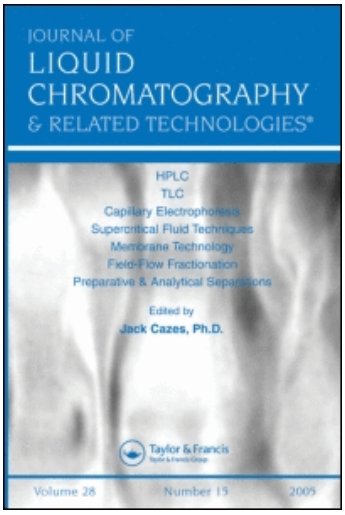


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Simultaneous Analysis of Nitrite and Nitrate in Whole Blood by Ion Chromatography

Osamu Suzuki[a]; Kanako Watanabe[a]; Naoko Okamoto[a]; Hideki Nozawa[a]; Akira Ishii[b]
[a] Department of Legal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan [b] Department of Legal Medicine, Fujita Health University School of Medicine, Toyoake City, Aichi, Japan

**To cite this Article** Suzuki, Osamu , Watanabe, Kanako , Okamoto, Naoko , Nozawa, Hideki and Ishii, Akira(2005) 'Simultaneous Analysis of Nitrite and Nitrate in Whole Blood by Ion Chromatography', Journal of Liquid Chromatography & Related Technologies, 28: 19, 3077 — 3085
**To link to this Article: DOI:** 10.1080/10826070500295179
**URL:** http://dx.doi.org/10.1080/10826070500295179

*Journal of Liquid Chromatography & Related Technologies*®, 28: 3077–3085, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500295179

# Simultaneous Analysis of Nitrite and Nitrate in Whole Blood by Ion Chromatography

**Osamu Suzuki, Kanako Watanabe, Naoko Okamoto, and Hideki Nozawa**
Department of Legal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan

**Akira Ishii**
Department of Legal Medicine, Fujita Health University School of Medicine, Toyoake City, Aichi, Japan



**Abstract:** A simple and modern method for simultaneous analysis of nitrite and nitrate in whole blood has been devised by ion chromatography with an autosuppressor and a conductivity detector. To our knowledge, this is the first report for such analysis dealing with whole blood as a specimen. A 1 mL volume of whole blood was mixed with 2 mL of Milli Q water, vortex-mixed for hemolysis, and ultrafiltered to obtain a clear extract solution. It was passed through double connected mini-cartridges of silver resin and a silver absorbing material to remove a high concentration of chloride ion usually present in whole blood. An aliquot of the filtrate was analyzed on a Dionex DX-500 instrument with a microbore IonPac® AS9-HC column ($2 \times 250$ mm). The present method gave very low backgrounds for blank whole blood at locations where nitrite should appear, and around the endogenous peak of nitrate. The spiked nitrite markedly decreased in whole blood, probably by the action of hemoglobin in vitro; it was only 10–20% 2 h after the addition. Therefore, quantitative validation had to be made only for nitrate. The recoveries of nitrate from whole blood were more than 90%; the linearity was confirmed in the range of 5–100 $\mu g\,mL^{-1}$ for whole blood. Detection limit of nitrate in whole blood was about 0.5 $\mu g\,mL^{-1}$. Coefficients of intra- and inter-day variations were 3.66–7.14%. Using the present method, nitrite and nitrate concentrations were measured for human blood specimens after sniffing isobutyl nitrite gas in two male subjects.

Address correspondence to Osamu Suzuki, Department of Legal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan. E-mail: suzuko@hama-med.ac.jp

The endogenous nitrate concentrations without such sniffing were 1.27 to 7.60 $\mu g\,mL^{-1}$ for seven subjects.

**Keywords:** Nitrite, Nitrate, Ion chromatography, Whole blood, Silver resin cartridge

## INTRODUCTION

Nitrite and nitrate compounds are being widely used for various purposes, such as coronary artery dilators, coloring reagents for meat, rust preventives, fertilizers, and explosives. Forensic chemists occasionally encounter fatalities due to methemoglobinemia caused by ingestion of nitrite[1] or nitrate.[2] Nitrite and/or nitrate in biomedical specimens is being analyzed by various types of ion chromatography,[3–8] by gas chromatography,[2] and by gas chromatography-mass spectrometry.[9,10] Among the above methods, ion chromatography is most common for simple analysis of nitrite and/or nitrate. In such analysis, the trace levels of nitrite is especially interfered by very high levels (about 5,500 $\mu g\,mL^{-1}$) of chloride anion, because both anions appear at similar retention times.[3,5–7] In this study, we have devised a new and simple technique for removal of the high levels of chloride in biomedical specimens.

