

Toxicological analysis of azide and cyanide for azide intoxications using gas chromatography

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Abstract

Azide is a highly toxic chemical agent to human being. Accidental, but also intentional exposure to azide occurs. To be able to confirm azide ingestion, we developed a method to identify and quantify azide in biological matrices. Cyanide was included in the method to evaluate suggested in vivo production of cyanide after azide ingestion. Azide in biological matrices was first derivatized by propionic anhydride to form propionyl azide. Simultaneously, cyanide was converted into hydrogen cyanide. After thermal rearrangement of propionyl azide, ethyl isocyanate was formed, separated together with hydrogen cyanide by gas chromatography (GC) and detected using a nitrogen phosphorous detector (NPD). The method was linear from 1.0–100 µg/mL for both analytes, and azide was stable in human plasma at –20°C for at least 49 days. Azide was measured in the gastric content of two cases of suspected azide ingestion (case 1:1.2 mg/mL, case 2:1.5 mg/mL). Cyanide was only identified in the gastric content of case 1 (approximately 1.4 µg/mL). Furthermore, azide was quantified in plasma (19 µg/mL), serum (24 µg/mL), cell pellet (21 µg/mL) and urine (3.0 µg/mL) of case 2. This method can be used to confirm azide and cyanide exposure, and azide concentrations can be quantified in several biological matrices.

KEYWORDS

azide, cyanide, gas chromatography, nitrogen phosphorous detector, toxicology

1 | INTRODUCTION AND BACKGROUND

Sodium azide (NaN₃) is a toxic chemical agent with applications in industry, agriculture, medicine, safety products and research laboratories. It has been used in automobile airbags,

explosives, for the production of rubber, but also in laboratories as a preservative for reagents.^{1,2} In the past, it has been used for the treatment of hypertension, but quickly abandoned because of severe side effects.^{3,4}

Exposure to azide is highly poisonous for human being. Work accidents in laboratories and the manufacturing industry are main causes of exposure to azide, but intentional exposure

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such as poisonings and suicides occur as well. The most common route of exposure is through oral ingestion followed by inhalation and direct skin contact. Azide is very soluble in water and is rapidly converted into the gas hydrazoic acid in an acidic environment. After exposure, absorption of azide or hydrazoic acid gas is fast. It is metabolized by the liver into nitrogen oxide, and azide is excreted by the kidneys. Early symptoms of exposure to azide are hypotension, tachycardia, dyspnoea, headache, a decreased mental status, nausea, diarrhoea and loss of vision. These symptoms occur within minutes after exposure. Severe and fatal effects include hypothermia, seizure, coma, cardiac arrhythmia, pulmonary oedema, metabolic acidosis and cardiopulmonary failure. These effects have a later onset and can occur between 1 hour up to days. The lowest reported lethal dose is 700 mg (10 mg/kg).^{5,6}

The exact mechanism of action is not fully understood. Azide is an inhibitor of cytochrome oxidase and forms a strong complex with haemoglobin preventing oxygen transport in blood. It induces hypotension by dilating the peripheral blood vessels, it is an inhibitor of platelet aggregation, and it stimulates the cardiac muscles which results in tachycardia. Besides the effects on the cardiovascular system, it causes an enhanced excitatory transmission in the central nervous system after conversion to nitric oxide. This modulates the cholinergic system resulting in neurotoxicity.⁷

In the past few years, the attention for azide as a suicide drug in The Netherlands is raising. Therefore, increasing numbers of cases of azide toxicity are expected. Accordingly, there is a growing relevance and importance to develop methods for the identification and quantification of azide. A few methods have been described in the literature for the determination of azide in biological matrices,⁸⁻¹⁷ but not for the combined analysis of azide and cyanide using the same procedure. Several reports found elevated levels of cyanide in blood or plasma after azide ingestion.^{9,10,14,18,19} The metabolic pathway and whether the production of cyanide took place in vivo or post-mortem is unknown. Moreover, most of these methods lack specificity, involve long procedures, interfere with other anions or use a headspace injection system for gas chromatography (GC), which is not widely available. We aimed to develop a GC method coupled to a nitrogen phosphorous detector (NPD) for the simultaneous detection of azide and cyanide in several biological matrices. Moreover, we aimed to apply the method for the measurement of azide and cyanide in biological samples in two lethal cases with suspected azide ingestion.

