Diagnosis of lethal cyanide poisoning. Analysis by Anion-Exchange Chromatography with Pulsed Amperometric Detection

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Abstract
Cyanide is a poison widely used in cases of suicide or homicide. Although various methods to identify and quantify this substance are reported in the literature, they are mainly validated on biological fluids (e.g., blood and urine). In the present study, the Anion-Exchange Liquid Chromatography with Pulsed Amperometric Detection (IC-PAD) method was validated on blood and, for the first time, on gastric content, and organs (brain, lung, and liver). For each matrix, linearity, accuracy, precision, limit of detection (LOD), lower limit of quantification (LLOQ), matrix interferences, and carryover were assessed. The samples were extracted by steam distillation in acid environment for the following analysis by IC-PAD. Furthermore, cyanide values found in two real poisoning cases are reported. For each investigated matrix, the analytical method satisfied all acceptance criteria for validation: it showed a good precision and accuracy, selectivity, and sensitivity with no carryover and matrix interference. The extraction by steam distillation in acid environment REDUCED the interference of the matrices and ALLOWED to perform the analysis with good precision and accuracy. In case #1, analysis showed a blood cyanide concentration of 0.99 μg/ml. In case #2, cyanide concentrations were 1.3 μg/g in brain, 0.8 μg/g in lung, 1.6 μg/g in liver, and 1.2 μg/g in gastric content. The cyanide concentrations found in the two reported cases have been suitable to cause death by poisoning.

KEYWORDS
blood, cyanide, forensic toxicology, gastric content, IC-PAD, organs, validation

Highlights
• Cyanide is currently used for homicide and suicide and diagnosis of lethal poisoning could sometimes be complex.
• Method validation by IC-PAD on blood, gastric content, and organs (brain, lung, and liver) was described.
• The analytical method satisfied all acceptance criteria for validation.
• Cyanide values found in two real poisoning cases are reported.
1 | INTRODUCTION

Cyanide (CN-) is the salt of hydrogen cyanide (HCN). Due to its extreme toxicity, cyanide is one of the most potent poisons; its LD$_{50}$ is approximately 1–2 mg/kg of body weight [1]. Potassium cyanide (KCN) and sodium cyanide (NaCN) are the most common ingested forms of cyanide. Once the cyanide ion reaches the extremely acidic environment of the stomach, CN- is converted to HCN, which passively diffuses across the gastric membrane [2].

After oral intake, the inhibition of psychophysical abilities occurs within a few seconds to 1 or 2 min. In some cases, a latency interval of 5–10 min or longer has been reported. Soon after loss of consciousness, convulsions, hypotension, apnea, shock, and, eventually, death from cellular asphyxia have been observed [3].

Cyanide is a poison widely used for homicide or suicide. Worldwide, recent fatal and non-fatal cases of cyanide poisoning have been reported [4–10]. In Seoul (Korea), 255 deaths from cyanide poisoning were described between 2005 and 2010; of these, 97.3% were suicides. The most frequent route of administration was oral one (98.8%) [11].

Cyanide is a relatively easy-to-obtain poison. In nature, cyano- genetic glycosides present in various plants release hydrogen cyanide by hydrolysis. Amygdalin is the most common cyanogenic glyco- side; it is contained in seeds or kernels of bitter almonds, apricots, peaches, cherries, and plums. It is also present in the leaves of the Prunus laurocerasus (also known as Cherry Laurel) and in other common plants [12].

It is used as fumigant, rodenticide, in agricultural products [13] and in jewelry electroplating [14]. Cyanide is easily available on the website [15], especially in the so-called "Deep Web" and "Dark Web" [16].

Moreover, hydrogen cyanide is a possible product of combustion processes: it is present in the exhaust of internal combustion engines, in cigarette smoke, and in fumes derived from the fusion of plastic materials containing nitrogen [17].

Diagnosis of lethal cyanide poisoning could sometimes be complex. The aims of the present study have been a) to develop and validate an analytical method by Anion-Exchange Liquid Chromatography with Pulsed Amperometric Detection to detect and quantify cyanide in blood, and, for the first time by this technique, in gastric content, and in organ samples and b) to report cyanide values found in two real forensic cases.

