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Analysis of methanol or formic acid in body fluids by headspace solid-phase microextraction and capillary gas chromatography

Xiao-Pen Lee[a], Takeshi Kumazawa[a,*], Kei Kondo[a], Keizo Sato[a], Osamu Suzuki[b]

[a]*Department of Legal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*
[b]*Department of Legal Medicine, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-3192, Japan*

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Abstract

Methanol and its metabolite formic acid have been found extractable from human whole blood and urine by headspace solid-phase microextraction (SPME) with a Carboxen/polydimethylsiloxane fiber. The headspace SPME for formic acid was carried out after derivatization to methyl formate under acidic conditions. The determinations of both compounds were made by using acetonitrile as internal standard (IS) and capillary gas chromatography (GC) with flame ionization detection. The headspace SPME–GC gave sharp peaks for methanol, methyl formate and I.S.; and low background noises for whole blood and urine samples. Extraction efficiencies were 0.25–1.05% of methanol and 0.38–0.84% formic acid for whole blood and urine. The calibration curves for methanol and formic acid showed excellent linearity in the range of 1.56 to 800 and 1.56 to 500 μg/0.5 ml of whole blood or urine, respectively. The detection limits were 0.1–0.5 μg/0.5 ml for methanol and 0.6 μg/0.5 ml for formic acid for both body fluids. The within-day relative standard deviations in terms of extraction efficiency for both compounds in whole blood and urine samples were not greater than 9.8%. By using the established SPME method, methanol and formic acid were successfully separated and determined in rat blood after oral administration of methanol.

Keywords: Methanol; Formic acid

1. Introduction

Methanol is one of the most popular solvents and available as a constituent of some antifreeze solutions, various paints, varnishes, gasoline additives and ethanol denaturants. Methanol is metabolized in vivo to formaldehyde by alcohol dehydrogenase and to formic acid by aldehyde dehydrogenase; and formic acid plays the major role for methanol poisoning symptoms in human [1,2]. Therefore, the assays of methanol and formic acid in biological fluids are important in examining of methanol poisoning.

Solid-phase microextraction (SPME) is a new technique developed by Pawliszyn and co-workers [3,4], which integrates sampling, extraction, concentration and sample introduction into a single procedure. A number of reports have recently appeared addressing the usefulness of SPME for the analyses of drugs and xenobiotic substances in various samples [5–16]. In this paper, we have

*Corresponding author. Tel.: +81-3-3784-8140; fax: +81-3-3767-6418.
E-mail address: kumazawa@akiha.hama-med.ac.jp (T. Kumazawa)

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established a recommendable procedure for analyzing methanol and formic acid from human whole blood and urine samples by use of headspace SPME and capillary gas chromatography (GC).

2. Experimental

2.1. Materials

Methanol, methyl formate, sodium formate, acetonitrile as internal standard (IS) and sulfuric acid (98%) were obtained from Wako (Osaka, Japan). The SPME devices and their fiber assemblies, 85-μm polyacrylate, 100-μm polydimethylsiloxane (PDMS), 65-μm PDMS/divinylbenzene (PDMS/DVB), 65-μm Carbowax/DVB and 75-μm Carboxen/PDMS fibers were purchased from Supelco (Bellefonte, PA, USA). Other common chemicals used were of analytical grade. Whole blood and urine were obtained from healthy subjects.

2.2. Conditioning of SPME fiber

New fibers of PDMS, PDMS/DVB and Carbowax/DVB were conditioned in the injection port of a gas chromatograph at 260°C for 60 min to remove fiber contaminants; polyacrylate and Carboxen/PDMS fibers were conditioned at 300°C for 120 min and 280°C for 30 min, respectively. Used fibers were cleaned by heating at the conditioning temperatures for 15 min before extraction.

