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Determination of nitrate in blood by gas chromatography and gas chromatography–mass spectrometry

Shigetoshi Kage[a], Keiko Kudo[b], Noriaki Ikeda[b,*]

[a]*Forensic Science Laboratory, Fukuoka Prefectural Police Headquarters, 7-7, Higashikoen, Hakata-ku, Fukuoka 812-8576, Japan*
[b]*Department of Forensic Pathology and Sciences, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

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Abstract

We devised a sensitive and simple method for determining nitrate in whole blood, using an extractive alkylation technique. Nitrate in whole blood was reduced to nitrite by hydrazine sulfate in the presence of Cu^{2+} and Zn^{2+} as catalysts, and alkylated with pentafluorobenzyl bromide using tetradecyldimethylbenzylammonium chloride as the phase-transfer catalyst. The obtained derivative was analyzed qualitatively by gas chromatography–mass spectrometry and quantitatively by gas chromatography with electron-capture detection. The detection limit of nitrate in whole blood was 0.01 m*M*. The calibration curve was linear over the concentration range from 0.02 to 1.0 m*M* for nitrate in whole blood. The accuracy and precision of the method were evaluated and the relative standard deviations were found to be within 10%. Using this method, the blood nitrate levels of two victims who committed suicide by inhaling automobile exhaust gas were determined.

Keywords: Nitrate

1. Introduction

Automobile exhaust gas contains carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (NO_x) and unburned hydrocarbons [1–3]. Many fatal cases of CO poisoning where people committed suicide by introducing exhaust gas into a car have been reported [4]. However, both exhaust gas from a gasoline engine in a warm start situation with well-functioning control and purification systems, and that from a diesel engine contain less than 0.06% CO [1–3], and therefore it is difficult to successfully complete a suicide attempt using such exhaust gas. There have been a few cases where the concentration of CO hemoglobin in the whole blood of the victim was below 10%, even though the victim was found dead in a car into which exhaust gas had been introduced. Therefore, a high level of NO_x in blood was expected to be a useful indicator of exhaust gas poisoning. Since NO_x, a mixture of nitrogen monoxide (NO) and nitrogen dioxide (NO_2), is rapidly converted to a mixture of nitrite and nitrate in whole blood, before being further oxidized to nitrate in 1 h [5–7], we attempted to determine nitrate levels in whole blood.

Numerous methods have been reported for determining nitrate in serum and plasma, including

*Corresponding author. Fax: +81-92-6426-126.
E-mail address: norii@forensic.med.kyushu-u.ac.jp (N. Ikeda)

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spectrometry [7–9], ion chromatography [6,10,11], liquid chromatography [12], gas chromatography (GC) [13,14], gas chromatography–mass spectrometry (GC–MS) [15,16] and capillary electrophoresis [17,18], however there are only a few methods for determining nitrate in whole blood [5,19,20].

We previously developed a sensitive and simple method to determine sulfide, cyanide, thiocyanate and polysulfides in whole blood by pentafluorobenzyl bromide as the alkylating agent using an extractive alkylation technique [21–23]. Using this technique, we tried to determine nitrate in whole blood.

2. Experimental

2.1. Reagents

Oxygen-free water was used throughout this study and was prepared by bubbling nitrogen into distilled water for 15 min.

A solution of internal standard (I.S.) was prepared by dissolving 1,3,5-tribromobenzene (TBB) in ethyl acetate to give a concentration of 0.01 m*M*. TBB was purchased from Wako Pure Chemical Industries (Osaka, Japan). An alkylating agent, pentafluorobenzyl bromide (PFBBr; Aldrich, Milwaukee, WI, USA), was dissolved in ethyl acetate with a concentration of 20 m*M*. Tetradecyldimethylbenzylammonium chloride (TDMBA), purchased from Tokyo Kasei Kogyo (Tokyo, Japan), was used as the counter-ion.

A stock standard solution of nitrate of 1000 ppm (16.13 m*M*) was obtained from Wako Pure Chemical Industries.

Hydrazine sulfate was purchased from Wako Pure Chemical Industries for use as the reduction agent of nitrate to nitrite. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) and cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Wako Pure Chemical Industries as catalysts for reduction. The other reagents used were of analytical grade.

2.2. Preparation of whole blood samples

Samples to be tested were prepared by adding the standard solution of nitrate to whole blood, which was collected from a healthy volunteer. A 0.4-ml volume of whole blood was mixed with 1.0 ml of acetonitrile, and the mixture was vortex-mixed and centrifuged in order to precipitate protein. The supernatant was evaporated to dryness, and the residue was dissolved in 0.4 ml of distilled water. The solution was centrifuged, and 0.2 ml of the supernatant was submitted to the following reduction and alkylation procedure.

