Journal of Chromatography, 442 (1988) 325–332
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 394

DETERMINATION OF TRICHLOROETHYLENE METABOLITES IN RAT LIVER HOMOGENATE USING HEADSPACE GAS CHROMATOGRAPHY

BENNY KØPPEN*,* and LARS DALGAARD
Department of Chemistry BC, Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)
and
JYTTE MOLIN CHRISTENSEN
Danish National Institute of Occupational Health, 73 Baunegaardsvej, DK-2900 Hellerup (Denmark)
(Received October 28th, 1987)

SUMMARY

An headspace gas chromatographic (HSGC) method for determination of trichloroethylene metabolites in rat liver homogenates is described. These metabolites are chloral hydrate (CH), trichloroethanol (TCE), trichloroacetic acid (TCA) and the glucuronic acid conjugate of trichloroethanol (TCE-β-glucuronide). The method is based on selective thermal conversion of CH and TCA into chloroform, which is determined together with trichloroethanol by HSGC using electron-capture detection. TCE-β-glucuronide was determined as the difference between free TCE and total TCE after enzymatic hydrolysis with β-glucuronidase. Synthesized TCE-β-glucuronide was used to compare the efficiency of enzymatic and acid hydrolysis of the conjugate. Enzymatic hydrolysis was found to be advantageous for determination of TCE-β-glucuronide.

INTRODUCTION

Trichloroethylene (TRI) is an organic solvent widely used in industry. Various disorders, including hepatotoxicity[1], have been related to exposure to TRI. Biotransformation of TRI leads to the formation of a number of metabolites, which have been identified by several investigators[2,3]. The main metabolites are chloral hydrate (CH), trichloroacetic acid (TCA) and trichloroethanol (TCE). TCE is partly conjugated to glucuronic acid.

These metabolites in liver tissue homogenates have been determined by a spectrophotometric method[4] based on the Fujiwara reaction. Similar spectrophotometric methods for the determination of TRI metabolites in urine and blood have been replaced by gas chromatographic (GC) methods[5–10] because of the poor sensitivity

* Present address: The State Chemical Supervision Service, 26 Mørkhøj Bygade, DK-2860 Søborg, Denmark.

0021-9673/88/$03.50

and selectivity of the former, but no GC method has been reported for the determination of these metabolites in liver tissue homogenates. The GC methods are based on either solvent extraction of the sample[5–7] or direct equilibrium headspace analysis[8–10]. The simplicity of headspace analysis has made it attractive compared to the other GC methods, which involve extraction of the rather polar metabolites into an organic solvent.

We now report an headspace gas chromatographic (HSGC) method for determination of these metabolites in rat liver homogenate. The analytical procedure involves selective decarboxylation of CH and TCA to chloroform followed by HSGC determination of chloroform and TCE using electron-capture detection.

MATERIALS AND METHODS

TCE (Fluka, Buchs, Switzerland) was dried over calcium hydride and distilled before use. TRI (Fluka) was purified by distillation before use. TCA (Merck, Darmstadt, F.R.G.), CH (Fluka), 1,1,2-trichloroethane (Fluka) and sodium dodecyl sulphate (SDS) from Serva (Heidelberg, F.R.G.) were of the highest purity available and were used as received. All other chemicals were of analytical grade and were used as received.

TCE-β-glucuronide was synthesized in this laboratory as described below. When stored desiccated at room temperature the salt was stable for at least 4 months. β-Glucuronidase (*Helix pomatia*, type H-2) was from Sigma (St. Louis, MO, U.S.A.). The crude enzyme solution contained *ca.* 30 U/ml (1 U hydrolyses 1 μmol substrate per min). The activity was assayed under standard conditions (37°C, pH 4.5) using *p*-nitrophenyl β-D-glucuronide as a substrate.

Nuclear magnetic resonance (NMR) spectra were obtained on a Jeol FX90Q spectrometer.