2 | CASE REPORTS

2.1 | Case 1

This patient (female, 26 years old) was found at home unconscious 1-2 hours after an alarming farewell text message and

the associated intake of an unknown toxic substance. During transport, rapid circulatory deterioration was observed. Upon arrival at the emergency department, circulatory arrest necessitated the initiation of cardiopulmonary resuscitation (CPR). Laboratory assessment revealed a combined acidosis with a pH of 6.7 (7.35-7.45), bicarbonate of 10.8 mmol/L (22-29 mmol/L), pCO₂ of 56 mm Hg (35-48 mm Hg), a plasma lactate level above the detection limit of 20 mmol/L (0.5-1.7 mmol/L) and a plasma potassium level of 11.2 mmol/L (3.5-5.0 mmol/L). Despite aggressive resuscitation, including the administration of calcium gluconate, insulin/glucose and sodium bicarbonate, spontaneous circulation did not return. Echocardiography did not demonstrate any cardiac movement. Further treatment was therefore withheld 1 hour after the initiation of CPR. The combination of rapid deterioration of vital parameters in combination with an extremely high lactate level prompted the medical team to consider toxicity of azide or another substance that interfered with the mitochondrial electron transport chain as the cause of death. The gastric content was collected shortly after death and sent for toxicological assessment.

2.2 | Case 2

This patient (female, 41 years old) was found at home after an alarming farewell e-mail. Upon arrival, several documents informed the paramedics that the patient had ingested azide. Shortly before and during transport, progressive unconsciousness and circulatory instability developed. Approximately 2 hours after ingestion, the patient arrived at the emergency department where she was intubated and subsequently transported to the intensive care unit. Resuscitation included the administration of isotonic crystalloids and high dosage adrenalin. On theoretical grounds, sodium thiosulphate (15 g) and hydroxocobalamin (5 g) were administered. Despite all treatments, the patient died approximately 4.5 hours after ingestion of azide. Plasma, serum, red blood cell pellet, urine and gastric content were collected at the intensive care (IC) 3 to 4.5 hours after the supposed azide intake.

3 | MATERIALS AND METHODS

3.1 | Materials

Sodium azide was purchased from Merck and sodium cyanide, propionitrile, ethyl isocyanate and propionic anhydride were purchased from Sigma-Aldrich. Tert-butyl methyl ether (TBME) (ULC-MS grade) was supplied from Biosolve Ltd, and water (WFI) was obtained from B.Braun. Control human K₂EDTA plasma was from BioIVT.

3.2 | Standards

Separate stock solutions of sodium azide and sodium cyanide were prepared in water at a concentration of 1.5 mg/mL. From the stock solutions, combined working solutions were prepared in water at concentrations of 20, 100 and 500 µg/mL for preparation of calibration standards and at concentrations of 20, 60 and 200 µg/mL for preparation of quality control samples. Working solutions were further diluted in blank human K₂EDTA plasma to obtain calibration standards at concentrations of 1, 5, 25 and 100 µg/mL and quality control samples at concentrations of 1, 3, 10 and 75 µg/mL. An internal standard stock solution was prepared by dissolving 13 µL propionitrile in 100 mL TBME which gave a concentration of 101.4 µg/mL (corrected for density). This stock solution was further diluted to obtain an internal standard working solution of 20 µg/mL in TBME. The prepared stock and working solutions were stored at -20°C, and calibration standards and quality control samples were stored at -70°C.