All previous reports dealt with only serum[3,4,6,7] or plasma[5] specimens as starting materials. However, it seems essential to test whole blood for analysis of nitrite and nitrate, because every drug or poison ingested is primarily absorbed into whole blood.

The present study provides a simple and modern method for simultaneous analysis of nitrite and nitrate in human whole blood by ion chromatography equipped with an autosuppressor, a conductivity detector, and a microbore anion exchanger column.



## EXPERIMENTAL

### Materials

Sodium nitrite and potassium nitrate were purchased from Aldrich (Milwaukee, WI, USA); isobutyl nitrite from Tokyo Kasei Kogyo (Tokyo, Japan); anion mixture standard solution IV from Kanto Chemical (Tokyo, Japan); centrifuge tubes for ultrafiltration (Amicon® Ultra-4) from Millipore (Bedford, MA, USA); 1 cc cartridges of OnGuard®II Ag and OnGuard®II H from Dionex (Sunnyvale, CA, USA). The Milli Q water was prepared with its device Milli-Q Labo (Millipore).

### Procedure

Two new Amicon® Ultra-4 filter tubes were washed by adding 2 mL each of Milli Q water to each upper compartment, centrifuging at 3,000 rpm for

10 min and discarding the filtrate water; this pretreatment was very useful to remove sulfate and chloride ions being included in the filter. A 1 mL volume of test whole blood was mixed with 2 mL of Milli Q water and vortex-mixed for 30 s. The 3 mL hemolysate was divided into two fractions (1.5 mL each); both were subjected to the ultrafiltration with the above washed filter tubes (4,000 rpm, 15 min). With this procedure, about 2 mL volume of the filtrate could be obtained in total, and was carefully passed through the double connected mini-cartridges (1 cc) of OnGuard® II Ag plus OnGuard® II H using a 2 mL plastic disposable syringe. Upon passage through the cartridges, care should be taken not to pour air bubbles into the cartridges. A 5 μL aliquot of the final filtrate was subjected to analysis by ion chromatography.

## Ion Chromatographic Conditions

Samples were analyzed on a Dionex DX-500 ion chromatographic system (Dionex) equipped with an IonPac® AG9-HC guard column (2 × 50 mm), an IonPac (AS9-HC separation column (2 × 250 mm), an autosuppressor, and a conductivity detector. The mobile phase was 9 mM $Na_2CO_3$ solution at a flow rate of 0.3 mL $min^{-1}$ in the isocratic mode. The SRS current was 30 mA.



## Calibration Curves

Quantitative analysis was made by the external calibration method using peak areas. Since nitrite was found unstable in whole blood, its solutions at various concentrations (1–100 μg $mL^{-1}$ g, 13 plots) were prepared using Milli Q water, followed by the pretreatment as described above. For nitrate, various concentrations (5–100 μg $mL^{-1}$, 11 plots) of it were spiked into whole blood samples and pretreated prior to ion chromatographic analysis.

## Whole Blood Specimens and Human Experiments

For sampling human whole blood specimens, vacuum glass tubes containing heparin were used. To measure endogenous levels of nitrate in whole blood, the specimens were drawn from 5 healthy males and 2 females between ages of 25–57 years old.

Two male volunteers (26 and 57 years) participated in human experiments. About 1 mL volume of isobutyl nitrite was put in each plastic bag (38 × 50 cm). Blank whole blood specimens were sampled from the cubital vein just before sniffing. The two subjects lightly sniffed the gas for 5 min with care not to affect their health. Blood specimens were sampled in the

same way immediately, and after 10 min of the end of sniffing. The dilution of the specimen and ultrafiltration were started just after each sampling.

## RESULTS

### Optimization of Analytical Conditions

Using an anion standard mixture, separation of anions, including chloride, nitrite, and nitrate, was tested for various types of microbore separation columns, such as IonPac® AS15, IonPac® AS12 and IonPac® AS9-HC (all purchased from Dionex). The IonPac® AS9-HC was the best for the separation between chloride and nitrite; it was difficult to separate them with the IonPac® AS15 column. Therefore, the IonPac® AS9-HC column was adopted in this study.