Preparation of ammonium 2,2,2-trichloroethyl-β-D-glucopyranosiduronate (TCE-β-glucuronide)

Methyl 2,2,2-trichloroethyl-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (I) was prepared according to Magnusson *et al.*[11], m.p. 159–160°C (160–161.5°C[11]). Compound I (500 mg, 1.1 mmol) was dissolved in 10 ml methanol, 2.0 ml 4 *M* potassium hydroxide were added and stirred at room temperature overnight. Methanol was evaporated *in vacuo* and after the addition of 10 ml water the solution was neutralized with Amberlite IRC-50 cation-exchange resin (H^+). The neutral solution was freeze-dried. The residue was dissolved in 2.0 ml water and applied to a column (25 cm × 1 cm) packed with Whatman DE-32 anion-exchange cellulose equilibrated with 0.05 *M* ammonium formate, pH 3.20. After elution with 0.15 *M* ammonium formate, pH 3.20, the fractions (5 ml) containing TCE-β-glucuronide (TLC) were pooled and freeze-dried. Dissolution of the residue in a few ml water and freeze-drying to constant weight left 220 mg (60%) of hygroscopic TCE-β-glucuronide, m.p. 155–157°C (decomp.). ^{1}H NMR [90 MHz, $(C^2H_3)_2SO$]: δ 3.06–3.29 (4H, m, CHOH), 4.24, 4.42 (2H, 2d, $CH–CCl_3$, J = 11.7 Hz), 4.47 (1H, d, anomeric proton, J = 7.1 Hz), 4–6 (broad, ^{2}HOH). Found: C 27.07, H 4.69, N 3.85, Cl 28.73; calc. for anhydrous $C_8H_{14}NCl_3O_7$: C 28.05, H 4.12, N 4.09, Cl 31.05%.

The purity of the TCE-β-glucuronide was estimated from the chloride content (28.73%) found by elemental analysis, which corresponds to a purity of 92.5%.

Preparation of liver homogenates

Male Sprague-Dawley rats (150–200 g) were treated with phenobarbital (0.1% in drinking-water) for 4 days and were killed by decapitation the following day. A 10% (w/v) crude homogenate of the liver in 1.15% potassium chloride–0.01 *M* phosphate buffer, pH 7.4, was prepared (4°C) using an Ultra-Turrax homogenizer. The crude homogenate was centrifuged (4°C) at 10 000 *g* for 10 min, and the supernatant was used as an enzyme source for the liver homogenate incubations.

Incubation

Incubation was performed in a glass-stoppered vessel placed in a thermostatted water-bath at 37°C. A 10.0-ml volume of liver homogenate was incubated for 10 min with 20.0 ml cofactor solution containing 6 μmol reduced nicotinamide-adenine dinucleotide phosphate (NADPH), 30 μmol glucose-6-phosphate, 25 μmol magnesium chloride, 15 μmol uridine diphosphoglucuronic acid and 30 μmol reduced nicotinamide-adenine dinugleotide (NADH). Metabolism was initiated by adding 2.0 ml aqueous solution containing TRI (12.6 m*M*) and 6% ethanol.

Determination of metabolites

Free TCE and CH. Metabolism was terminated by adding 500 μl sample to 500 μl 2% SDS. A 500-μl volume of this mixture was added to 1000 μl 0.2 *M* phosphate buffer, pH 8.0, and 200 μl 2% SDS in a 5-ml glass vial. After addition of 200 μl 1,1,2-trichloroethane (1.15 μg/ml) as an internal standard (I.S.), the vial was closed with a PTFE-lined rubber septum and incubated at 60°C for 90 min before HSGC analysis.

Total TCE and TCA. A 500-μl volume of the sample was added to 500 μl solution containing β-glucuronidase (0.3 U/ml) and 25 μmol formaldehyde in 0.02 *M* acetate buffer, pH 4.5. The mixture was incubated for additional 2 h at 37°C. A 500-μl volume of this incubation mixture was added to 1000 μl saturated ammonium sulphate solution and 200 μl conc. sulphuric acid in 5-ml glass vials. After addition of 200 μl I.S., the vial was closed with a PTFE-lined rubber septum and incubated at 90°C for 90 min before HSGC analysis.