2 | MATERIAL AND METHOD

2.1 | Chemicals and reagents

Potassium cyanide KCN (≥98.0%) was purchased from Sigma Aldrich. Ultrapure water (UHPLC–MS/MS), acetic acid glacial (≥99.5%), sodium hydroxide 0.1 M (≥99.9%), and sodium hydroxide 1.0 M (≥99.9%) were obtained from Carlo Erba Reagents. EDTA disodium salt (≥98.0%) was purchased from VWR Chemicals. Lead sulfate salt (≥98.5%) was obtained from Thermo Scientific.

2.2 | Sample storage and extraction

Soon after autopsy, aliquots of 1 ml of blood or 1 g of brain, lung, liver, and gastric content were homogenized with 5 ml of NaOH 0.1 M and stored at −20°C until analysis. The samples were diluted up to 30 ml with ultrapure water. Each sample was added with 30 mg of lead sulfate used to reduce the concentration of sulfide. Each sample was transferred into distillation flasks, and 500 μl of acetic acid were added. The samples were extracted by steam distillation using a BÜCHI Distillation Unit B-324; the mobile phase flow rate was 0.7 ml/min. The steam was condensed and collected in a 10-ml plastic tube containing 1 ml of NaOH 1 M, up to the total volume of 10 ml. Then, 1 ml of each extract was further diluted up to 10 ml with NaOH 0.1 M (final dilution: 1:100 v/v).

2.3 | Sample analysis

The analysis was conducted by Anion-Exchange Liquid Chromatography with Pulsed Amperometric Detection (IC-PAD). For this purpose, an Ion Chromatograph Metrohm 761 Compact IC (software Metrohm MagIC Net) with Metrohm Metrosep A Supp 10–100/4.0 polystryrene- divinylbenzene column with particle size of only 4.6 μm and Metrohm 945 Professional Detector Vario amperometric detector were used. Characteristic amperometric wave for cyanide and sulfide was used. Potential profile: 0.0 V for 900 ms, followed by −0.5 V for 100 ms and, finally, 0.0 V for 100 ms. Detector temperature: 35.0°C. Mobile phase: 0.1 M sodium hydroxide solution with EDTA. Flow rate: 1 ml/min.

Injection volume: 10 μl.

2.4 | Method validation

Analytical validation was performed according to Laudani et al. [18]. It was performed on blank pig blood and on organ samples (brain, lung, and liver). Validation was carried out on pig because, as well known, it represents the best modeling studies since it shows several anatomic and physiologic similarities with humans [19]. Blank gastric content sample was prepared by homogenizing 10 g of meat, 10 g of vegetables, and 30 ml of HCl 0.1 M.

Linearity, accuracy, precision, limit of detection (LOD), lower limit of quantification (LLOQ), matrix interferences, and carryover were assessed for each tested matrix:

- Linearity was evaluated with Mandel test using a 6-points calibration curve replicated five times (0.25–0.5–1.0–2.5–5.0–7.5 μg/ml or μg/g);
- Sensitivity was determined by evaluation of limits of detection (LOD) and lower limit of quantification (LLOQ). LOD and LLOQ were measured by evaluating the signal/noise (S/N) ratio for each matrix. LOD was fixed at the concentration with a S/N > 3. The concentrations of analyte with a S/N > 10 were chosen as LLOQ; moreover, we have established that LLOQ had to be less than 0.26 μg/ml or μg/g, that is, lower than the lowest concentration reported as toxic [20];
• Precision (as relative standard deviation RSD%) and accuracy (as percent recovery R%) were calculated for two concentration levels (1.0 and 5.0 μg/ml or μg/g) for each matrix. Six aliquots of each sample were extracted and analyzed. For blood, RSD% and the recovery should be between 80% and 120% (i.e., accuracy up to 20%). For organs and gastric content, due to the complexity of these matrices, it was established that the precision had to be up to 30% while the recovery had to be between 70% and 130% (i.e., accuracy up to 30%);
• Matrix interferences were assessed using three blank pig blood and organ samples to verify that no other component (i.e., sulfide), except the analyte, contributes to the result;
• Carryover was assessed by six analyses of extracted blank samples, following the analysis of samples at 5.0 μg/ml or μg/g.

