Sensitive Analysis of Alkyl Alcohols as Decomposition Products of Alkyl Nitrites in Human Whole Blood and Urine by Headspace Capillary GC with Cryogenic Oven Trapping

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Abstract

The abuse of alkyl nitrites is becoming a serious social problem worldwide. In this report, a simple and sensitive method is presented for the determination of *n*-butyl alcohol, isobutyl alcohol, and isoamyl alcohol as decomposition products of alkyl nitrites in human whole blood and urine samples using capillary gas chromatography (GC) with cryogenic oven trapping. After heating a whole blood or urine sample containing each alkyl alcohol and *t*-butyl alcohol [the internal standard (IS)] in a 7-mL vial at 55°C for 15 min, 5 mL of the headspace vapor is drawn into a gas-tight syringe and injected into a GC inlet port. The vapor is introduced into an Rtx-BAC2 medium-bore capillary column in the splitless mode at 0°C oven temperature in order to trap the entire analytes, and then the oven temperature is programmed up to 240°C for the GC measurements by flame ionization detection. These conditions give sharp peaks for each compound and the IS and low background noise for whole blood or urine samples. The detection limits of the analytes are 10 ng/mL for whole blood and 5 ng/mL for urine. Linearity and precision are also tested to confirm the reliability of this method. Isobutyl alcohol and methemoglobin could be determined from the whole blood samples of three male volunteers who had sniffed isobutyl nitrite.

Introduction

Alkyl nitrites are being widely abused by inhalation among young people for their aphrodisiac and euphoric effects (1–5). Because these drugs are not legally controlled by governments, it is very easy to get them at common markets or by internet sales. The most popular alkyl nitrite being abused is isobutyl nitrite, followed by isoamyl nitrite and *n*-butyl nitrite. Alkyl nitrites easily decompose to alkyl alcohols and inorganic nitrite by light or chemical hydrolysis in aqueous or biological matrices (6,7). The inorganic nitrite reacts with hemoglobin to produce methemoglobin; the cause of death after the ingestion of large amounts of alkyl nitrites is considered to be methemoglobinemia (8,9).

There are a few reports of the analysis of alkyl alcohols as the decomposition products of alkyl nitrites by gas chromatography (GC) (4,7,10). Two reports dealt with the qualitative analysis of isobutyl alcohol as the product of isobutyl nitrite in adulterated coffee drink (7) and with the qualitative analysis of cyclohexanol as the product of cyclohexyl nitrite in the liquid of video head cleaners in a bottle (4). Only one report presented the analysis of *n*-butyl alcohol as the decomposition product of *n*-butyl nitrite in a biological matrix (whole blood) by headspace solid-phase microextraction (SPME) (10). SPME is a useful technique, but requires some skill in the handling of the SPME device.

In this study, we present a new, simple, and sensitive analytical method for the determination of three alkyl alcohols as the decomposition products of alkyl nitrites in human whole blood and urine using headspace GC with cryogenic oven trapping.

Experimental

Materials

Isobutyl nitrite, *n*-butyl nitrite, and isoamyl nitrite (with a purity of over 90%) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). *t*-Butyl alcohol, isobutyl alcohol, *n*-butyl alcohol, and isoamyl alcohol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other common chemicals used were of the highest purity commercially available. An Rtx-BAC2 medium-bore capillary column (30-m × 0.32-mm i.d., 0.25-µm film thick-

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ness) was purchased from Restek (Bellefonte, PA). Whole blood and urine samples were obtained from healthy volunteers.

Procedure

Stock solutions (20 and 100 µg/mL and 1 mg/mL) of *t*-butyl alcohol [the internal standard (IS)], isobutyl alcohol, *n*-butyl alcohol, and isoamyl alcohol were prepared by dissolving them in distilled water. One-milliliter whole blood or urine samples containing various amounts of each compound and 1 µg of the IS were added to a 7.0-mL vial containing 0.5 g sodium sulfate. The vial was rapidly sealed with a silicone-septum cap and placed on an aluminum block heater. After heating the vial at 55ºC for 15 min, 5 mL headspace vapor was drawn with a gas-tight syringe (10-mL volume with a 23-G needle) and injected into the GC inlet port in the splitless mode at an oven temperature of 0ºC.

GC conditions

GC analysis was carried out on a Shimadzu GC-15A GC equipped with flame ionization detection and a cryogenic oven temperature device (Shimadzu, Kyoto, Japan). The column temperature was 0ºC to 240ºC (held for 1 min at 0ºC, then from 0ºC to 120ºC at 10ºC /min, and then from 120ºC to 240ºC at 20ºC/min); the injection and detection temperature was 240ºC; and the helium flow rate was 3 mL/min. The vapor was injected in the splitless mode, and the splitter was opened 1 min after the completion of the injection. For GC quantitation, the peak areas of each compound were used.