For deproteinization by ultrafiltration, various filters, such as Ultrafree-CL-LCG, Centricon YM-10, Microcon-10 and Amicon® Ultra-4 (all from Millipore), were tested for diluted whole blood solutions. In view of the price, time required, and rotation speed, the Amicon® Ultra-4 gave the best results.

Human body fluids usually contain very high concentrations of chloride anion, which interfere with the peak of nitrite. To remove the chloride ion, silver acetate or silver fluoride was added to the diluted whole blood solution; but both gave intense peaks of acetate or fluoride overlapping the nitrite peak and also other peaks. To overcome this problem, the use of double-connected mini-cartridges (1 cc) of OnGuard®II Ag plus OnGuard® II H was found very useful to remove chloride anion from blood extract. The OnGuard® II H cartridge was used to remove $Ag^+$ leaking from the first cartridge; without the latter cartridge, the leakage of $Ag^+$ caused serious contamination of the separation column and the autosuppressor.

### Reliability of the Method

Figure 1 shows ion chromatograms obtained by analysis just after and 2 h after spiking nitrite ion ($NO_2^-$) into blood at the concentration of 10 $\mu g\,mL^{-1}$. Immediately after the spiking, distinct peaks due to nitrite and nitrate ions appeared at 7.2 and 11.4 min of retention times, respectively (Fig. 1, upper panel); the nitrate peak may be composed of the endogenous ion and newly produced ion from nitrite. After leaving the spiked blood at room temperature for 2 h, the nitrite peak became very small and the nitrate peak larger (Fig. 1, lower panel). Therefore, we followed the changes of nitrite and nitrate ions after spiking 10 $\mu g\,mL^{-1}$ nitrite ion into human whole blood at room temperature. The results are shown in Fig. 2. It seemed evident that nitrite rapidly converted into nitrate in whole blood in vitro.

Because of such rapid conversion of nitrite into nitrate, it was difficult to make further quantitative analysis for the nitrite ion by spiking it into whole