2.3. Headspace SPME procedure for methanol

To a 4-ml vial containing 0.5 ml of human whole blood or urine which had been spiked with 200 μg of methanol and 2 μg of I.S., were added 0.5 ml of distilled water, 0.6 g of $(NH_4)_2SO_4$ and a small PTFE-coated stirring bar. The vials were rapidly sealed with silicon-septum caps and put on an aluminum block heater (Reacti-Therm Heating/Stirring Model, Pierce, Rockford, IL, USA) for heating and stirring. After heating at 60°C for 5 min, the septum piercing needle of the SPME device was passed through the septum. The pretreated fiber was pushed out from the needle and exposed to the headspace of the vial at 60°C for 10 min to allow adsorption of the compounds. The fiber was withdrawn into the needle and pulled out from the vial. It was immediately injected into the GC port and exposed in the injection port for 1.5 min for complete desorption of the compounds.

2.4. Derivatization and headspace SPME procedure for formic acid

Headspace SPME of formic acid from human samples was carried out after its derivatization to methyl formate under acidic conditions; methyl formate was produced according to a slightly modification of the method of Abolin et al. [17]. Briefly, to a 7.5-ml vial containing a small PTFE-coated stirring bar, 0.5 ml of human whole blood or urine which had been spiked with 54 μg of formic acid and 20 μg of I.S., were added 0.3 ml of sulfuric acid, and cooled thoroughly for 5 min with ice. After stirring vigorously with a vortex mixer for 1 min, 20 mg of methanol were added for formation of methyl formate. The vial was rapidly sealed with a silicon-septum cap and heated at 35°C with stirring on the aluminum block heater. After heating for 5 min, the septum piercing needle of the SPME device was passed through the septum. The pretreated fiber was pushed out from the needle and exposed to the headspace of the vial at 35°C for 10 min to allow adsorption of the compounds. The fiber was withdrawn into the needle and pulled out from the vial. It was immediately injected into the GC port and exposed in the injection port for 1.5 min for complete desorption.

2.5. GC conditions

GC analyses were carried out on a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detection (FID) system (Shimadzu, Kyoto, Japan). A Supelcowax 10 fused-silica capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm) which had been kindly donated by Dr. N. Fukunaga, Supelco Division, Sigma–Aldrich Japan (Tokyo, Japan) was used. The GC conditions were: column temperature 35–135°C (6 min hold at 35°C, 20°C/min) for methanol and 30–145°C (3 min hold at 30°C, 25°C/min from 30 to 105°C and 10°C/min from 105 to 145°C) for methyl formate; the injection

and detector temperature 280°C; and helium flow-rate 0.7 ml/min. In the case of direct injection of the authentic compounds dissolved in acetone, a 0.1-μl aliquot (80 ng of I.S., 200 ng of methanol or 300 ng of methyl formate on-column) was injected into the GC port. The samples were injected in the splitless mode at a column temperature of 35°C for methanol and 30°C for methyl formate, and the splitter was opened after 1.5 min.

2.6. Mass spectrometric conditions

Mass spectra in the positive ion electron impact mode were recorded on a HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled to a JEOL JMS-AX505H mass spectrometer (Tokyo, Japan) with a computer-controlled data analysis system. The mass spectrometric conditions were electron energy 70 eV; accelerating voltage 3.0kV; ionization current 300 μA; separator temperature 200°C and ion source temperature 200°C. GC separation was made with the above Supelcowax 10 capillary column. The GC conditions for the GC–mass spectrometry (MS) system were the same as those described for GC–FID.

2.7. Animal experiments

Male Wistar rats, weighing about 280 g, were obtained from Saitama Experimental Animal Supply (Saitama, Japan). Animals were kept in an animal room for at least one week prior to the experiments. Methanol in a 30% aqueous solution was administered orally at a single dose of 3.5 g methanol/kg by use of a gavage needle. The cardiac blood samples were collected 4 h after methanol administration under pentobarbiturate anesthesia, and stored frozen at −40°C until analysis.