Human experiments for sniffing isobutyl nitrite

Three male scientists in our laboratory participated in the experiments after informed consent was given. Special care was taken not to affect their health. They sniffed, together with air, the vapor of isobutyl nitrite soaked on a gauze placed in a 28- × 20-cm-sized polyethylene bag for 2 min. Blood samples were taken immediately and then 10 min after the sniffing. Isobutyl alcohol was measured using *t*-butyl alcohol as the IS soon after each sampling by this method. Methemoglobin in the whole blood was also measured soon after each sampling by a spectrophotometric method (11).

Table I. Conversion of Alkyl Nitrites into Corresponding Alkyl Alcohols in Different Matrices

Alkyl nitrite added*	Alkyl alcohol formed	Matrix	Amount of alkyl alcohol found (nmol/mL)†
Isobutyl nitrite	Isobutyl alcohol	Distilled water	8.77 ± 0.21
		Whole blood	8.97 ± 0.99
		Urine	8.77 ± 0.38
n-Butyl nitrite	*n*-Butyl alcohol	Distilled water	8.94 ± 0.70
		Whole blood	9.24 ± 1.81
		Urine	9.43 ± 0.30
Isoamyl nitrite	Isoamyl alcohol	Distilled water	9.58 ± 0.36
		Whole blood	10.1 ± 3.45
		Urine	9.65 ± 0.29

* Amount added = 10 nmol/mL.
† The values are means ± standard deviation of three experiments.

Results and Discussion

Conversion of alkyl nitrites into alkyl alcohols

In our preliminary experiments, alkyl nitrites very rapidly decomposed into alkyl alcohols in water, whole blood, and urine in less than 10 min after mixing each alkyl nitrite with each matrix. Thus, we carefully examined the conversion of 10-nmol/mL alkyl nitrites (1.03 µg/mL each for isobutyl nitrite and *n*-butyl nitrite and 1.17 µg/mL for isoamyl nitrite) into alkyl alcohols in distilled water, whole blood, and urine.

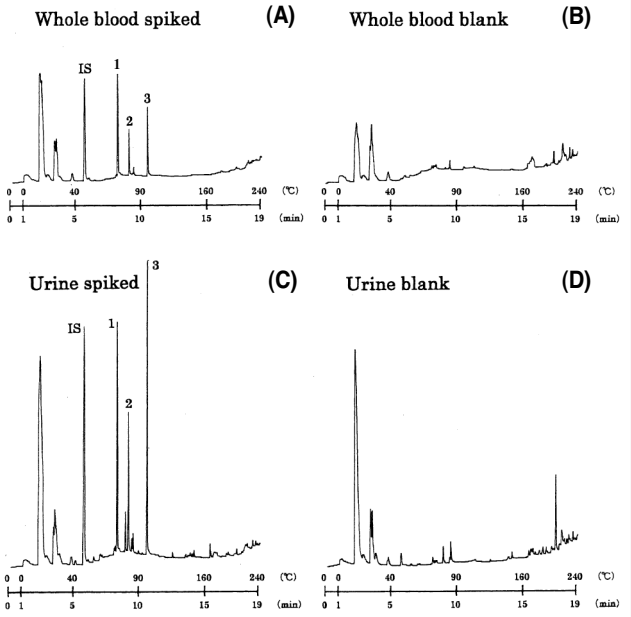

Figure 1. Headspace capillary gas chromatograms with cryogenic oven trapping for whole blood and urine samples spiked or not spiked with alkyl alcohols: (1) isobutyl alcohol, (2) *n*-butyl alcohol, and (3) isoamyl alcohol and the IS. One microgram each of four compounds was spiked into 1 mL whole blood or urine.

Table II. Extraction Efficiencies of Alkyl Alcohols and IS in Human Whole Blood and Urine for Their Measurements by GC with Cryogenic Oven Trapping*

Concentration (µg/mL)	Extraction efficiency (mean ± standard deviation, %) Isobutyl alcohol	*n*-Butyl alcohol	Isoamyl alcohol	*t*-Butyl alcohol
Whole blood (*n* = 10)				
0.2	11.2 ± 1.6	9.06 ± 3.03	4.05 ± 1.06	–
1.0	8.48 ± 0.56	3.03 ± 0.42	3.88 ± 0.47	19.2 ± 0.9
10.0	10.8 ± 0.7	5.53 ± 0.44	6.41 ± 0.62	–
Urine (*n* = 10)				
0.2	26.3 ± 2.6	15.7 ± 2.6	14.1 ± 2.0	–
1.0	18.9 ± 1.1	10.8 ± 0.8	15.9 ± 1.1	35.1 ± 0.8
10.0	23.5 ± 1.1	13.8 ± 0.8	22.4 ± 1.1	–

* Each compound and IS (1 µg) was added to 1 mL of human whole blood or urine samples. The extraction efficiencies were calculated by comparing the peak areas obtained from the headspace gas of the spike whole blood or urine samples with those obtained from nonextracted authentic compounds directly injected into the GC port.

