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Short Communication

Simultaneous Determination of Methanol, Ethanol and Formic Acid in Serum and Urine by Headspace GC-FID

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A simple, cost-effective headspace gas chromatography (GC) method coupled with GC with flame ionization detection for simultaneous determination of methanol, ethanol and formic acid was developed and validated for clinical and toxicological purposes. Formic acid was derivatized with an excess of isopropanol under acidic conditions to its volatile isopropyl ester while methanol and ethanol remained unchanged. The entire sample preparation procedure is complete within 6 min. The design of the experiment (the face-centered central composite design) was used for finding the optimal conditions for derivatization, headspace sampling and chromatographic separation. The calibration dependences of the method were quadratic in the range from 50 to 5,000 mg/L, with adequate accuracy (89.0–114.4%) and precision (<12%) in the serum. The new method was successfully used for determination of selected analytes in serum samples of intoxicated patients from among those affected by massive methanol poisonings in the Czech Republic in 2012.

Introduction

Methanol poisoning is a relatively infrequent medical emergency and may result in significant morbidity and mortality if untreated and represents challenges for both clinicians and toxicological laboratories. Methanol is used as a solvent component of some antifreeze solutions, paints, gasoline additives and ethanol denaturants. However, most massive methanol poisonings (Argentina 1993, Norway 2002, Czech Republic 2012 and Libya 2013) were caused by the ingestion of adulterated alcoholic beverages (1–3).

Methanol is a toxic substance and is metabolized to formaldehyde by alcohol dehydrogenase and is then converted to formic acid by aldehyde dehydrogenase in the liver. Formic acid is the principle toxic metabolite that can cause severe problems at high concentrations (>200 mg/L) and warrants hemodialysis treatment (4). Therefore, the initial stage of methanol intoxication is typically asymptomatic. During the later stage (6–12 h after ingestion), serious symptoms caused by methanol and formic acid can occur. The symptoms specific to high serum concentrations of formic acid include visual impairment, damage to the optical nerve, abdominal discomfort, nausea and headache. Without medical intervention, the high serum concentrations of formic acid can cause respiratory and renal failure, coma and eventually death. In the late phase, the diagnostic hallmark of methanol poisoning is the presence of a high anion gap, osmolal gap and metabolic acidosis. These indicators are unfortunately not specific and can be caused by other conditions, for instance diabetic ketoacidosis or multiple organ failure (5). Thus, measurement of serum methanol, and ideally formic acid, its major toxic metabolite in serum, is required to differentiate the cause of the patient's condition.

Despite the toxicity at elevated serum concentrations of formic acid, it is normally present in mammals due to the degradation of amino acids and external sources like the diet, alcoholic beverages or inhalation of methanol vapors. In previous studies, the serum formic acid concentration in living subjects has ranged up to 44 mg/L (6, 7).

Gas chromatography (GC) is a precise and reliable method for the determination of low-molecular weight alcohols in blood and other biological fluids and has become a reference method in forensic and clinical toxicology. Previously published methods employed different sample preparation techniques, including direct injection, static and dynamic headspace or headspace injection using solid-phase microextraction (HS-SPME) (8, 9). Headspace GC (HS-GC) is now the most widely used technique for the detection of volatile organic compounds in biological fluids, mainly for its ability to detect trace levels of volatiles without the need for complex sample preparation.

Formic acid is commonly analyzed after derivatization with methanol to methyl formate or with ethanol to ethyl formate under acidic conditions (6, 8, 10). HS-GC or HS-SPME-GC are the methods of choice, although other variations of these techniques have been employed (7, 11). Enzymatic methods with colorimetric detection exhibit good selectivity, but the use of rather expensive reagents precludes their widespread application in clinical laboratories. Formate determination by capillary electrophoresis has been reported, but despite excellent separation efficiency and short separation times, this method is limited to the analysis of small ions only and is rarely used in clinical laboratory practice worldwide (12).

Unfortunately, there is a lack of appropriate methods for simultaneous determination of methanol and formic acid. The simultaneous measurement of toxic formic acid along with parent methanol is clearly desirable for both enhanced clinical service and the correct diagnosis.

The goal of this work is simultaneous, simple and fast determination of methanol, ethanol and formic acid in biological specimens by headspace GC with flame ionization detection. Previously published methods for the determination of formic acid were based on its derivatization with an excess of either methanol or ethanol, which prohibited the analysis of these alcohols along with formic acid in a single analytical procedure (6, 8). In our new method, derivatization of formic acid was achieved by using an excess of isopropanol, allowing for the simultaneous determination of methanol, ethanol and formic acid. This approach is suitable for monitoring all the analyte concentrations in a single measurement during antidotal therapy (ethanol) or hemodialysis treatment.