3. Results and discussion

3.1. Optimization of conditions for SPME

The first step for the development of an SPME method is the selection of a fiber coating best suited for analysis of a biological sample. In the present study, five different fibers, PDMS, PDMS/DVB, polyacrylate, Carbowax/DVB and Carboxen/PDMS, were evaluated for extraction efficiencies of methanol and methyl formate from human body fluids. The Carboxen/PDMS fiber showed the highest efficiencies for methanol and methyl formate from human whole blood; the relative efficiencies (Carboxen/PDMS: 100%) for methanol were only 21, 6.4, 5.8 and 3.6% with Carbowax/DVB, PDMS/DVB, PDMS and polyacrylate fibers, respectively. In the case of methyl formate, the efficiency values for Carbowax/DVB, PDMS/DVB, PDMS and polyacrylate fibers were less than 1.2% compared with the Carboxen/PDMS fiber. We had also tested these fibers for extraction efficiencies of methanol and methyl formate from human urine, and the Carboxen/PDMS fiber also gave the best results. Thus the Carboxen/PDMS fibers were used in further experiments.

The addition of salting-out agents, such as $(NH_4)_2SO_4$ and/or NaCl, to the sample solutions improved extraction efficiency for some drugs and poisons in biological samples [7,11,13–15]. In the present study, this has been also true for methanol; the peak areas of methanol in whole blood and urine samples were more than 3–4-times better than those without the salts (Fig. 1). In the case of methyl formate, the addition of salts did not improve its efficiencies in both samples (data not shown). Thus methanol and methyl formate were extracted by

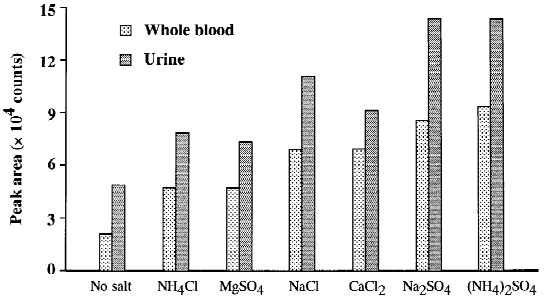

Fig. 1. Effects of various salts on the headspace SPME of methanol from human whole blood and urine samples with use of a Carboxen–PDMS fiber. Two hundred micrograms of methanol was added to the vial containing 0.5 ml of whole blood or urine, 0.5 ml of distilled water and 0.6 g of each salt. After pre-heating at 60°C for 5 min, the fiber was exposed to the headspace of the vial at the same temperature for 10 min. Each column represents the mean of duplicate determinations.

headspace SPME with $(NH_4)_2SO_4$ and without any salt, respectively.

The effect of temperature on headspace SPME was examined; suitable temperatures for headspace SPME of methanol and methyl formate were 60 and 35°C, respectively, for both whole blood and urine samples. The equilibria of them were reached within 10 min of exposure for both compounds. We thus exposed the fiber to the headspace for 10 min.

3.2. Validation of the method

Fig. 2 shows gas chromatograms for non-extracted authentic methanol (200 ng on-column) and I.S. (80 ng on-column) dissolved in acetone and for headspace SPME extracts from human whole blood and urine, to which 200 μg methanol and 2 μg I.S. had been added; the blank chromatograms without addition of both compounds are also presented (Fig. 2, lower panels). Methanol and I.S. were well separated from each other and from impurities, and gave sharp peaks.

Fig. 3 shows gas chromatograms for non-extracted authentic methyl formate (300 ng on-column) and I.S. (80 ng on-column) dissolved in acetone and for headspace SPME extracts after derivatization from human whole blood and urine, to which 54 μg formic acid and 20 μg I.S. had been added. Methyl formate and I.S. were well separated from each other and from impurities, and gave sharp peaks. The blank chromatograms gave a few small impurity peaks and a large methanol peak (Fig. 3, lower panels).

To confirm that the detected peaks from human whole blood and urine in the headspace SPME are methanol, methyl formate and I.S., we measured the positive ion electron impact mass spectra for each peak. Molecular peaks at m/z 32, 60 and 41 (base peak) for methanol, methyl formate and I.S., respectively, were observed in the spectra; there were fragment ions at m/z 31 (base peak), 30 and 28 for methanol, m/z 44, 31 (base peak), 29 and 15 for methyl formate, and m/z 40, 39 and 38 for I.S. The spectra for whole blood and urine extracts were almost identical to those of the authentic compounds, confirming the identities of the compounds in the body fluids.

