15	1.31±0.05	1.41±0.08

[a] Data represent the mean±standard deviation from replicates (n=3).

chemical rearrangements of the metabolites. These complications are especially pronounced if the metabolites are polar and contain hydroxylated groups [14]. In addition, background levels of TCE species in solvents used for extraction, or derivatization reagents, may also contribute to interferences. Adoption of the phenylmethyl siloxane column facilitated the use of the temperature programme while achieving high separation efficiency of TCEOH and the acetate without peak broadening as often observed with hydroxy groups [15,16]. The results are shown in Fig. 2 and Table 1. This enabled the chromatography to be linked up with mass spectrometry and to allow the validation of the chemical identity of chromatographic peaks (Table 2).

In conclusion, the major metabolites of TCE in rat urine have been identified. The methods used were quite sensitive, and minimize ambiguities in identification of metabolites. Simple procedures have been developed for extraction and derivatization of the metabolites in rat urine. This methodology will greatly facilitate the pharmacokinetic study TCE metabolism. The major metabolites identified in this study have also been clearly established. The enzymatic pathways for the formation of the metabolites can be understood through the identification of the type of metabolites in rat urine. Major pathways for their generation can be characterized. In summary, an efficient sample preparation protocol is reported for the determination of the major TCE metabolites in urine. A simple GC–MS method is also reported that can be applied to achieve high efficiency separation and identification of TCEOH and TCA. The method achieves quantitative recovery of the metabolites from urine. Good recovery and sensitivity of the method enabled the limit of quantitation to be set under 2 ng/ml for the compounds.

Acknowledgements

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