Furthermore, the complete sample preparation time is shortened to nearly 6 min. The quantitative serum results are available

within 30 min (two serial measurements), which corresponds well with the usual emergency medicine turnaround time.

The derivatization procedure as well as headspace and separation parameters were optimized using a chemometric approach design of experiment, replacing the common one-factor-at-a-time procedure because it is not time-effective and does not take into account possible interactions among the individual parameters (13, 14).

Experimental

Chemicals

Methanol (Chromasolv), isopropanol (LC–MS Chromasolv), 3-methyl-2-pentanone (99%) and formic acid (95%) were purchased from Sigma-Aldrich (Germany). Sulfuric acid (97%) was obtained from Merck (Germany) and ethanol (96%) was purchased from Penta (Czech Republic). Deionized water for preparation of all the solutions was purified (18.2 MW) using a Mille-Q Plus (Merck Millipore, Billerica, MA, USA).

Preparation of calibrators

Blank human serum for method validation was purchased from ACQ Science GmbH (Germany). Blank urine samples were provided by three healthy male and three healthy female volunteers from our department and stored at +4°C until use.

Seven concentration levels of the calibrators in aqueous samples were prepared to yield the following final concentrations for methanol, ethanol and formic acid: 50, 100, 200, 500, 1,000, 3,000 and 5,000 mg/L (five replicates of each concentration point). Human whole blood or urine samples were delivered from various hospitals with a request for quantification, mainly for emergency purposes.

Sample preparation

Whole blood was centrifuged at 10,000 rpm for 30 s to obtain serum sample for analysis (urine was used without any pretreatment). One hundred microliters of 10% (v/v) aqueous solution of isopropanol (derivatizing agent) with internal standard (3-methyl-2-pentanone, 40 mg/L) was placed in a 10 mL glass crimped headspace vial (Agilent Technologies, USA), then 5 μL concentrated sulfuric acid was added as a catalyst for the formation of isopropyl formate and 100 μL of serum or urine was added. The glass vial was sealed with a Teflon-lined silicone septum and an aluminum cap (Agilent Technologies). The prepared solution was lightly mixed manually and placed in the headspace autosampler.

Headspace conditions

The automated headspace autosampler HT200H (HTA, Italy) was interfaced with the GC-FID for sample preparation and sample introduction into the GC.

The samples were incubated in the oven for 5 min at 80°C without shaking. The headspace injection was performed with a 2.5 mL gas-tight syringe that was heated to 110°C. A 300 μL headspace aliquot was sampled for the analysis with a fill speed of 35 mL/min. To obtain homogenous sampling, a fill stroke count of 3 was used. The sample was injected into the GC at an injection speed of 50 mL/min. After each analysis, the syringe was flushed with nitrogen for 5 min. The run cycle time was set at 12 min.

GC-FID conditions

All analyses were performed on Shimadzu GC-2010 Plus and operated by a computer running the GCsolution version 2.41.00 SU1 (Kyoto, Japan). The chromatograph was equipped with a single injection connected with two different parallel columns RTX–BAC 1-fused silica column (30 m × 0.32 mm ID × 1.8 μm film thickness, Restek, USA) and RTX–BAC 2 Plus (30 m × 0.32 mm ID × 0.6 μm film thickness, Restek) and two FIDs, operated under the same conditions. Nitrogen (purity 99.9992%) was employed as a carrier gas. The oven temperature was held at 50°C for 4 min following injection and then raised to 180°C at 65°C/min (2 min); the total run time was 8 min. The instrument parameters were as follows: 215°C inlet temperature, 250°C detector temperature and carrier gas linear velocity 25 cm/s. All the injections were done in the split mode (1 : 20).

Statistical software

The construction and analyses of the experimental design and the response surfaces were carried out using the Minitab 16 statistical package (Minitab Inc., USA). The calculations were performed with Microsoft Excel 2007 (Microsoft, WA, USA). Chromatogram was constructed in Origin 9.1 (OriginLab Corporation, MA, USA).