The extraction efficiencies and their within-day relative standard deviations (RSDs) measured by the method for methanol, methyl formate and I.S. in human body fluid samples are presented in Table 1. The extraction efficiencies for methanol were 0.25 and 0.38% for whole blood and urine, respectively, and those of I.S. were 8.86–12.4% for both samples. The RSD values were satisfactory and not greater than 9.8% for both body fluids. In the case of formic acid, the efficiencies were 1.55% for whole blood, 1.24% for urine; those of I.S. were 0.9% for both samples, the RSD values were not greater than 3.3%.

The calibration curves for methanol and formic acid in human whole blood and urine samples were constructed by the present method. They were drawn by plotting six different concentrations according to the peak-area ratios with acetonitrile as I.S. They showed good linearity in the range of 1.56 to 800 μg/0.5 ml for methanol and 1.56 to 500 μg/0.5 ml for formic acid in whole blood and urine. The equations and r values for the curves of methanol were: $y=0.0108x-0.0587$ and $r=0.997$ for whole blood, and $y=0.0093x+0.001$ and $r=0.999$ for urine, respectively. In the case of formic acid, the equations and r values for the curves were: $y=0.0214x+0.113$ and $r=0.999$ for whole blood, and $y=0.0161x+0.142$ and $r=0.999$ for urine, respectively. The detection limits (signal-to-noise ratio=3) under optimal conditions for methanol were 0.5 μg/0.5 ml for whole blood and 0.1 μg/0.5 ml for urine; those for formic acid 0.6 μg/0.5 ml in both fluids.

The low extraction efficiencies (Table 1) are not surprising because they are just a result of the partition of the compounds among the stationary phase of the SPME fiber, headspace vapor and the sample solution [3]. In spite of the low extraction efficiencies, small variation (Table 1) and excellent quantitativeness could be achieved by the present headspace SPME.

3.3. Actual measurements of methanol and formic acid in rat blood after methanol intake

In addition to the above spiked human whole blood and urine samples, we have measured the levels of methanol and formic acid in rat blood after oral administration of methanol. Fig. 4 shows headspace SPME–GC chromatograms of methanol (left panel) and formic acid (right panel) extracted from