The results are shown in Table I. Almost all of the alkyl nitrites were converted into the corresponding alcohol products in all of the matrices.

Stability of alkyl alcohols

In view of the detectability of alkyl alcohols as the decomposition products of alkyl nitrites in actual death cases involving alkyl nitrite inhalation, we tested the stability of alkyl alcohols (1 µg/mL each) in human whole blood by storing the sample at room temperature for 7 days and at 4ºC for 14 days. The amount of the spiked alkyl alcohols decreased to 30–40% on the third day at room temperature, but storage at 4ºC gave almost no change of the alcohol levels up to 14 days.

Table III. Intra- and Interday Relative Standard Deviations for Alkyl Alcohols Spiked into Human Whole Blood and Urine

Concentration (µg/mL)		Relative standard deviation (%)*		
		Isobutyl alcohol	*n*-Butyl alcohol	Isoamyl alcohol
Whole blood (n = 5)				
0.2	Intraday	12.6	15.5	12.7
	Interday	6.61	17.6	14.6
1.0	Intraday	3.79	9.65	7.57
	Interday	13.3	6.65	8.06
10.0	Intraday	5.59	5.29	7.96
	Interday	10.5	12.6	12.5
Urine (n = 5)				
0.2	Intraday	9.18	8.03	2.28
	Interday	3.76	10.0	9.18
1.0	Intraday	1.41	1.81	0.86
	Interday	2.91	3.18	3.03
10.0	Intraday	5.67	3.40	4.22
	Interday	2.00	1.56	2.34

* Relative standard deviations were obtained after calculation with each calibration curve for each compound spiked into whole blood or urine.

Table IV. Calibration Curves for Alkyl Alcohols in Human Whole Blood and Urine

Compound (concentration range)	Whole blood			Urine		
	Equation* ($y = ax + b$)		Correlation coefficient (r)	Equation* ($y = ax + b$)		Correlation coefficient (r)
	a	b	r	a	b	r
Isobutyl alcohol (0.05–1 µg/mL)	0.426	+0.018	0.973	0.557	+0.016	0.999
(1–10 µg/mL)	0.444	+0.010	0.996	0.589	–0.067	1.000
n-Butyl alcohol (0.05 µg/mL)	0.166	+0.015	0.973	0.318	+0.011	0.998
(1–10 µg/mL)	0.218	–0.021	0.995	0.358	–0.070	1.000
Isoamyl alcohol (0.05–1 µg/mL)	0.268	+0.006	0.964	0.611	–0.014	0.999
(1–10 µg/mL)	0.305	–0.072	0.987	0.650	–0.109	1.000

* The data were subjected to linear regression analysis of peak area ratios (y) of each compound to IS (1 µg/vial) against the spiked concentrations (x). Seven plots for low concentrations (0.05–1 µg/mL) and six plots for high concentrations (1–10 µg/mL) were used for each compound.

Optimization of conditions

Various conditions for the headspace extraction of isobutyl alcohol, *n*-butyl alcohol, isoamyl alcohol, and the IS from whole blood were tested. Vials were heated at 40ºC, 50ºC, 55ºC, and 60ºC for 10, 15, 20, and 30 min; it was found that the maximal extraction into the headspace was attained at 55ºC for 15 min.

Various initial oven temperatures (30ºC, 0ºC, –10ºC, –20ºC, –30ºC, –40ºC, –50ºC, and –60ºC) were tested for trapping each compound vapor. At 30ºC, the alkyl alcohol peaks were relatively broad and became sharper upon lowering the oven temperature down to 0ºC. The peaks did not become sharper below 0ºC, and some impurity peaks appeared at –20ºC. Therefore, we selected 0ºC as the initial oven temperature for trapping the alkyl alcohol vapor.

We added various amounts (0.1, 0.3, 0.5, and 0.7 g) of sodium sulfate to 1 mL whole blood in order to increase the extraction efficiencies of alkyl alcohols. The peaks were highest and gave a 3.6–5.1 times increase in the peak areas with 0.5 g sodium sulfate; this amount was adopted in this method.

Reliability of the method

Figure 1 shows the gas chromatograms for isobutyl alcohol, *n*-butyl alcohol, isoamyl alcohol, and *t*-butyl alcohol (IS) spiked into human whole blood or urine (1 µg/mL each) (Figure 1A and 1C) and for extracts in the absence of each compound (Figure 1B and 1D). There were some small impurity peaks visible in the blank chromatograms at 5–15 min of retention time, but they gave no problems.