3.3 | Sample pretreatment

Prior to sample pretreatment, samples were thawed and vortex-mixed at room temperature. The sample pretreatment was performed in a fume hood. A volume of 10 µL propionic anhydride was added to 200 µL of calibration standard, quality control sample and study samples and quickly closed. Samples were set at room temperature for 15 minutes for the derivatization reaction to take place. Afterwards, 200 µL internal standard working solution, containing propionitrile and the extraction solvent TBME, was added to all samples, and 200 µL TBME was added to the double blank calibration standard. Samples were mixed by vortex-mixing, centrifuged at 23 100 × *g* for 5 minutes and snap-frozen in an ethanol/dry ice bath. The supernatant was transferred to an autosampler vial with insert and quickly crimp-capped. A volume of 1 µL was injected onto the GC.

3.4 | Chromatography and detection

An Agilent 6890N GC with split injector equipped with an 7683 GC autosampler (Agilent Technologies) was coupled to a Nitrogen Phosphorous Detector (NPD) for analysis of azide and cyanide. The temperature of the inlet and detector was maintained at 250 and 280°C, respectively. A temperature gradient was applied to the oven starting at 40°C for 2 minutes rising gradually to 150°C at 13 minutes. The split mode was turned on with a split ratio of 2.5. Chromatographic separation was acquired on a DB-ALC2 column (Agilent Technologies, 30 m × 0.53 mm, 2 µm) with a constant helium flow of 7.1 mL/min. Data were processed using Chromeleon (v. 7.2.5 9507) (Thermo Fisher Scientific).

3.5 | Validation

A validation procedure was followed based on guidelines from the Scientific Working Group for Forensic Toxicology (SWGTOX).²⁰ We used a fit-for-purpose strategy and included the calibration model, lower limit of quantitation (LLOQ), limit of detection (LOD), accuracy, precision, carry-over, cross-analyte interference, dilution integrity and stability.

4 | RESULTS AND DISCUSSION

4.1 | Development

Concentration ranges were chosen based on the literature. Reported azide and cyanide concentrations in blood or plasma after suspected azide ingestion ranged from not detected to 262 µg/mL for azide^{9-11,14,16,21} and not detected to 9 µg/mL for cyanide.^{9,10,19} Azide concentrations in the gastric content were much higher (up to 19 000 µg/mL).¹⁸ A calibration range was chosen from 1.0-100 µg/mL for both analytes (sodium salt), and dilution integrity was included during the validation for sodium azide to be able to quantify high concentrations in the gastric content.

The developed method was based on the method developed by Meatherall et al.⁹ The use of a headspace injection system can avoid the loss of volatiles in biological samples; however, this system was not available in our laboratory. Therefore, we made some adjustments to the method to make it applicable for our laboratory. The method is based on the derivatization of the azide anion with propionic anhydride and the protonation of cyanide in an acidic environment.²² As a result, propionate and the volatile propionyl azide are formed as well as hydrogen cyanide. In the heated GC injector, propionyl azide is thermally decomposed based on the Curtius rearrangement to form ethyl isocyanate and this compound is detected by the NPD.

Samples were handled in a fume hood. The duration of the derivatization reaction was optimized. After 20 minutes, the ratio ethyl isocyanate/propionitrile decreased (Figure 1A); therefore, 15 minutes derivatization time was chosen. The decrease in ratio was a result of decrease in ethyl isocyanate area, since the internal standard area remained unchanged (Figure 1B). Subsequently, analytes were extracted from the matrix using TBME and samples were snap-frozen after centrifugation in an ice-cold ethanol bath using dry ice. Hydrogen cyanide will be mainly present in the liquid phase instead of the gaseous phase, since the temperature is far below its boiling point of 27.7°C.²² The ice-cold supernatant was transferred to an autosampler vial with insert and injected into the GC system. The back inlet temperature was maintained at 250°C to form hydrogen cyanide in its gaseous form and ethyl

isocyanate after thermal rearrangement of propionyl azide.⁹ This resulted in reproducible ethyl isocyanate responses and sufficient sensitivity; therefore, no further optimization was performed. Separation took place by gas chromatography using a temperature gradient. Ethyl isocyanate, hydrogen cyanide and the internal standard propionitrile were detected using a NPD. Propionate and propionic anhydride lack a nitrogen atom and are therefore not detected by NPD.