Gas chromatography

The instrumentation and conditions for GC were as described previously[12].

RESULTS AND DISCUSSION

The determination of TCA, in biological materials, as chloroform formed upon thermal decarboxylation has been described by several authors[9,13–15]. Preliminary experiments with rat liver homogenates spiked with TCA showed that the rate of decarboxylation was practically independent of the pH of the sample. However, the decarboxylation rate was temperature dependent and to achieve complete conversion into chloroform an incubation time of 90 min at 90°C was necessary, in accord with the findings of Herbolsheimer and Funk[14]. In strongly acidic solution (pH < 1) some loss of chloroform occurred. In spite of that, determination of TCA in the presence of strong acid was chosen, because the percentage conversion of TCA into chloroform was reproducible under the chosen conditions as reported by Christensen *et*

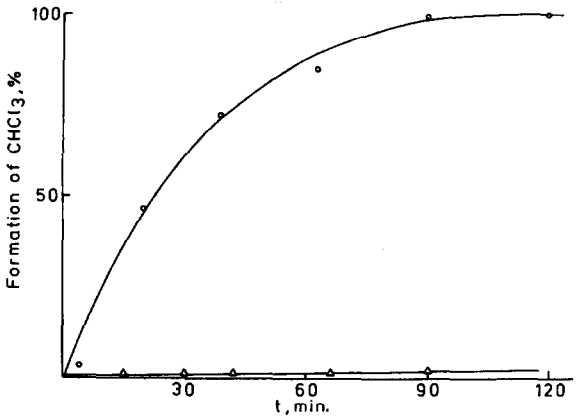

Fig. 1. Conversion of trichloroacetic acid (TCA) and chloral hydrate (CH) into chloroform. The formation of chloroform was measured by HSGC following incubation of 1.0 μg/ml TCA (△) and CH (○) in 0.2 *M* phosphate buffer, pH 8.0, at 60°C. Mean values of duplicates.

al.[12], and the strong acid was necessary to inhibit conversion of CH into chloroform as discussed later.

The effect of various parameters on the rate of decarboxylation of CH was examined. Preliminary experiments showed that the rate increased with increasing incubation temperature and with increasing pH. Increasing the temperature also increases the interference from TCA present in the sample. Fig. 1 shows the rates of decarboxylation of CH and TCA at 60°C (pH 8.0), which was chosen as the optimum temperature for the determination of CH. While achieving complete conversion of CH into chloroform during 90 min of incubation, 2.5% of the TCA present is decarboxylated. Thus interference from TCA becomes significant only when the samples contain large amounts of TCA compared to the amount of CH.

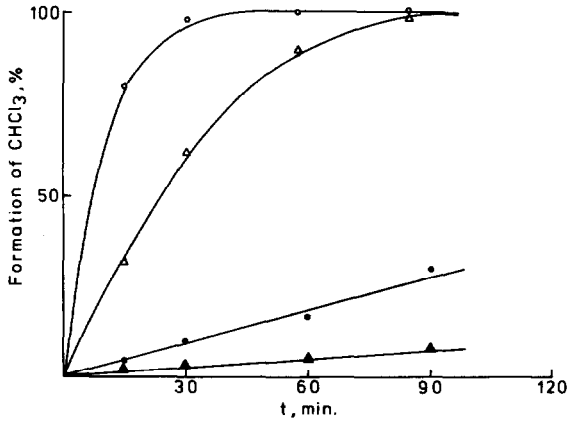

Fig. 2. Effect of pH on the conversion of chloral hydrate (CH) into chloroform. The formation of chloroform was measured by HSGC following incubation of 1.0 μg/ml CH at 60°C in 0.2 *M* phosphate buffer at pH 6.0 (▲), 7.0 (●), 8.0 (△) and 9.0 (○). Mean values of duplicates.