2.3. Reduction and alkylation procedure

A 0.2-ml volume of 1 *M* sodium hydroxide was added to the supernatant in a 10-ml glass-stoppered test tube, followed by 0.2 ml of the solution containing 2 μg/ml Cu^{2+} and 40 μg/ml Zn^{2+}, and 0.1 ml of 0.14% hydrazine sulfate. The mixture was kept at 60°C in a water bath for 20 min for reduction of nitrate to nitrite.

To the above mixture containing nitrite, a 0.5-ml volume of 20 m*M* PFBBr solution in ethyl acetate, 2.0 ml of ethyl acetate containing 0.01 m*M* of I.S. (TBB) and 0.8 ml of 5 m*M* TDMBA solution in oxygen-free water saturated with sodium tetraborate were added for extractive alkylation. The preparation was vortex-mixed for 1 min at room temperature, maintained at 60°C in a water bath for 30 min, and then centrifuged at 1400 *g* for 15 min. A 0.1-ml aliquot of the supernatant was diluted with 2.0 ml of *n*-hexane to prevent any decrease in sensitivity with a concentrated solution of PFBBr, and a 0.2-μl aliquot of the solution was injected into the GC–electron-capture detection (ECD) apparatus. For GC–MS analysis, a 1-μl aliquot of the supernatant was used without dilution. GC–ECD was used for quantitative determination, while GC–MS was used for confirmation.

2.4. Preparation of calibration graphs

Whole blood samples were prepared to contain nitrate at concentrations of 0.02–1.0 m*M*. These samples were extracted and derivatized in the same manner as described above. Calibration graphs were obtained by plotting the peak-area ratio of the reaction product of nitrate to the I.S. versus the concentration of nitrate, using GC.

2.5. GC conditions

The apparatus used was a Shimadzu Model GC-14AE gas chromatograph (Kyoto, Japan), equipped with a ^{63}Ni ECD system, connected to a computerized recorder Shimadzu Model C-R5A Chromatopac. The column was a glass tube of 2.1 m×3 mm I.D. packed with 5% OV-225 on Uniport HP, 60–80 mesh. The temperatures of the column, the injection port and the detector were kept at 160, 220 and 220°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

2.6. GC–MS conditions

GC–MS was carried out on a Hewlett-Packard HP 5790A gas chromatograph (Palo Alto, CA, USA) interfaced to a JEOL AX505A mass spectrometer (Tokyo, Japan). The column was a J&W fused-silica capillary tube of DB-225 (30 m×0.32 mm I.D., 0.25 μm film thickness). A splitless injection mode was selected with a valve off-time of 1.5 min. The initial temperature of the column was held at 80°C for 3 min, and then programmed to rise at 10°C/min to 220°C. The injection port, separator and ion source were kept at 220, 200 and 220°C, respectively. Helium was used as the carrier gas at a flow-rate of 2 ml/min. The ionization energy of the positive-ion electron ionization (EI) condition was 70 eV. The ionization energy and reagent gas of the negative-ion chemical ionization (CI) conditions were 200 eV and isobutane, respectively.

3. Results

3.1. Analysis by GC

A gas chromatogram of alkylated extracts from whole blood adding 0.2 m*M* of nitrate is shown in Fig. 1.

Sharp and symmetrical peaks of the reaction product of nitrate and I.S. were observed, with retention times of 3.8 and 6.3 min, respectively.

The calibration curve was linear within the concentration range from 0.02 to 1.0 m*M* for nitrate in whole blood, with correlation coefficients of 0.999. The recoveries of nitrate in the whole blood at three different concentrations, 0.2, 0.4, 0.8 m*M*, were

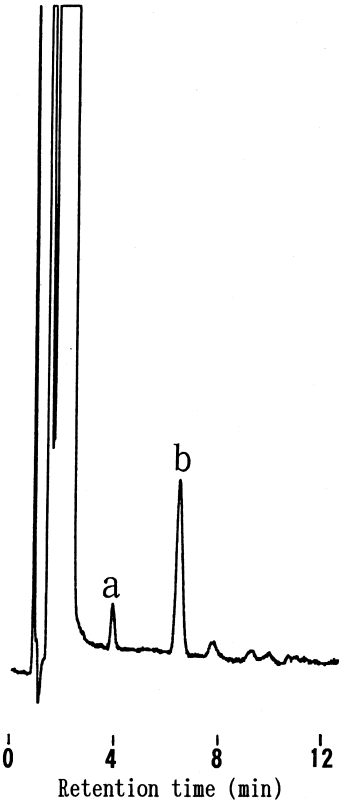

Fig. 1. Gas chromatogram of the alkylated extracts obtained from blood to which nitrate at the concentration of 0.2 m*M* had been added. (a) The derivative of nitrate, (b) TBB (I.S.).