Separation of hydrogen cyanide, ethyl isocyanate, propionitrile and some contaminants was necessary for the reliable identification and quantification of cyanide and azide. Chromatographic conditions were optimized by making adjustments to the helium flow rate, make-up flow rate, split ratio, temperature gradient of the oven, temperature of the detector and temperature of the inlet. GC-NPD chromatograms of a blank plasma sample, a plasma sample spiked with sodium azide and sodium cyanide at LLOQ and ULOQ concentrations and the gastric content sample of case 1 are shown in Figure 2.

4.2 | Validation

We validated the method in human K₂EDTA plasma based on the SWGTOX guidelines.²⁰ Post-mortem whole blood samples were not submitted for analysis in our laboratory, and therefore, this matrix was not validated. The linearity of the calibration model was determined by analysing four calibration standards in three analytical runs on three separate days. Linear regression of the analyte/IS peak area ratio vs concentration (x) was used. To obtain the lowest absolute and total bias across the calibration ranges, a weighting factor of $1/x^2$ was used, where x is the analyte concentration. The method was linear for sodium

azide and sodium cyanide from 1–100 µg/mL with a correlation coefficient of 0.987 (± 0.012) for sodium azide and 0.980 (± 0.003) for sodium cyanide. The LLOQ was set to 1.0 µg/mL for both analytes and showed accuracy and precision within $\pm 20\%$ and $\leq 20\%$, respectively (Table 1). The signal-to-noise ratio was ≥ 14.4 for sodium azide and ≥ 4.4 for sodium cyanide. The LOD was estimated according to the guidelines from SWGTOX²⁰ using the y intercept and slope of the linear calibration curve. The LOD was 0.09 µg/mL for sodium azide and 0.40 µg/mL for sodium cyanide.

The accuracy and precision were determined for each analyte by analysing five replicates of QC LLOQ, low, medium and high in two analytical runs on two separate days. Additionally, five replicates of a QC sample above the upper limit of quantification (ULOQ) with a concentration of 1500 µg/mL were diluted 100 times in human K₂EDTA plasma prior to sample processing, to investigate dilution integrity (Table 1). The inter- and intra-assay accuracy and precision were calculated with the equations described by Herbrink et al²³ Except for two results, the accuracy and precision were acceptable according to the SWGTOX guidelines (acceptance criteria: must not exceed $\pm 20\%$ and $\leq 20\%$). Accuracy (bias) for sodium cyanide shows large (-24.4% to $+16.6\%$), but for most calibration points still acceptable deviations. The volatile propionyl azide and hydrogen cyanide partitions between the gas and liquid phase. Opening of the tubes after addition of the acidic derivatizing agent could have influenced accuracy and precision. Since the assay was developed for toxicological purposes, identification and semi-quantification were more essential. The 100 times diluted samples had deviations and CV values below 20%, and, therefore, dilution integrity was demonstrated.

