II.8.5 Nitrate and nitrite compounds

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Introduction

Nitrate and nitrite compounds are being used for various purposes, such as coronary artery dilators, coloring reagents for meat, rust preventives, fertilizers and explosives. When a nitrate salt is ingested by a human, nitric acid is converted into nitrous acid by the action of nitric acid-reducing bacteria inhabiting the upper digestive tract; the nitrous acid oxidizes the divalent ferrous ion of hemoglobin to produce methemoglobin [a] with the trivalent ferric ion. When methemoglobin is produced, its reduction into hemoglobin starts by the action of methemoglobin reductase (identical with cytochrome b_5 reductase requiring NADH as a cofactor) present in mammalian red cells. The concentration of methemoglobin in blood of normal subjects is not higher than 1–2 % [1]; in case of higher concentrations of methemoglobin, it is called methemoglobinemia. Especially in newborn babies and small infants, methemoglobinemia can easily take place, because of lower methemoglobin reductase activities in these periods.

Methemoglobin is not capable of transporting oxygen. When methemoglobinemia becomes severe, it causes cyanosis, headache, easy fatigability, respiratory and conciousness disturbances and finally death. The fatal methemoglobinemia is rare, but fatal ones after ingestion of nitrate or nitrite were reported [2–6].

For analysis of nitrate or nitrite in blood plasma or serum, the methods by ion chlomatography [7] and GC/MS [8] were reported. In this chapter, a method for GC analysis of nitrate and nitrite in blood using wide-bore and medium-bore capillary columns after their derivatization with mesitylene [b] is presented.

Reagents and their preparation

- Potassium nitrate is dried at 110 °C for 4 h; 6.35 mg of the resulting compound is dissolved in 10 mL purified water. The concentration of nitrate ion in this solution is 0.4 mg/mL.
- Sodium nitrite is dried by keeping it in a desiccator for 24 h; 4.5 mg of the compound is dissolved in 10 mL purified water to prepare 0.3 mg/mL (as nitrite ion) solution.
- A 1-mg aliquot of 4-nitro-*o*-xylene (Aldrich, Milwaukee, WI, USA) is dissolved in 1 mL acetone to give internal standard (IS) solution.
- A 1-mg aliquot of 2-nitromesitylene (Aldrich) is dissolved in 1 mL acetone to serve as stock standard solution for the final derivatized product of nitrate or nitrite.
- Silver acetate is dissolved in purified water to prepare its saturated solution.
- Anhydrous sodium carbonate, hydrogen peroxide and sulfuric acid to be used are of atomic absorption spectrometry grade or even purer.
- The purified water being used for the above preparations is prepared with the Milli Q device (Millipore Corp., Bedford, MA, USA). The nitrate or nitrite working standard solution should be prepared just before use.

GC conditions

Column: a DB-1 fused silica wide-bore capillary column (15 m × 0.53 mm i.d., film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA) and a DB-1 fused silica medium-bore capillary column (30 m × 0.25 mm i. d., film thickness 0.25 μm, J&W Scientific).

GC conditions; instrument: HP5890 Series II and HP6890 Series gas chromatographs[c] (Agilent Technologies, Palo Alto, CA, USA) with nitrogen-phosphorus detectors (NPD); injection volumes: 1 μL for the HP5890 Series II and 4 μL for the pulsed splitless injection with the HP6890 Series.

Conditions for the wide-bore capillary column:

Column (oven) temperature[d]: 100 °C → 5 °C/min → 125 °C (2 min); injection temperature: 250 °C; detector temperature: 270 °C; carrier gas: He; flow rate: 30 mL/min.

Conditions for the medium-bore capillary column:

Column (oven) temperature[d]: 110 °C (3 min) → 10 °C/min → 200 °C (3 min); injection temperature: 280 °C; detector temperature: 280 °C; carrier gas: He; flow rate: 2.1 mL/min.