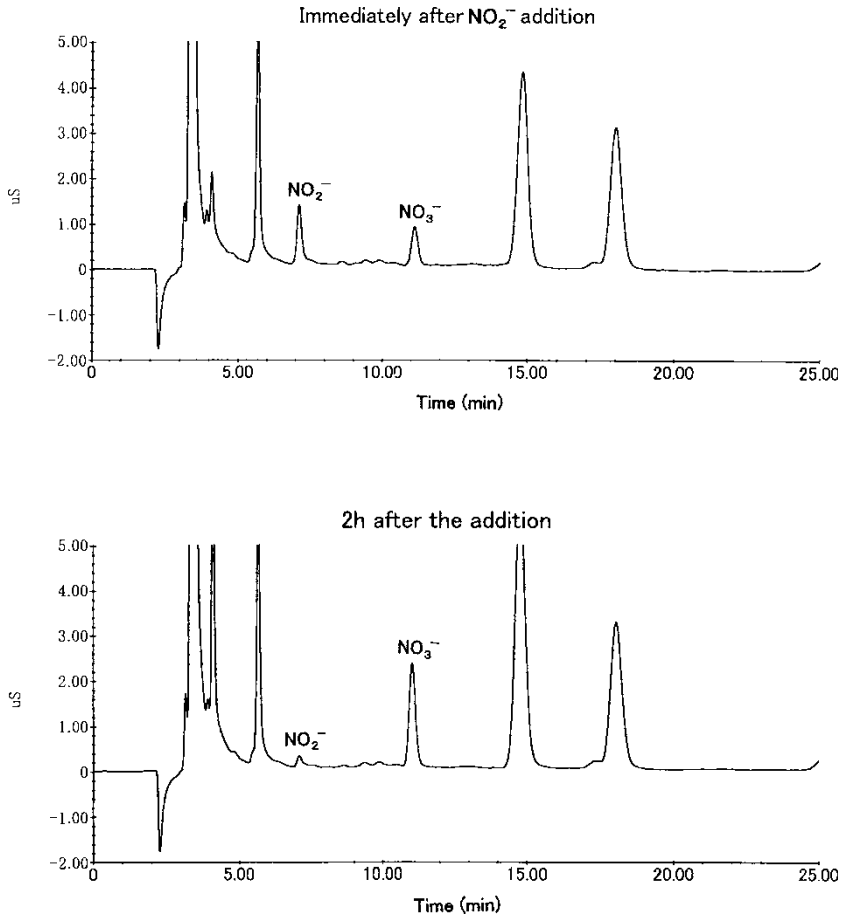




***Figure 1.*** Ion chromatograms for extracts of whole blood, into which 10 $\mu g\,mL^{-1}$ of $NO_2^-$ had been spiked. Upper panel: analytical procedure was started just after the spiking; lower panel: the procedure started after leaving the spiked whole blood at room temperature for 2 h.

blood. For nitrate ion, however, its recovery rates from whole blood were 94.4 $\pm$ 8.9 and 93.8 $\pm$ 8.1% ($n = 10$ for both) at the concentrations of 5 $\mu g$ and 50 $\mu g\,mL^{-1}$, respectively.

A calibration curve was constructed after pretreatment of different 13 concentrations of the authentic nitrite dissolved in Milli Q water in the range of 1–100 $\mu g\,mL^{-1}$; its equation and correlation coefficient were: $y = 2.72 \times 10^4\,x - 5.71 \times 10^4$ and $r = 0.996$, where $y$ is the peak area unit of our data analysis system; $x$ the concentration of nitrite anion expressed as $\mu g\,mL^{-1}$. The corresponding curve for nitrite in whole blood was not tested, because of its instability in the matrix.

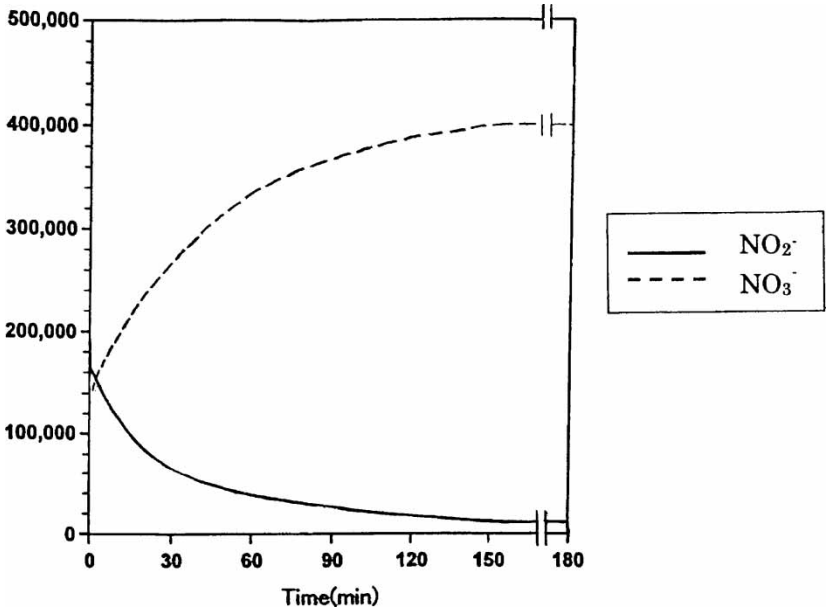


***Figure 2.*** Time courses of the levels of nitrite and nitrate in whole blood after spiking $10\,\mu g\,mL^{-1}$ of nitrite anion. The vertical axis was expressed as peak area units of our data analysis system.



The equations and correlation coefficients for calibration curves of nitrate dissolved in Milli Q water and whole blood were: $y = 2.68 \times 10^4 x - 4.33 \times 10^4$ and $r = 0.997$ ($1–100\,\mu g\,mL^{-1}$, 13 plots); $y = 1.96 \times 10^4 x + 1.45 \times 10^4$ and $r = 0.991$ ($5–100\,\mu g\,mL^{-1}$, 11 plots), respectively.

Detection limits (signal-to-noise ratio = 3) were about $20\,ng\,mL^{-1}$ for both nitrite and nitrate anions present in Milli Q water with the same pretreatment procedure, and about $0.5\,\mu g\,mL^{-1}$ for nitrate in whole blood.