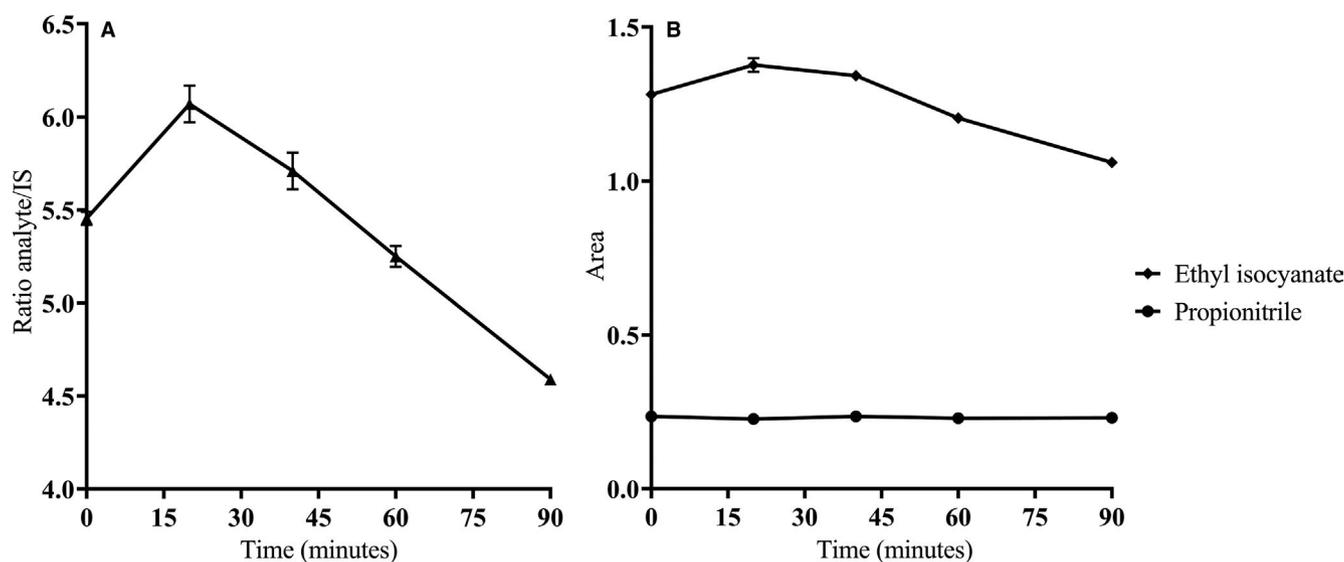


FIGURE 1 The ratio ethyl isocyanate/propionitrile (A) and area of ethyl isocyanate and propionitrile (B) at several time-points after addition of propionic anhydride