Procedures

i. Analysis of nitrate

i. A 0.2-mL volume of a test specimen, 20 μL of IS solution and 0.2 mL of the saturated silver acetate solution[e] are placed in a 1.5-mL volume polypropylene tube with a cap, vortex-mixed and centrifuged at 3,000 rpm for 5 min.
ii. A 0.2-mL volume of the supernatant solution is transferred to another polypropylene tube of the same type, and 0.5 mL of concentrated sulfuric acid[f] is added to the solution slowly.
iii. A 0.5-mL volume of mesitylene (Aldrich) is added to the mixture, capped and vortex-mixed for 2 min.
iv. It is centrifuged at 3,000 rpm for 5 min. A 0.2-mL volume of the resulting upper layer is transferred to a new 1.5-mL volume polypropylene tube with a cap, followed by the addition of 20 mg of anhydrous sodium carbonate and vortex-mixed for 1 min.
v. After centrifugation at 3,000 rpm for 2 min, 1- or 4-μL of the upper layer is injected into GC.
vi. Various known amounts of nitrate and a fixed amount of IS are added to blank specimens and processed in the same way to construct a calibration curve. The peak area ratio of nitrate to IS obtained from a test specimen is applied to the calibration curve to calculate its concentration.

ii. Analysis of nitrite

i. A 0.2-mL volume of a test specimen and 0.1 mL of 0.1 M hydrogen peroxide solution are placed in a 1.5-mL volume polypropylene tube with a cap and vortex-mixed for 3 min.
ii. After centrifugation, the supernatant solution is decanted into another polypropylene tube of the same type; 20 μL of IS solution and 0.2 mL of the saturated silver acetate solution[e] are added to the above solution, vortex-mixed and centrifuged at 3,000 rpm for 5 min.
iii. A 0.2-mL volume of the supernatant solution is transferred to a new polypropylene tube of the same type, followed by addition of 0.5 mL of concentrated sulfuric acid[f] and 0.5 mL of mesitylene.

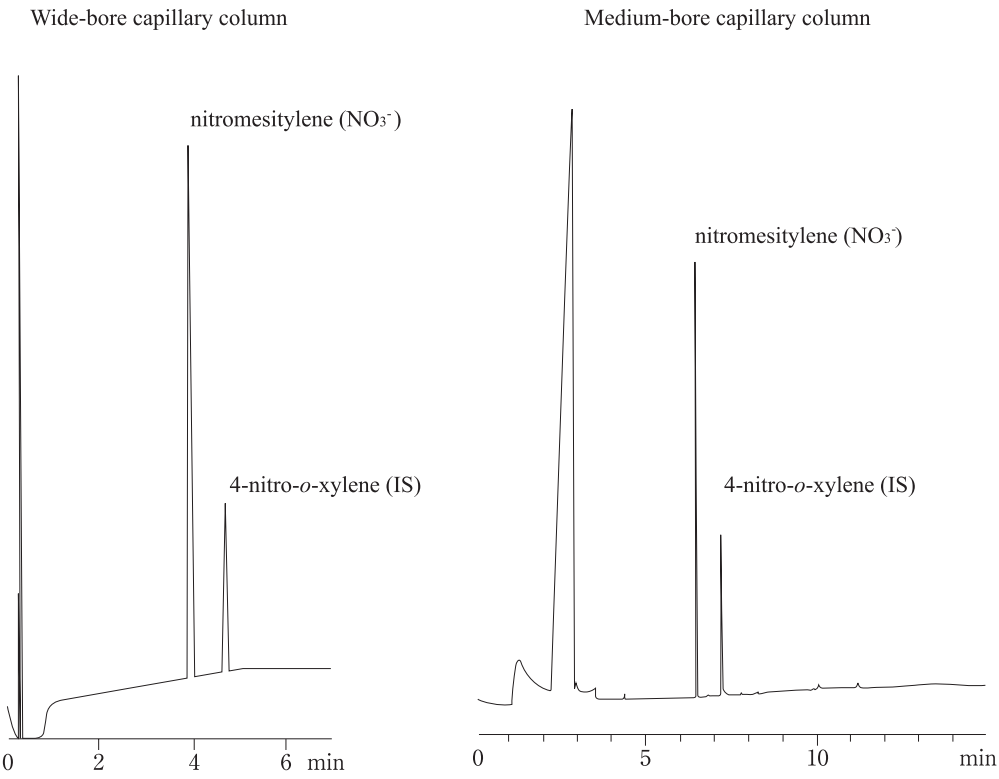
iv. The mixture is vortex-mixed for 2 min and centrifuged at 3,000 rpm for 5 min.
v. A 0.2-mL volume of the upper layer is transferred to a new polypropylene tube containing 20 mg anhydrous sodium carbonate and vortex mixed for 1 min.
vi. After centrifugation at 3,000 rpm for 2 min, 1- or 4-μL of the supernatant solution is injected into GC.
vii. The above procedure consists of the oxidation of nitrite into nitrate and the same extractions as those for nitrate. A calibration curve for nitrite using blank specimens and IS is also prepared for quantitation in the same way.