The precision for nitrate spiked into whole blood was tested as shown in Table 1. The relative standard deviations were less than 10% for every concentration.

***Table 1.*** Precision data for nitrate spiked into whole blood measured by the present method

| Amount spiked ($\mu g\,mL^{-1}$) | Relative standard deviation (%) | |
|---|---|---|
| | Intra-day | Inter-day |
| 0 | 9.21 | 5.13 |
| 5.0 | 3.83 | 7.14 |
| 50 | 3.88 | 3.66 |

*Table 2.* Endogenous nitrate levels in whole blood of some healthy volunteers

| Subject no. | Sex | Age | $NO_3^-$ concentration ($\mu g\,mL^{-1}$) |
|---|---|---|---|
| 1 | M | 57 | 2.62 |
| 2 | M | 38 | 3.58 |
| 3 | M | 26 | 7.60 |
| 4 | M | 53 | 2.85 |
| 5 | F | 33 | 1.27 |
| 6 | F | 41 | 1.70 |
| 7 | M | 25 | 1.74 |
| Mean $\pm$ SD | | | 3.05 $\pm$ 2.16 |

## Actual Measurements of Nitrite and Nitrate in Human Whole Blood



Table 2 shows endogenous nitrate levels in whole blood of seven healthy subjects. They ranged from 1.27 to 7.60 $\mu g\,mL^{-1}$ with a mean value of 3.05 $\mu g\,mL^{-1}$. Nitrite levels were below the detection limit (about 0.2 $\mu g\,mL^{-1}$) in every sample.

Since alkyl nitrites easily decompose to alkyl alcohols and inorganic nitrite,[11] two male volunteers lightly sniffed the vapor of isobutyl nitrite. Whole blood was sampled before the sniffing, immediately after, and 10 min after the end of sniffing. As shown in Table 3, nitrate levels increased for both subjects just after the sniffing; 10 min after the end of sniffing they decreased slightly. For nitrite, it appeared in whole blood obtained immediately after the sniffing only for Subject 1; but it disappeared in 10 min. For Subject 3, nitrite could not be detected in all specimens.

*Table 3.* Levels of nitrate and nitrite in whole blood after sniffing the vapor of isobutyl nitrite in two volunteers

| Subject no. | Sampling time | Concentration ($\mu g\,mL^{-1}$) | |
|---|---|---|---|
| | | $NO_3^-$ | $NO_2^-$ |
| 1 | Before sniffing | 2.62 | ND[a] |
| | Immediately after | 3.90 | 3.23[b] |
| | 10 Minutes after | 3.73 | ND |
| 3 | Before sniffing | 7.60 | ND |
| | Immediately after | 10.3 | ND |
| | 10 Minutes after | 9.68 | ND |

[a]ND: not detectable.

[b]The value was tentatively obtained using a calibration curve with Milli Q water.

## DISCUSSION

In the present paper, we have presented the most modern ion chromatographic method for simultaneous analysis of nitrite and nitrate in whole blood; it employs an autosuppressor and a microbore separation column. The system is useful for getting low and stable backgrounds and better resolution ability. In addition, it has enabled us to detect a small peak of nitrite by the conductimetric method after removing chloride ion abundantly present in human specimens, with use of small cartridges of silver resin and silver remover. There are a few reports dealing with ion chromatographic methods for simultaneous analysis of nitrite and nitrate in human serum;[3,6,7] their peaks were exclusively detected by ultraviolet absorption detectors, because closely appearing chloride ion shows almost no absorption. However, the chromatograms in these reports[3,6,7] suffered from high or unstable background levels due to impurities.

Although nitrite is stable in human serum or plasma, this is not the case in whole blood. When ingested nitrite is absorbed into the blood stream, it reacts with oxyhemoglobin to form a hemoglobin-nitrite complex.[12] Within this complex, oxygen from oxyhemoglobin reacts with nitrite to form nitrate with concomitant oxidation of ferrous heme iron ($Fe^{2+}$) into the ferric form ($Fe^{3+}$). Therefore, nitrite is very unstable in the presence of hemoglobin; this is the reason why there are no reports on the methods for analysis of nitrite in whole blood. On the other hand, it should be also mentioned that nitrate is converted into nitrite by intestinal bacteria.[13] Therefore, interconversion between nitrite and nitrate can take place in the human body. There were forensic cases, in which both nitrite and nitrate could be detected from whole blood specimens, especially when large amounts of nitrite or nitrate were ingested;[1,2] however, the data on nitrite levels should be regarded as being only qualitative.