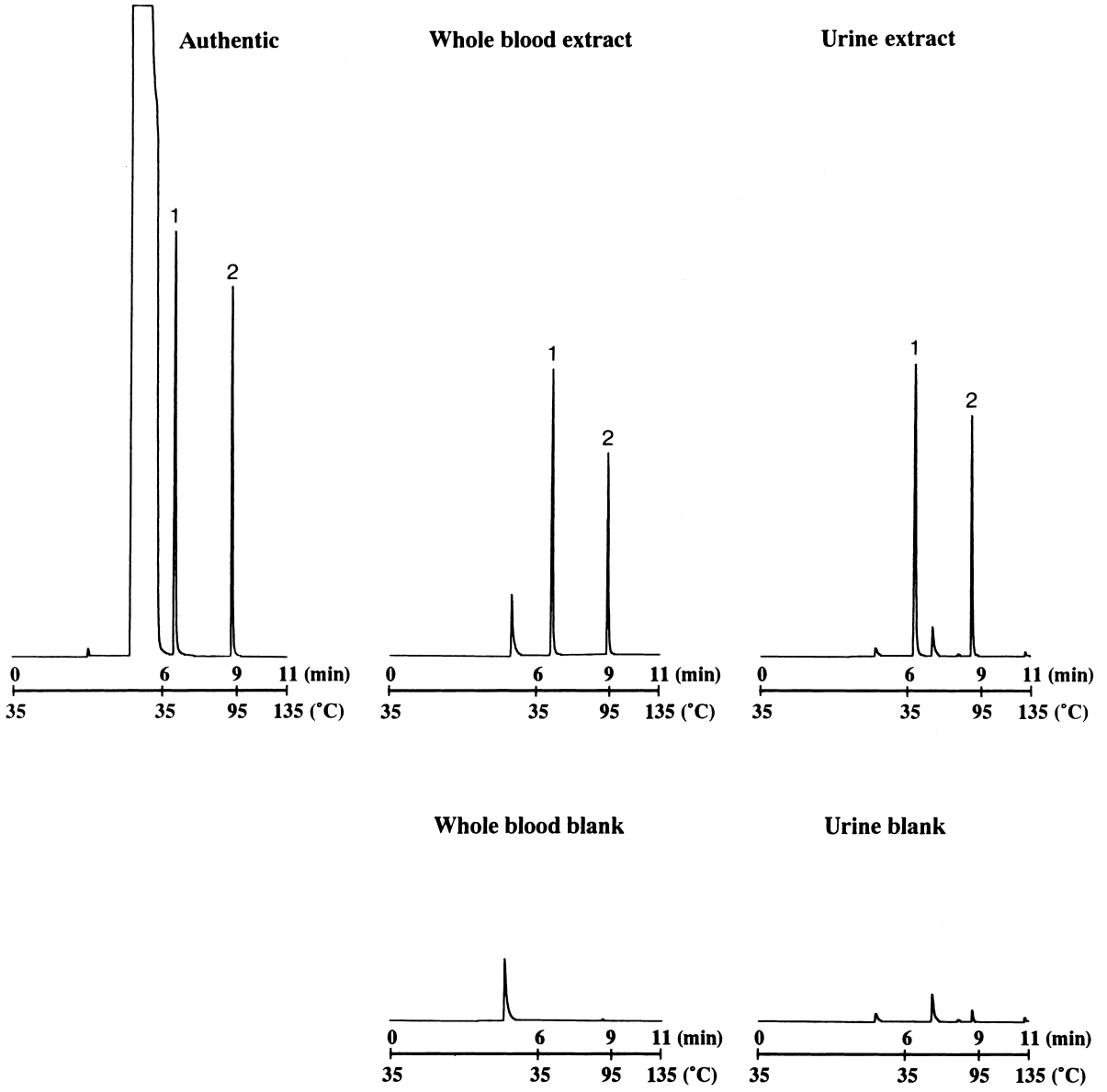

Fig. 2. Capillary GC–FID chromatograms for methanol and acetonitrile (IS) extracted from human whole blood and urine samples by the headspace SPME with use of a Carboxen–PDMS fiber. The amounts of methanol and I.S. spiked to 0.5 ml of both samples, containing 0.5 ml distilled water and 0.6 g $(NH_4)_2SO_4$, were 200 and 2 μg, respectively. The vertical scale of the chromatogram for the authentic compounds (200 ng methanol and 80 ng I.S. on-column) was two-fold larger as compared to that of whole blood or urine sample. Peaks: 1=methanol; 2=I.S. The big front peak appearing in the authentic chromatogram is due to acetone used as a vehicle.

0.5-ml rat whole blood samples after oral administration of methanol. Methanol and formic acid could be detected as intense peaks on gas chromatograms with use of the present method. Mass spectra for blood extracts of the rats were almost identical to those of the authentic compounds (data not shown). After confirmation by GC–MS, the same samples were subjected to GC–FID for quantitation. The blood