The effect of pH on the decarboxylation rate of CH was investigated and the results are shown in Fig. 2. Complete conversion of CH into chloroform at 60°C was achieved within 90 min at pH 8.0. Increasing the pH to 9.0 further reduces the incubation time needed for complete conversion. The time needed for TCE to reach equilibrium in the headspace at 60°C was about 35 min, while equilibration for chloroform was more rapid (< 10 min). The incubation time for the simultaneous determination of TCE and CH must therefore be at least 35 min. For practical reasons (automatic sampling), it was convenient to have the same incubation time throughout the analysis, and 90 min was therefore chosen for both the determination at 60°C and that at 90°C. A pH of 8.0 was then chosen for the determination of CH and TCE. Chloroform is unstable in alkaline solution, but no measurable loss of chloroform, TCE or the internal standard occurred upon incubation at pH 8.0 (60°C) for 90 min.

Increasing the ionic strength in the incubation solution by addition of a salt is known to increase the sensitivity of headspace analysis by "salting out" the volatiles. Saturated ammonium sulphate was used to increase the headspace concentration of chloroform and TCE[12], and we used that procedure for determination of TCA and total TCE in liver homogenate. Increasing the headspace concentration of the volatiles by addition of ammonium sulphate or sodium chloride was also attempted in the determination of free TCE and CH, but an high ionic strength was found to reduce the rate of conversion of CH into chloroform. Fig. 3 shows the conversion of CH into chloroform at 60°C (pH 8.0) where different concentrations of sodium chloride were added during the incubation. The determination of free TCE and CH was therefore performed without addition of any "salting out" agent.

TCE-β-glucuronide is usually determined as the amount of TCE liberated upon hydrolysis of the conjugate. For liver tissue homogenate, acid hydrolysis has been used[4], but for blood and urine hydrolysis was performed either using the enzyme β-glucuronidase from various sources[5,7,9] or strongly acidic conditions[6,10,16]. Eval-

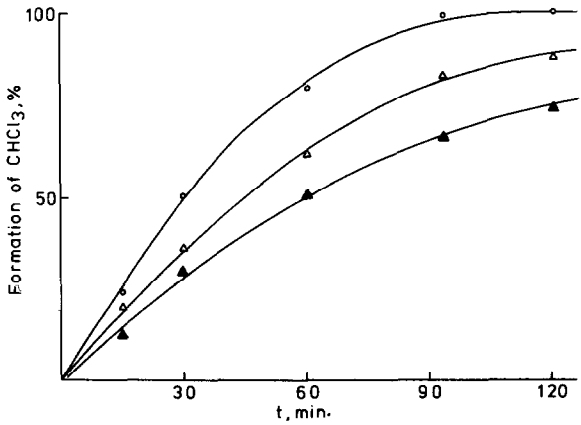

Fig. 3. Effect of ionic strength on the conversion of chloral hydrate (CH) into chloroform. The formation of chloroform was measured by HSGC following incubation of 1.0 μg/ml CH at 60°C, pH 8.0, in 0.2 *M* phosphate buffer and 0.1 (○), 0.5 (△) and 2.0 *M* (▲) sodium chloride. Mean values of duplicates.

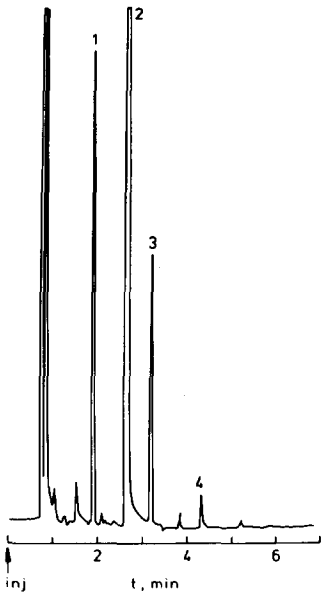

Fig. 4. Typical chromatogram from HSGC analysis of a rat liver incubation to which was added trichloroethylene (0.8 m*M*). Peaks: 1 = chloroform; 2 = trichloroethylene; 3 = internal standard and 4 = trichloroethanol.

uation of the optimum hydrolysis conditions was difficult, since TCE-β-glucuronide was not available for preparation of standards.