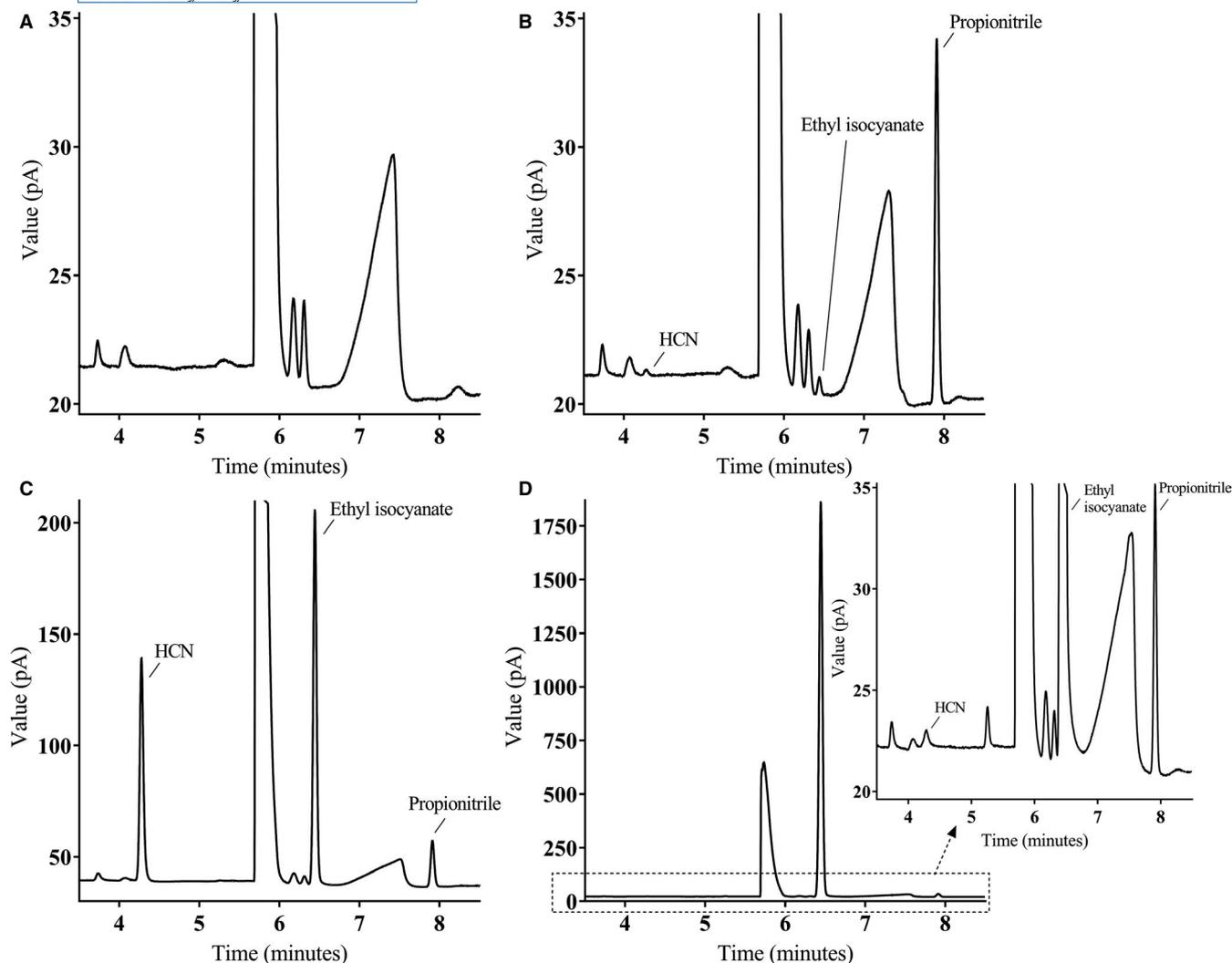


FIGURE 2 GC-NPD chromatograms of a blank human K₂EDTA plasma sample (A), a human K₂EDTA plasma sample spiked with 1 µg/mL (B) and 100 µg/mL (C) sodium azide and sodium cyanide and the gastric content sample from case 1 (D). Hydrogen cyanide RT = 4.30 min, ethyl isocyanate RT = 6.46 min, propionitrile RT = 7.90 min

Since several case reports mention elevated concentrations of cyanide after azide ingestion, analysis of azide must be free of cyanide interference. No cross-analyte interference was observed for azide, cyanide and the internal standard propionitrile. Carry-over was investigated by injection of the highest calibration standard (100 µg/mL) followed by a blank sample in three separate runs. No carry-over was observed.

The stability of azide was investigated in human plasma and the gastric content. Since the accuracy varied for cyanide, accurate determination of the stability of cyanide in biological matrices was not possible. Stability data for cyanide were available in the literature and show a rapid decrease when stored at room temperature in serum and plasma.²⁴ Therefore, all samples were stored at -70°C and it is advised to measure the samples as soon as possible. The stability of azide was investigated in human plasma at different concentrations and different conditions (Table 2). According to the literature, azide is not stable in biological matrices, but the stability data

were inconsistent. At room temperature, the half-life of azide in blood was 4.5 days,¹³ while another paper found a decrease in azide of 48% after 3 days at 4°C .²⁵ This suggests azide is more stable at room temperature, which is very implausible. Ohmori et al²⁵ investigated the influence of red blood cells on the stability of azide by comparing the stability in the plasma fraction to the red blood cell fraction. Azide was stable in plasma up to 48 hours at 4°C , while a decrease of 50% was seen in the red blood cell fraction, which suggests azide rapidly disappears in the presence of red blood cells. These results are in agreement with the results of Ohmori et al²⁵ Azide is stable in human plasma at several conditions (Table 2), except at room temperature and long storage in the freezer (1 year). Therefore, collected blood samples should be centrifuged to obtain plasma, stored in the freezer and analysed as quickly as possible. Stability in the gastric content was investigated by analysing the gastric content sample from case 1 at two time-points. After 1 year, the concentration