determined by comparing the peak-area ratios of the reaction product of nitrate to TBB in blood samples with those in water samples, using GC. The gross recovery of nitrate from the whole blood was 60%. The lower limit of detection for nitrate in whole blood, based on a concentration giving a signal three-times stronger than the average noise intensity, was ca. 0.01 m*M*. Within- and between-day precisions were obtained using four different concentrations (0, 0.2, 0.4 and 0.8 m*M*) by adding nitrate to blank whole blood. The relative standard deviations (RSDs) ranged from 3.7 to 8.1% (Table 1).

3.2. Analysis by GC–MS

The derivatized extracts were also analyzed by GC–MS. Sharp and symmetrical peaks of the reaction product from nitrate and I.S. were observed, with retention times of 8.9 and 11.2 min, respective-

Table 1
Accuracy and precision of blood nitrate determination[a]

NO_3 added (m*M*)	Within-day (n=5)		Between-day (n=5)	
	Detected (m*M*) (mean±SD)	RSD (%)	Detected (m*M*) (mean±SD)	RSD (%)
0.00	0.11±0.008	7.9	0.09±0.007	8.1
0.20	0.29±0.020	7.1	0.29±0.021	7.2
0.40	0.48±0.038	8.0	0.49±0.033	6.6
0.80	0.90±0.033	3.7	0.88±0.041	4.6

[a] SD=Standard deviation, RSD=relative standard deviation.

ly. The mass spectra of the reaction product from nitrate and I.S. using positive-ion EI and negative-ion CI modes are shown in Figs. 2 and 3, respectively. Using the positive-ion EI mode, the molecular ion

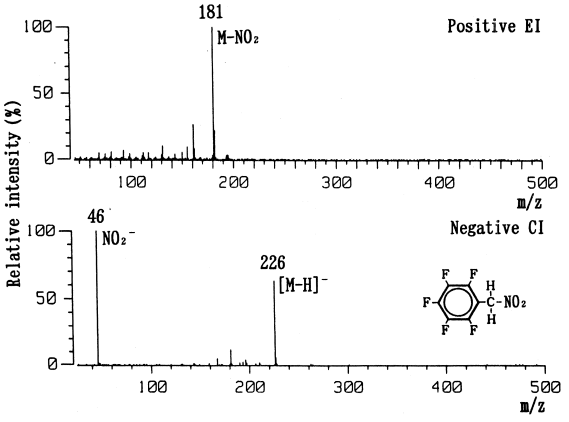

Fig. 2. Mass spectra of the derivative of nitrate.

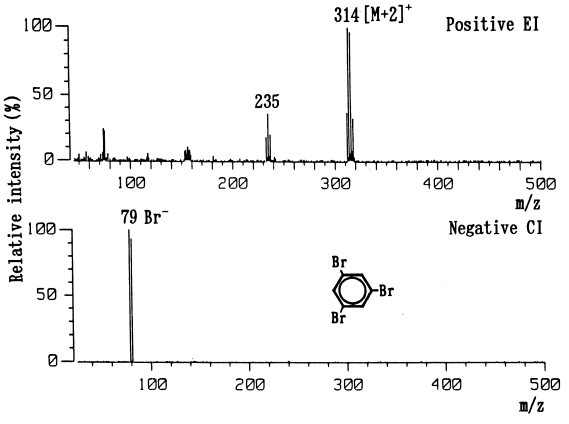

Fig. 3. Mass spectra of the internal standard, TBB.

of the reaction product from nitrate was not observed, but the base peak ion was observed at m/z 181 $[M-NO_2]^+$. Using the negative-ion CI mode, the base peak ion of the reaction product from nitrate was NO_2^- at m/z 46, the other ion being observed at m/z 226 $[M-H]^-$. The mass spectral pattern indicated that the obtained derivative was PFB-NO_2. The most abundant ion of TBB was observed at m/z 314 $[M+2]^+$, and the fragment ion was observed at m/z 235 $[M+2-Br]^+$ using the positive-ion EI mode. Using the negative-ion CI mode, the base peak ion of TBB was observed at m/z 79 $[Br]^-$.