In fatal nitrite or nitrate poisoning cases, the direct cause of death is methemoglobinemia; when more than 50% of total hemoglobin is converted into methemoglobin, there is a danger for life,[14] because methemoglobin is not capable of transporting oxygen. However, it should be pointed out that many other compounds, such as chlorate, chlorite, nitroglycerin, nitrobenzene, and arylamine derivatives, can also cause methemoglobinemia.[15] To finally judge a causative poison, the present ion chromatographic method for nitrite and nitrate seems useful, because it is simple, rapid, and reliable.

## REFERENCES

1. Standefer, J.C.; Jones, A.M.; Street, E.; Insera, R. Death associated with nitrite ingestion: report of a case. J. Forensic Sci. **1979**, *24*, 768–771.
2. Saito, T.; Takeichi, S.; Osawa, M.; Yukawa, N.; Hung, X.-L. A case of fatal methemoglobinemia of unknown origin but presumably due to ingestion of nitrate. Int. J. Legal Med. **2000**, *113*, 164–167.

3. Michigami, Y.; Yamamoto, Y.; Ueda, K. Determination of nitrite, sulphate, bromide and nitrate in human serum by ion chromatography. Analyst **1989**, *114*, 1201–1205.
4. Salas-Auvert, R.; Colmenarez, J.; de Ledo, M.H.; Colina, M.; Gutierrez, E.; Bravo, A.; Soto, L.; Azuero, S. Determination of anions in human and animal tear fluid and blood serum by ion chromatography. J. Chromatogr. A **1995**, *706*, 183–189.
5. Everett, S.A.; Dennis, M.F.; Tozer, G.M.; Prise, V.E.; Wardman, P.; Stratford, M.R.L. Nitric oxide in biological fluids: analysis of nitrite and nitrate by high-performance ion chromatography. J. Chromatogr. A **1995**, *706*, 437–442.
6. Monaghan, J.M.; Cook, K.; Gara, D.; Crowther, D. Determination of nitrite and nitrate in human serum. J. Chromatogr. A **1997**, *770*, 143–149.
7. El Menyawi, I.; Looareesuwan, S.; Knapp, S.; Thalhammer, F.; Stoiser, B.; Burgmann, H. Measurement of serum nitrite/nitrate concentrations using high-performance liquid chromatography. J. Chromatogr. B **1998**, *706*, 347–351.
8. Helaleh, M.I.H.; Korenaga, T. Ion chromatographic method for simultaneous detemination of nitrate and nitrite in human saliva. J. Chromatogr. B **2000**, *744*, 433–437.
9. Kage, S.; Kudo, K.; Ikeda, N. Determination of nitrate in blood by gas chromatography and gas chromatography-mass spectrometry. J. Chromatogr. B **2000**, *742*, 363–368.
10. Kage, S.; Kudo, K.; Ikeda, N. Simultaneous determination of nitrate and nitrite in human plasma by gas chromatography-mass spectrometry. J. Anal. Toxicol. **2002**, *26*, 320–324.
11. Watanabe-Suzuki, K.; Nozawa, H.; Suzuki, O.; Ishii, A. 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. J. Chromatogr. Sci. **2003**, *41*, 63–66.
12. Rodkey, F.L. A mechanism for the conversion of oxyhemoglobin to methemoglobin by nitrite. Clin. Chem. **1976**, *22*, 1986–1990.
13. Comly, H.H. Cyanosis in infants caused by nitrates in well water. J.A.M.A **1945**, *129*, 112–116.
14. Saito, T.; Takeichi, S.; Yukawa, N.; Osawa, M. Fatal methemoglobinemia caused by liniment solutions containing sodium nitrite. J. Forensic Sci. **1996**, *41*, 169–171.
15. Roth, M. Inorganic Anions. In *Analytical Toxicology for Clinical, Forensic and Pharmaceutical Chemists*; Brandenberger, H., Maes, R.A.A., Eds.; Walter de Gruyter: Berlin, 1997; 269–280.

Received March 31, 2005
Accepted May 17, 2005
Manuscript 6623