Assessment of the method

Figure 5.1 shows gas chromatograms using wide-bore and medium-bore capillary columns. With the medium-bore capillary column, a big and broad peak due to mesitylene appears.

The detection limits are about 0.3 and 0.1 μg/mL for wide-bore and medium-bore capillary columns, respectively. Since a fatal blood concentration of nitrite was reported to be 0.55 μg/mL [2], the present method is sensitive enough to detect it with either column.

Figure 5.1



Detection of nitrite by wide-bore and medium-bore capillary GC after its derivatization into nitromesitylene. The concentration of nitrite was 5 μg/mL for both chromatograms.

Except GC-NPD, GC-ECD can be used for analysis of nitrite and nitrate; but the latter detector may suffer from appearance of many interfering peaks. By GC/MS in the EI mode, it is difficult to detect the derivative, because of its lower sensitivity. Although there is a report [9] dealing with sensitive GC/MS analysis for nitrate in the NICI mode, it does not seem recommendable in view of the time required for stabilizing the instrument for reproducible analysis.

Toxic and fatal concentrations

In *Table 5.1*, clinical symptoms according to the concentrations of methemoglobin are presented.

Table 5.1

Clinical symptoms appearing according to methemoglobin concentrations

Methemoglobin concentration	Symptom(s)
not higher than 1–2 %	normal
10–15 %	cyanosis
not lower than 20 %	headache, dyspnea, tachypnea, tachycardia and hypertension
40–50 %	mental derangement, listlessness and metabolic acidosis
not lower than 50 %	coma, convulsive attack and hypotension
70 %	death

A poisoning case [4]

An unidentified male was found naked from the waist up in a park at about 10:00 p. m.; he was suffering from some difficulty in breathing. He was sent to a nearby clinic and diagnosed as exsiccosis; he was brought to a hospital, but he was found dead in the bed of the hospital in the next morning 7 h after admission. On the day of his death, autopsy was performed; there are no notable disorders, but the colors of all organs and blood were chocolate-brown. The blood methemoglobin concentration was thus measured; it was as high as 78 %. Since the fatal concentration of methemoglobin was reported to be about 70 % [1, 2, 5], the cause of his death was diagnosed as methemoglobinemia. The poison causing his methemoglobinemia was analyzed by the present method; it was disclosed that he had ingested nitrate. The analytical results for nitrite and nitrate in his blood and stomach contents are shown in *Table 5.2*. An example of the gas chromatograms in the present case is also shown in *Figure 5.2*. The blood nitrite concentration exceeded the fatal level (about 0.5 μg/mL), but nitrite could not be detected from stomach contents; a high concentration of nitrate is detected from stomach contents.