TABLE 1 Accuracy and precision of sodium azide and sodium cyanide in human K₂EDTA plasma

| Analyte | Nominal concentration (µg/mL) | Intra-assay (n = 10) | | Inter-assay (n = 10) | |
|----------------|-------------------------------|----------------------|---------------|----------------------|--------------|
| | | Bias (%) | CV (%) | Bias (%) | CV (%) |
| Sodium azide | 1.0 | 7.4 | ≤6.9 | 7.2 | ^a |
| | 3.0 | -7.0 | ≤ 20.4 | -2.1 | 1.8 |
| | 10 | -7.1 | ≤7.9 | -5.0 | 0.7 |
| | 75 | 11.6 | ≤2.9 | 7.0 | 5.9 |
| | 1500 ^b | -1.3 | 4.5 | NA | NA |
| Sodium cyanide | 1.0 | -2.9 | ≤7.8 | -0.3 | 2.6 |
| | 3.0 | -24.2 | ≤7.2 | -19.1 | 8.4 |
| | 10 | -19.5 | ≤2.9 | -16.3 | 5.2 |
| | 75 | 16.6 | ≤1.4 | 15.0 | 1.9 |

Note: Values ± 20% in bold.

Abbreviations: CV, coefficient of variation; NA, not applicable.

^aInter-run precision could not be calculated (mean square between group is less than mean square within groups).

^b100 times diluted in human K₂EDTA plasma (n = 5).

TABLE 2 Concentration of sodium azide (azide) and sodium cyanide (cyanide) in biological matrices of two lethal cases

| Matrix | Case 1 | | Case 2 | |
|-------------------------|------------------------------------|--|------------------------------------|--|
| | Concentration sodium azide (azide) | Concentration sodium cyanide (cyanide) | Concentration sodium azide (azide) | Concentration sodium cyanide (cyanide) |
| Gastric content (mg/mL) | 1.9 (1.2) | 0.0027 (0.0014) | 2.3 (1.5) | <LOD |
| Plasma (µg/mL) | ^a | ^a | 30 (19) | <LOD |
| Serum (µg/mL) | ^a | ^a | 37 (24) | <LOD |
| Cell pellet (µg/mL) | ^a | ^a | 32 (21) | <LOD |
| Urine (µg/mL) | ^a | ^a | 4.7 (3.0) | <LOD |

Abbreviation: LOD, limit of detection.

^aMatrix was not available.

sodium azide (azide) decreased with 25.5% from 1.9 (1.2) mg/mL to 1.4 (0.9) mg/mL when stored at -70°C.

4.3 | CLINICAL APPLICATION

Samples from two lethal cases were analysed. The results are shown in Table 3. In both cases, a small gastric content sample was provided (30 mL). With the developed method, azide ingestion could be confirmed and thus the supposed cause of death in both cases.

Plasma, serum, red blood cell pellet and urine were available only from case 2. Sodium azide was measured with concentrations of 30 µg/mL (19 µg/mL azide) in plasma, 37 µg/mL (24 µg/mL azide) in serum and 32 µg/mL (21 µg/mL azide) in the cell pellet. Since concentrations in these matrices were similar, azide probably distributes into the red blood

cells. In line with this, Ohmori et al have suggested that azide is transported from plasma into red blood cells and ultimately destroyed by oxyhemoglobin (O₂-Hb).²⁵ In case 2, the blood sample was collected 3-4.5 hours after the estimated time of ingestion, thus around the t_{max} of azide. Notably, blood concentrations in samples collected during the distribution phase of azide are high. These high concentrations may not predict the toxic effects of azide, since concentrations in blood do not reflect concentrations in tissue.⁹ Undetectable azide concentrations in blood can also not exclude azide ingestion. In one lethal case, no azide was detected in blood, but azide ingestion was confirmed by analysing the gastric content.¹⁸ Since elimination of azide is quick, blood samples taken after 12 hours of ingestion contain low or no azide concentrations. In this case, autopsy was performed 2 days after death which explains why no azide concentrations were detected in blood. Quick elimination of azide is probably the reason