To the best of our knowledge, no comparison has been made between enzymatic and acid hydrolysis of TCE-β-glucuronide in liver homogenates. Using synthesized TCE-β-glucuronide, we have compared the efficiency of two different hydrolysis procedures. A liver homogenate spiked with TCE-β-glucuronide (10 μg/ml) was treated with either acid or enzyme: incubation for 90 min at 90°C in the presence of 2 *M* sulphuric acid, or 120 min at 37°C (pH 4.5) with added β-glucuronidase (*H. pomatia*). 12.0% of TCE-β-glucuronide was hydrolysed during treatment with the acid, while 96.4% was hydrolysed by β-glucuronidase under these conditions. This incomplete acidic hydrolysis in in accord with the results of Sedivec and Flek[16], who investigated conditions for the acidic hydrolysis of TCE-β-glucuronide in urine samples. They found that maximum hydrolysis was achieved using 6–7.5 *M* sulphuric acid, but were not able to calculate the efficiency due to the lack of TCE-β-glucuronide for preparing standards. Since, in our procedure, TCE-β-glucuronide and TCA are determined simultaneously, and we found that strongly acidic conditions resulted in less efficient conversion of TCA into chloroform, no attempts were made to investigate the efficiency of TCE-β-glucuronide hydrolysis using 6–7.5 *M* sulphuric acid. Enzymatic hydrolysis of TCE-β-glucuronide using β-glucuronidase was chosen, since it was efficient and could be performed under mild conditions.

To samples that were incubated with β-glucuronidase before HSGC analysis was also added 25 m*M* formaldehyde, which was found to inhibit enzymatic reduction of CH during incubation. With other samples, metabolism was terminated by adding 2% SDS, which was found to denature enzymes immediately.

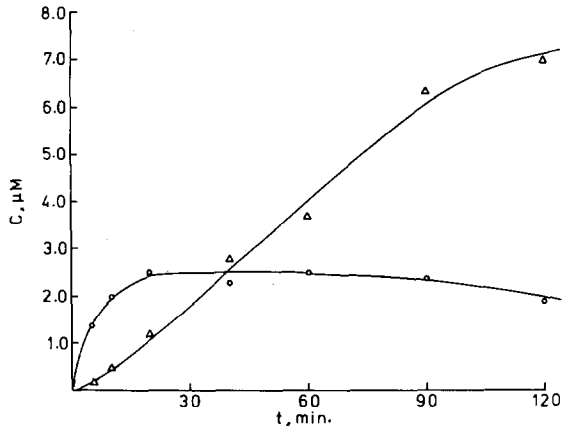

Fig. 5. Time course of trichloroethylene (TRI) metabolite formation in rat liver homogenate incubation. Metabolism was initiated by the addition of 0.8 m*M* TRI. The formation of chloral hydrate (○) and trichloroethanol (△) was measured by HSGC as described in the text. Mean values of duplicates.

Linearity, detection limits and precision

Calibration graphs obtained from spiked samples of liver homogenates were found to be linear over the concentration range 0–1000 ng/ml. The detection limits were 0.06 μg/ml for TCE and TCE-β-glucuronide and 0.02 μg/ml for CH and TCA. The precision of replicate measurements (R.S.D., six determinations) was 5–7% for all four metabolites.

Application

This method has been applied to the determination of TRI metabolites in incubations containing rat liver homogenate. A typical chromatogram from a rat liver incubation to which TRI (0.8 m*M*) had been added is shown in Fig. 4.

The formation of metabolites during incubation is shown in Fig. 5. Only CH and free TCE were formed under these conditions. As expected, CH was the primary metabolite, appearing within 5 min. TCE-β-glucuronide and TCA could not be detected, but no efforts were made to optimize conditions for their formation, *e.g.* incubation of endogenous β-glucuronidase, which probably is necessary to ensure formation of these metabolic end-products.

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