When a large amount of nitrite is ingested, a victim dies in a short time due to methemoglobinemia [2]. When nitrate is ingested, the compound cannot directly convert hemoglobin into methemoglobin; for such conversion, nitrate should be reduced to nitrite in a human body. In the present case, the victim had ingested a preparation including a large amount of nitrate, which was reduced to nitrite by the action of enteric bacteria; the nitrite resulted in accumulation of methemoglobin gradually. Similar methemoglobinemia cases had been sometimes observed after

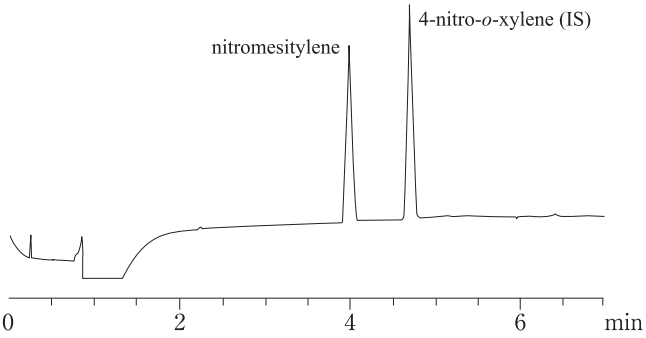
Table 5.2

Concentrations of nitrite and nitrate in blood and stomach contents obtained at autopsy

Specimen	Concentration (μg/mL)	
	nitrite	nitrate
blood	0.76	1.56
stomach contents	ND	20.3

ND = not detectable.

Figure 5.2



Gas chromatogram for nitrite plus nitrate in human blood obtained at autopsy. The blood specimen was oxidized to convert nitrite into nitrate, which was then derivatized for analysis by wide-bore capillary GC.

drinking well water containing high concentrations of nitrate salt [3, 10–12]. However, in the area where the victim was found, no similar poisoning cases were reported. Except such well water, a fertilizer product containing nitrate salt can be considered. However, a form or a product containing nitrate ingested could not be specified in the present poisoning case.

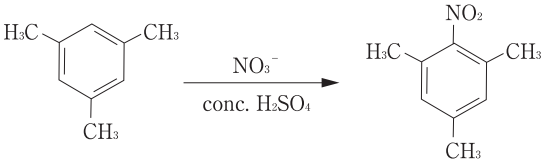
Notes

a) When nitrite is present in blood, methemoglobin is formed immediately. In severe poisoning cases, the concentrations of methemoglobin are more important than those of nitrate or nitrite. There is no problem when methemoglobin concentrations can be immediately measured with a CO oximeter. However, when the analysis has to be made later, it is necessary to store blood specimens at – 80 °C or even lower temperature until analyzed [9].

b) *Figure 5.3* shows derivatization reaction for nitrate being used in this method; 2-nitromesitylene formed is analyzed by GC. The same reaction method is used for analysis of nitrite after its conversion into nitrate. Therefore, when nitrate and nitrite coexist in a specimen, the difference in concentration between a total value after oxidation and a nitrate value shows the concentration of nitrite.

c) Any gas chromatograph for a capillary column equipped with a nitrogen-phosphorus detector can be used, regardless of its manufacturer and type.

Figure 5.3



mesitylene nitromesitylene

Derivatization reaction for nitrite and nitrate used in this method.

d) In this procedure, nitrate is derivatized during extraction procedure; the column temperatures are not so high, causing contamination of the column by impurities with high boiling points. When many specimens are analyzed, the column temperature should be frequently elevated to its maximum to remove impurities.
e) In this method, chloride can be considered as the most prominent interfering substance in human specimens. It is absolutely necessary to remove chloride by its precipitation at an early step; for this purpose, the saturated silver acetate solution is added.
f) When sulfuric acid is added to the mixture, appreciable heat is produced. The addition of the acid should be as slow as possible, followed by airtight capping of the polypropylene tube. Upon nitrification with vortex-mixing, rubber gloves should be worn, considering the rare accident of leakage of the mixture due to the exothermic phenomenon. However the author et al. have no experience of such accidents.

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