TABLE 3 Stability data of sodium azide in human plasma at various storing conditions

| Condition | | Nominal concentration (µg/mL) | Bias (%) |
|------------------|-------|-------------------------------|----------|
| Room temperature | 24 h | 3.0 | -19.0 |
| | | 80 | -10.9 |
| 2-8°C | 24 h | 3.0 | -9.5 |
| | | 80 | -8.4 |
| -20°C | 49 d | 3.0 | -4.8 |
| | | 80 | -11.4 |
| | 358 d | 3.0 | -42.0 |
| | | 80 | -34.1 |
| -70°C | 49 d | 3.0 | 0.0 |
| | | 80 | -12.1 |

why fatal blood concentrations vary and range from not detected-262 µg/mL.⁹ To obtain an informative blood sample, timing is important.

Azide is metabolized by the liver into nitrogen oxide and azide is eliminated by the kidneys.⁶ In urine of case 2, a concentration of 4.7 µg/mL sodium azide (3.0 µg/mL azide) was measured, similar to other reported concentrations of 7.5²⁶ and 3.7 µg/mL.²⁷ In most cases, no azide was found in urine. The ingested dose, time of blood withdrawal and storage time and conditions may have an impact. As stated, azide poisoning cannot be excluded even in the absence of azide concentrations in blood and/or urine. Therefore, the concentration in the gastric content is more informative and could be used to confirm azide poisoning if blood and/or urine concentrations are low or absent.

To date, no antidote is available for azide poisoning. Sodium thiosulfate and hydroxocobalamin were administered in case 2. Both are antidotes for cyanide poisoning and intravenously administered. Hydroxocobalamin forms a complex with cyanide which is cleared by the kidneys. Sodium thiosulfate is a substrate for the enzyme rhodanese and necessary for the conversion of cyanide into the less toxic thiocyanate. In several cases, cyanide concentrations in blood could be demonstrated upon azide ingestion without any indication of concomitant cyanide ingestion.^{9,10,14,18,19} The origin of these cyanide concentrations is still unknown, but formation probably occurs in vivo which has been demonstrated in vitro by Lambert et al¹⁴ Interestingly, a concentration of 2.7 µg/mL sodium cyanide (1.4 µg/mL cyanide) was found in the gastric content of case 1. Blood samples were not available for this case. Administration of sodium thiosulfate and hydroxocobalamin could have contributed to the fact that no cyanide was detected in the samples from case 2. Normal levels of cyanide in human plasma are between no cyanide to 0.107 µg/mL.²⁸ It is described that thiocyanate (SCN) is present in plasma which can be converted into cyanide in the presence

of haemoglobin.²⁴ Levels higher than 0.2 µg/mL have been associated with cyanide poisoning, and cyanide levels above 1.0 µg/mL are lethal.²⁸ High levels of cyanide were measured in the gastric content indicating that cyanide is produced in vivo or cyanide is ingested together with azide.

5 | CONCLUSION

We developed an easily applicable GC-NPD method for the simultaneous determination of azide and cyanide. We were able to confirm azide ingestion in two lethal cases. Azide could be quantified in plasma, serum, red blood cell pellet, urine and the gastric content. Cyanide was detected in the gastric content of one case. In conclusion, the developed method is able to confirm azide and cyanide exposure and azide concentrations can be quantified in several biological matrices.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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