2.5 Case reports

#1. A 72-year-old man was found dead in his home, lying in bed. The subject was a watchmaker; he was affected by depression with previous suicide attempts. A letter was found on the bedside table in which the man communicated his intention to commit suicide by cyanide. A glass was found over a handkerchief, on which it was written "cyanide poison"; a plastic bag, with the same indication, was found on the floor. His clothes did not show blood stains or lacerations. The Magistrate disposed only a postmortem external examination and femoral blood sampling. On external examination, cyanosis of the face, initial rigor mortis, body temperature of 28°C (external temperature of 23°C), and a cherry-red postmortem lividity were registered; no transformative phenomena and no traumatic lesions were present. Rest of the external examination was unremarkable.

#2. A 45-year-old man was found dead in his house. The body of the man was found lying on the ground. His face was cyanotic. The medical personnel who intervened declared the death for natural cause; no description of scene was reported. At first, the death was attributed to an acute cardiac event, although the man was in good health. However, few months later, the wife's lover was questioned by the police. He reported that the man's wife killed her husband by poisoning him with a drink containing cyanide and warfarin. The exhumation of the body was disposed; organs and gastric content were collected for toxicological analysis. The gastric content was found to be dry and its total weight was 650g. The autopsy and histopathological investigations did not show acute or chronic cardiovascular diseases, as well as hemorrhagic phenomena of internal organs that could be caused the death.

For both cases, cyanide analyses were carried out within 15 days after the samples collection; moreover, the routinary toxicological investigations were performed. Further, in case #2, LC–MS/MS analysis of warfarin was carried out on brain, liver, kidney, and gastric content.

3 RESULTS

3.1 Validation of method

In Table 1, for each matrix, calibration range and related correlation coefficient ($r^2$), LOD and LLOQ are reported. All calibration curves showed a linear coefficient higher than 0.98.

The performance of method is schematized in Table 2. For each matrix, mean value, deviation standard, relative deviation standard RSD%, and percent recovery R% have been calculated.

### TABLE 1 Calibration ranges (n = 5) and related curve correlation coefficients ($r^2$), LOD, and LLOQ

| Units   | n. replication | Range   | $r^2$  | LOD | LLOQ |
|---------|----------------|---------|--------|-----|------|
| Blood  | μg/ml          | 5       | 0.25-7.5 | 0.9915 | 0.05 | 0.13 |
| Lung   | μg/g           | 5       | 0.25-7.5 | 0.9833 | 0.09 | 0.24 |
| Brain  | μg/g           | 5       | 0.25-7.5 | 0.9886 | 0.08 | 0.21 |
| Liver  | μg/g           | 5       | 0.25-7.5 | 0.9837 | 0.07 | 0.19 |
| Gastric content | μg/g | 5       | 0.25-7.5 | 0.9844 | 0.07 | 0.17 |

### TABLE 2 Method performance parameters at 1.0 and 5.0 μg/ml or μg/g

| Units   | Spiked concentration | Mean   | Standard Dev. | RSD%  | R%   |
|---------|----------------------|--------|---------------|-------|------|
| Blood  | μg/ml                | 1.0    | 0.92          | 0.13  | 14.1 | 92.0 |
|         |                      | 5.0    | 4.63          | 0.69  | 14.9 | 92.6 |
| Lung   | μg/g                 | 1.0    | 0.79          | 0.19  | 24.1 | 79.0 |
|         |                      | 5.0    | 4.35          | 1.20  | 27.6 | 87.0 |
| Brain  | μg/g                 | 1.0    | 0.82          | 0.19  | 23.2 | 82.0 |
|         |                      | 5.0    | 4.45          | 0.82  | 18.4 | 89.0 |
| Liver  | μg/g                 | 1.0    | 1.09          | 0.28  | 25.7 | 109.0 |
|         |                      | 5.0    | 4.71          | 1.15  | 24.4 | 94.2 |
| Gastric content | μg/g | 1.0    | 1.15          | 0.26  | 22.6 | 115.0 |
|         |                      | 5.0    | 4.76          | 0.98  | 20.6 | 95.2 |
Precision and accuracy fulfilled the acceptance criteria (<30%) for all matrices, at each concentration. The RSD% ranged between 14.1% (blood) and 27.6% (lung), with a mean value of 21.6%. The R% ranged between 79.0% (lung) and 115.0% (gastric content), with a mean value of 93.5%.