The negative-ion CI mode proved to be useful for identification of the derivative based on its sensitivity and selectivity. The lower limit of detection for nitrate in whole blood was 0.005 m*M* using the negative-ion CI mode.

4. Discussion

Nitrite and nitrate in plasma or serum can be determined simultaneously by ion chromatography, however, ion chromatography provides only a retention time with which to identify these compounds, and therefore it is not suitable for forensic toxicological examinations. Derivatization of nitrate and/or nitrite followed by GC–MS analysis is a superior technique for the identification of these compounds. Several derivatization procedures for nitrate have been reported using benzene [14], 1,3,5-trimethoxybenzene [15] and mesitylene [13]. In these methods, nitrite was converted to nitrate with an oxidant prior to derivatization, and the total amount of nitrite and nitrate in serum or plasma was measured. Therefore, only nitrite in the sample must be determined using a different method.

As well as nitrate, nitrite can be also derivatized using an alkylating agent [16,24,25]. Tsikas and co-workers [16,25] reduced nitrate in human urine and plasma by cadmium to nitrite, and then nitrite was alkylated using PFBBr in acetone heated at 50°C for 60 min without using any counter-ion. Their method, however, produces harmful cadmium waste from the reductant. Chen et al. [24] reported a sensitive derivatization procedure for nitrite in human saliva with PFBBr by extractive alkylation with a counter-ion. Their method, however, requires

a long reaction time of over 3 h. In order to overcome the above problems, our extractive alkylation procedures for sulfide, polysulfides, cyanide and thiocyanate [21–23] were applied to nitrate analysis. In our method, nitrate in whole blood was reduced to nitrite by hydrazine sulfate [26,27], and alkylated in the same tube. The reaction time of derivatization was reduced to 30 min. Furthermore, as our method can directly alkylate nitrite in whole blood, the nitrite concentration in whole blood can be also determined. Therefore, the analytical problems described above were practically overcome.

5. Practical applications

We examined two blood samples collected from a 52-year-old woman and a 43-year-old man who had died after inhaling exhaust gas from a gasoline vehicle and a diesel vehicle, respectively. They were each found dead in a car, into which exhaust gas had been introduced from the exhaust pipe of the car via a vinyl hose. The concentrations of CO hemoglobin and ethanol in the blood samples were below 10% and not detected, respectively. The nitrate concentration in whole blood was 0.19 m*M* in the former, and 0.44 m*M* in the latter case; the levels were 1.9- and 4.4-times higher than the mean level of nitrate in healthy volunteers ($n=5$), 0.10 m*M* in blood, respectively. Nitrite was not detected in either of the samples from the victims nor in any of the samples from the volunteers.

Nitrate and nitrite concentrations in plasma, serum or whole blood in healthy volunteers are variously reported depends on the methods used; 0.044 and below 0.001 m*M* by high-performance liquid chromatography (HPLC)–UV [5], 0.0197 and 0.0042 m*M* by spectrometry [7], 0.061 and 0.0043 m*M* by ion chromatography [10], 0.068 and 0.0037 m*M* by GC–MS [16], 0.092 m*M* and not detected (below 0.017 m*M*) by capillary electrophoresis [17], respectively. Recently, a further analytical method based on HPLC has been reported by EI Menyawi et al. [12] in which the lowest level for the sum of nitrate and nitrite in serum of healthy volunteers, 0.0011 m*M*, was presented. In regard to the extremely low nitrate levels, discussions were done between Tsikas et al. [28] and EI Menyawi et al. [29].

Jungersten et al. reported that the plasma nitrate level was elevated from 0.029 to 0.313 m*M* within 60 min in volunteers ($n=8$) who took 500 mg potassium nitrate orally [30]. Automobile exhaust gas contains NO_x [1–3,31], at the levels of 50–60 ppm in a gasoline engine [31] and 120–220 ppm in a diesel engine [1,2,31]. Although there have been no reports on the levels of nitrate after inhaling NO_x in automobile exhaust, the high levels of nitrate in our cases were considered to be derived from inhaling the NO_x present in automobile exhaust. The cause of death of both cases were thought to be CO_2 accumulation and/or decrease of oxygen in the interior compartment of vehicles [1–3].

From our results, we conclude that the nitrate level in whole blood is useful as an indicator of having inhaled automobile exhaust gas, especially that from a diesel vehicle.

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