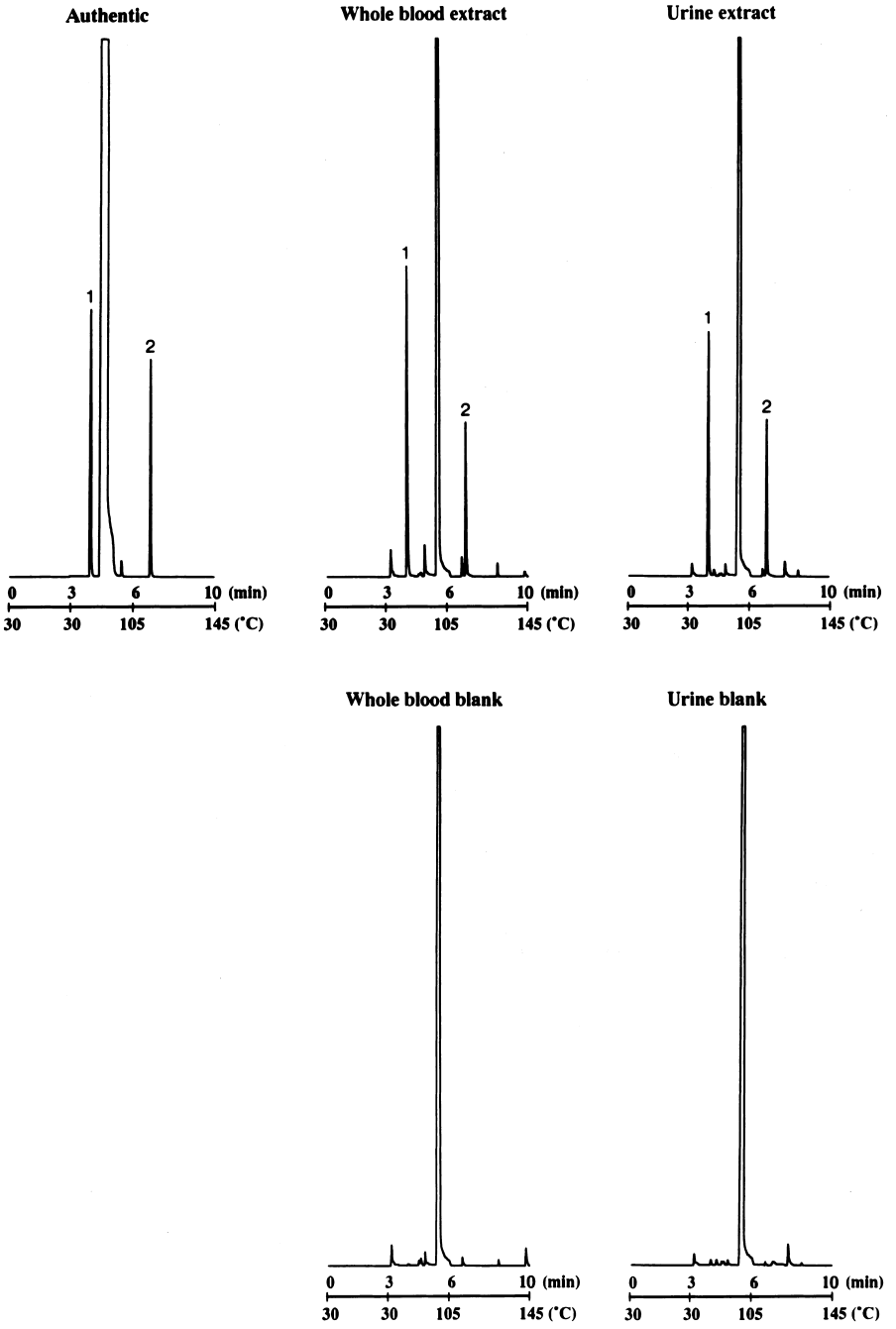

Fig. 3. Capillary GC–FID chromatograms for formic acid and acetonitrile (IS) extracted from human whole blood and urine samples by the headspace SPME with use of a Carboxen–PDMS fiber. The amounts of formic acid and I.S. spiked to 0.5 ml of both samples were 54 μg and 20 μg, respectively. For the authentic sample, the amounts of methyl formate and I.S. were 300 and 80 ng on-column, respectively. The vertical scale of chromatogram for the authentic compounds was two-fold larger as compared to that of whole blood or urine sample. Peaks: 1=methyl formate; 2=I.S. The big peak in the middle of each extract chromatogram is due to methanol added for derivatization; that in the authentic chromatogram due to acetone used as a vehicle.