Design of experiment

Chemometric approach (experimental design) was used for optimization of the new method. The experimental parameters were divided into the three groups (A, derivatization; B, headspace conditions; C, separation) (Table I) and optimized using the face-centered central composite design (13) (see

Table I

Experimental Parameters and Their Levels Used in Face-Centered Central Composite Design for Finding the Optimal Experimental Conditions of the New Method

Parameters	Level		
	Low	Central	High
A: Derivatization			
Volume of sulfuric acid (μL)	5	503	1,000
Concentration of isopropanol (%, v/v)	1	5	10
Reagent sequence	B/SA/I	SA/B/I	I/SA/B
B: Headspace			
Incubation time (min)	5	32.5	60
Temperature of incubation (°C)	60	90	120
Volume of headspace aliquot (μL)	100	550	1,000
Fill speed (mL/min)	10	55	100
Injection speed (mL/min)	10	55	100
Shaking (min)	0	2.5	5
C: Separation			
Split ratio	1:10	1:55	1:100
Injection temperature (°C)	150	200	250
Initial temperature (°C)	40	50	60
Carrier gas linear velocity (cm/s)	10	20	30

B, biological material (serum, urine); SA, sulfuric acid; I, isopropanol solution.

Supplementary data, Tables S1–S3). The optimal responses, defined as the relative peak area, resolution and peak symmetry of the selected analytes (methanol, ethanol and formic acid), were sought.

The optimum values of the parameters were found using the desirability function (15) and the following results were obtained: (A) volume of sulfuric acid 5 μL, concentration of isopropanol in water 10%, reagent sequence: isopropanol solution, sulfuric acid and biological sample; (B) incubation time and temperature in the headspace autosampler 5 min at 80°C, volume of the headspace aliquot 300 μL, fill speed 35 mL/min, injection speed 50 mL/min, without shaking; (C) split ratio 1 : 20, carrier gas linear velocity 25 cm/s, injection temperature 215°C and initial temperature 50°C.

Results and discussion

Analytical performance

Calibration curves were constructed and processed as described in the Preparation of calibrators section. The equations for the standard curves were obtained by plotting the analyte to internal standard peak area ratios against the analyte concentrations. The calibration curves were quadratic and coefficients of determination R^2 were range from 0.9992 to 0.9999.

The limit of detection (LOD) and quantitation (LOQ) were set at 50 mg/L, respectively. At this concentration level, the precision (relative standard deviation, RSD, 10.8%) and accuracy (range 91.3–104.0%) for the serum samples (within-day and between-day values) fulfilled the acceptance criteria for LOQ defined as the lowest concentration of a sample that can still be quantified with acceptable precision (20%) and accuracy (±20%) (16) (Table II). This selected LOD and LOQ should be sufficient for emergency cases since antidote treatment (ethanol, fomepizole and hemodialysis) is recommended at a serum concentration of methanol and formic acid starting at 200 mg/L for both compounds (17). Moreover, formic acid is an endogenous compound in the human body and methanol could be present in alcoholic beverages as an impurity.

The precision was evaluated by means of the RSD values for each compound. RSD values detected for within- and between-day repeatability at three concentration levels (low—50 mg/L, medium—100 mg/L and high—1,000 mg/L) were analyzed in six replicates for six consecutive days and the results for the serum samples are summarized in Table II. The method performed well in terms of precision over the selected concentration range, with all the results being within the appropriate range of RSD 12% in serum. As proposed earlier, the acceptable RSD limit for middle and high concentrations was ± 15%. The accuracy was assessed as the percentage difference of the mean calculated concentration at each concentration level from the corresponding nominal concentration (16). The evaluated ranges of accuracy were from 89.0 to 114.4% in serum (Table II).

The human samples, serum and urine were spiked with a group of volatile organic substances (acetaldehyde, acetone, acetonitrile, 1-butanol, diethylether and ethyl acetate) to verify the selectivity of the new method. The mentioned analytes did not exhibit any interference with methanol, ethanol, formic acid or with internal standard (Supplementary data, Table S5). Moreover, two different parallel capillary columns (RTX—BAC 1 and RTX—BAC 2 Plus) with different stationary phases were used for confirmation of the selected analytes. The method was found to be selective enough to reliably differentiate and quantify methanol, ethanol and formic acid.

The analytical validation (selectivity, precision and accuracy) of the method was also performed for urine samples with satisfactory results (Supplementary data, Table S4).

Application of real samples

The newly developed method was used for quantitation of methanol, ethanol and formic acid in serum samples from four patients from the massive methanol poisoning in the Czech Republic in 2012 (patients nr. 1–4, Table III) (3). In addition, a serum and urine samples from a patient intoxicated with methanol after ingestion of badly distillated spirit were obtained and measured in January 2015 (patient nr. 5). The results correlated well with the clinical course of intoxication, e.g., 12 h lapsed from the time of

Table III
Concentrations of Methanol, Ethanol and Formic Acid in Serum and Urine from Five Patients with Methanol Intoxication

Patient	*c* (methanol) mg/L	*c* (ethanol) mg/L	*c* (formic acid) mg/L
1	420	–	380
2	910	1,410	650
3	280	120	60
4	1,540	900	480
5	1,250	–	790
5[a]	1,540	–	4,660

[a]Urine, data rounded with respect to precision.