Table II shows the extraction efficiencies of each compound in whole blood or urine at 0.2, 1, and 10 µg/mL (n = 10 each). The extraction efficiencies for each compound were better for urine than for whole blood, as expected.

The intra- and interday relative standard deviations for each compound in whole blood and urine (n = 5) are shown in Table III. The majority of values were < 13% and the largest value was 17.6% for whole blood, and the values were < 5% and the largest value was 10% for urine.

Calibration curves for each compound in human whole blood and urine were drawn for both low and high concentration ranges. The equations and r values for the plots are listed in Table IV. Detection limits (signal-to-noise ratio = 3) were estimated to be 10 ng/mL for whole blood and 5 ng/mL for urine. Seto et al. (7) have recently reported that the detection limit of isobutyl alcohol in coffee solution is 1.9 µg/mL using common headspace capillary GC. The higher sensitivity obtained by our method was probably because of the cryogenic trapping resulting in focusing and thus yielding much sharper peaks.

Actual measurements of isobutyl alcohol and methemoglobin in whole blood after sniffing isobutyl nitrite gas

Figure 2 shows an example of the chromatograms for isobutyl alcohol and the IS (1.0 µg/mL) in the whole blood of a volunteer who sniffed isobutyl nitrite gas with samplings imme-

diately and then 10 min after the sniffing. It was confirmed that no isobutyl nitrite peaks appeared in these chromatograms. Concentrations of isobutyl alcohol and methemoglobin in whole blood obtained from three volunteers are listed in Table V. From this table it is clear that the amount of isobutyl alcohol in blood rapidly decreases, probably by the action of liver alcohol dehydrogenase or elimination through breathing if the subject survives after the end of sniffing. However, methemoglobin tended to increase 10 min after sniffing in two subjects. However, because there are many victims who die during inhalation or after the ingestion of large amounts of alkyl nitrites (3), the blood levels of alkyl alcohols formed seems to remain high in such fatal cases in contrast to the cases of survivors.

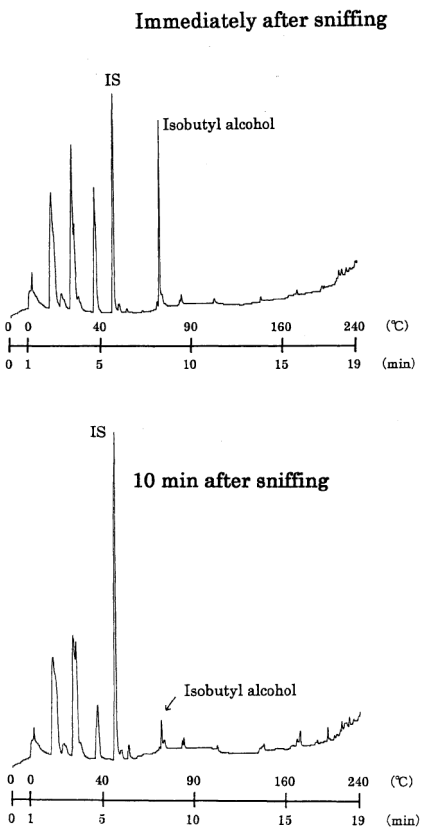

Figure 2. Headspace capillary gas chromatograms with cryogenic oven trapping obtained from the 1-mL human whole blood of a 55-year-old volunteer who sniffed isobutyl nitrite gas. The samplings of blood were made immediately (A) and then 10 min (B) after sniffing.

Table V. Concentrations of Isobutyl Alcohol and Methemoglobin in Whole Blood of Healthy Volunteers Who Sniffed Isobutyl Nitrite Gas*

Individual	Sex	Age	Time after sniffing (min)	Isobutyl alcohol (μg/mL)	Methemoglobin (%)
1	M	27	0	0.425	1.05
			10	0.066	ND†
2	M	36	0	0.345	4.00
			10	0.101	6.06
3	M	55	0	0.749	2.19
			10	0.063	4.08

* 754 cm^2 guaze (26 × 29 cm) soaked with 5 mL isobutyl nitrite placed in 28- × 20-cm polyethylene bag was used for sniffing for 2 min.
† ND, not detectable (lower than 0.01 μg/mL).

Conclusion

In this study, we have presented a detailed procedure for the sensitive assay of alkyl alcohols in whole blood and urine by headspace GC, which is coupled with cryogenic oven trapping. The latter device can be cheaply attached to any modern type of GC instruments and no special operation is needed. We can recommend this method for the detection of alkyl nitrite abuse in forensic science practice because of its high sensitivity and simplicity.

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