Table 1
Extraction efficiencies and their within-day relative standard deviation (RSD) for methanol, formic acid and acetonitrile as internal standard added to human whole blood and urine samples using headspace SPME[a]

	Compound	Amount added to 0.5-ml sample (μg)	Efficiency (%)	RSD (%)
Methanol measurements				
Whole blood	Methanol	200	0.25±0.01	4.0
	Acetonitrile	2	8.86±0.13	1.5
Urine	Methanol	200	0.38±0.01	2.6
	Acetonitrile	2	12.4±1.2	9.8
Formic acid measurements				
Whole blood	Formic acid	54	1.55±0.02	1.3
	Acetonitrile	20	0.91±0.03	3.3
Urine	Formic acid	54	1.24±0.03	2.4
	Acetonitrile	20	0.90±0.02	2.2

[a] The values are mean±SD of three experiments. The efficiencies were calculated by comparing the peak areas obtained from the extracts of the spiked human body fluid samples with those obtained by direct GC injection of non-extracted authentic compounds dissolved in acetone.

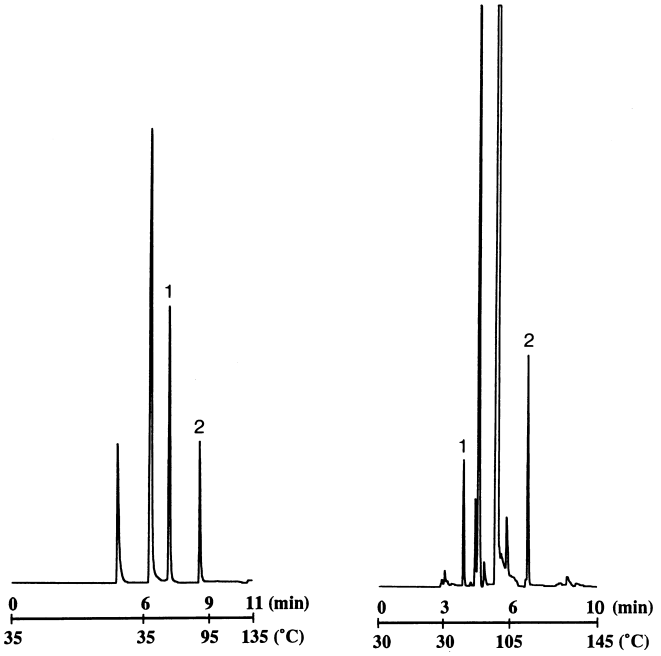

Fig. 4. Capillary GC–FID chromatograms for methanol (left panel) and formic acid (right panel) extracted from rat blood 4 h after oral administration of 3.5 g methanol/kg body mass. In each vial, 2 μg and 20 μg of acetonitrile were spiked as I.S. for extraction of methanol and formic acid, respectively. Peaks for left panel: 1=methanol; 2=I.S. Peaks for right panel: 1=methyl formate; 2=I.S.

concentrations of methanol and formic acid 4 h after a single dose of 3.5 g methanol/kg body mass were determined to be 1.44±0.17 mg/0.5 ml (mean±SD, $n=7$) and 13.6±5.9 μg/0.5 ml (mean±SD, $n=7$), respectively.

Toxic effects of methanol are usually associated with blood concentrations greater than about 0.1 mg/ml [18]; the post-mortem concentration of methanol in blood after its poisoning was reported to be 0.2–3.2 mg/ml [18–20]. In the case of formic acid, blood level of formic acid was reported to be 90–1210 μg/ml for methanol poisoning case [2]. The detection limits by our method sufficiently meet measurements of toxic levels of the methanol and formic acid.

4. Conclusion

We have been able to extract and detect methanol and formic acid in human and rat body fluids by headspace SPME with a Carboxen–PDMS fiber and capillary GC–FID. Our method is recommendable for extraction of these compounds from biological specimens in the fields of forensic and clinical toxicology.

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