Table II
Within- and Between-Day Method Precision and Accuracy for Methanol, Ethanol and Formic Acid in Human Serum (6 Days, Six Replicates)

	Methanol			Ethanol			Formic acid		
Within-day									
$c_{nominal}$ (mg/L)	50	100	1,000	50	100	1,000	50	100	1,000
$c_{measured}$ (mean ± SD)	50 ± 4	111 ± 9	1,144 ± 131	49 ± 3	101 ± 8	1,140 ± 122	48 ± 5	94 ± 7	1,033 ± 103
Precision (RSD %)	7.3	7.8	11.5	6.2	7.6	10.7	9.6	7.8	10.0
Accuracy (%)	100.3	111.4	114.4	99.8	100.6	113.9	97.4	94.2	103.3
Between-day									
$c_{nominal}$ (mg/L)	50	100	1,000	50	100	1,000	50	100	1,000
$c_{measured}$ (mean ± SD)	49 ± 5	110 ± 10	1,037 ± 118	52 ± 5	101 ± 11	1,015 ± 122	46 ± 4	89 ± 5	1,042 ± 116
Precision (RSD %)	10.8	9.4	11.4	9.7	10.5	12.0	8.8	5.4	11.1
Accuracy (%)	99.8	109.8	103.7	104.0	101.0	101.5	91.3	89.0	104.2

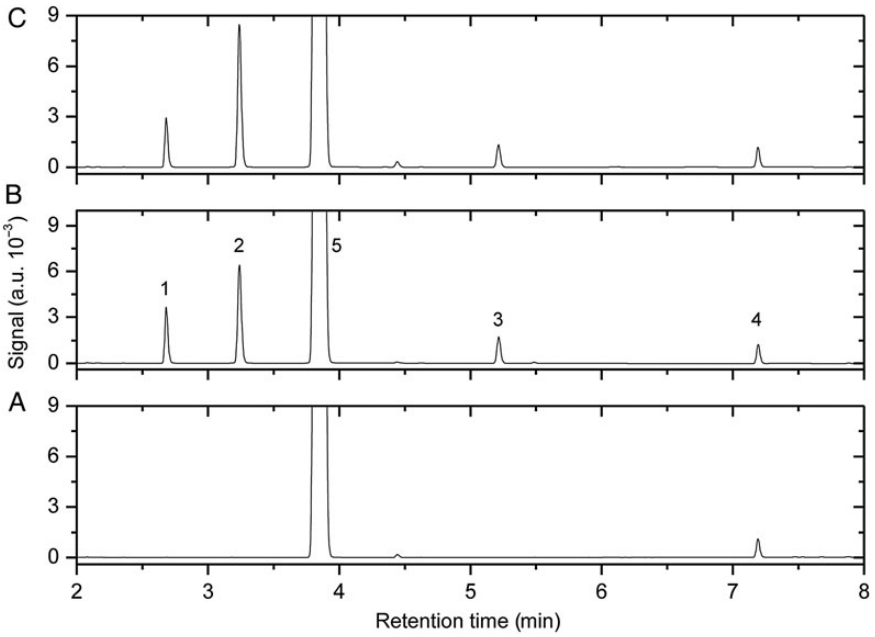

Figure 1. Representative GC-FID chromatograms from Rtx—BAC 1 column for methanol (1), ethanol (2), formic acid isopropyl ester (3), internal standard 3-methyl-2-pentanone (4) and isopropanol (5) in serum sample: (A) a blank human serum spiked with internal standard; (B) a blank human serum spiked with 1,000 mg/L of all analytes; (C) a human serum sample of patient no. 2.

methanol ingestion until symptoms of visual disturbance (snowfield vision) and dizziness appeared. Figure 1 shows the typical GC-FID chromatogram for methanol, ethanol and formic acid in serum separated on the RTX−BAC 1 column (separation on column RTX−BAC 2, Supplementary data, Fig. S1).

Conclusion

This article describes a simple and fast derivatization method for simultaneous determination of methanol, ethanol and formic acid in human serum and urine using headspace GC-FID. This method is suitable for monitoring all of the analyte concentrations in a single measurement during antidotal (ethanol) or hemodialysis treatment. The conditions of the new method were optimized using an experimental design resulting in minimized sample pretreatment and turnaround time of ∼30 min, which is required for timely effective support of diagnosis of suspected methanol poisoning and to initiate adequate treatment. The assay was successfully applied to measurements in our routine clinical practice and the results were also presented.

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Supplementary data

Supplementary data are available at *Journal of Analytical Toxicology* online.

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