Extracted blank samples showed no matrix interference and no cyanide concentration above LODs (Figures 1 and 2).

3.2 | Case reports

Case #1: a blood cyanide concentration of 0.99 μg/ml was detected. Case #2: cyanide was found in brain (1.3 μg/g), lung (0.8 μg/g), liver (1.6 μg/g), and gastric content (1.2 μg/g); warfarin was also found in brain (0.9 μg/g), liver (8.3 μg/g), kidney (6.6 μg/g), and gastric content (23.9 μg/g).

Figures 3 and 4 show the chromatograms of blood sample of case #1 and brain sample of case #2, respectively. The cyanide concentrations found in samples of both real cases are summarized in Table 3. In both cases, no other substances of toxicological interest were found.

4 | DISCUSSION

The analytical method reported here, satisfied all acceptance criteria for validation. The RSD% ranged between 14.1% (blood) and 27.6%
(lung) with a mean value of 21.6%. The R% ranged between 79.0% (lung) and 115.0% (gastric content) with a mean value of 93.5%. Blood analysis showed the best precision and accuracy for both examined concentrations.

The use of internal standard would be useful to improve precision and accuracy, especially for organs and gastric content. However, there is no compound that can be used as internal standard in this technique [21].

The method showed a good selectivity and sensitivity. No carryover and any matrix interferences were detected. The extraction method in acid environment by steam current distillation reduced matrices interferences and allowed to carry out the analysis with good precision and accuracy.

Anion-Exchange Liquid Chromatography with Pulsed Amperometric Detection proved to be an excellent technique for cyanide and sulfide separation as shown in Figure 4. As the amperometric wave used here is characteristic for the detection of cyanide and sulfide, the chromatographic separation of these two compounds was crucial to evaluate the resolution and the selectivity of this technique.

Although various methods to identify and quantify this substance are reported in the literature, as colorimetry, spectrophotometry, near-infrared spectroscopy, electrochemical detection, chromatography coupled with mass spectrometry, capillary electrophoresis, fluorometry, chemiluminescence, and atomic absorption spectrometry, they have been mainly applied to biological fluids (i.e., blood, urine, and saliva) [22].

Sim et al. (2021) [21] and Jaszczak et al. (2017) [23] applied this method to blood, urine, saliva, and sweat. In our study, the IC-PAD technique was also validated and applied to organs and gastric content, in addition to blood matrix. Its application to unconventional matrices, as organs and gastric content, is the only option in forensic investigation to ascertain death from cyanide poisoning in those cases where biological fluids are not available, as in our case #2.

The sample extraction reported here is different than that described by Sim et al. Our procedure of distillation in acid environment was developed from the classic one reported by Gettler and Golbaum [24]. Unlike to Sim et al. opinion, a total cyanide detection gives useful information on the amount of cyanide present in the body at the time of death. These data are very important especially when death occurs slowly or when there is a long interval of time between death and autopsy.

We agree with Sim et al. to consider the chromatographic techniques (GC), which require cyanide derivatization, too problematical and expensive; moreover, if the analyzed matrix is more complex than blood (as, for example, organs) this approach could be low accurate.

In the present study, we described and validated a simple, rapid, and inexpensive method. It can be used both to assess a cyanide acute fatal intoxication and, without the dilution here used, to detect low concentrations due to environmental or smoke exposure.

Cyanide does not always have lethal effects, depending on the concentrations it reaches in the body. Discriminate lethal from non-lethal cyanide concentrations is important in forensic investigation. In active smokers, cyanide concentrations are generally 20–100 times lower than fatal ones. Blood cyanide concentrations of healthy non-smokers ranged from 0.0027 to 0.0102 μg/ml [25]. In smokers, blood cyanide concentrations ranged from 0.00962 μg/ml to 0.0208 μg/ml; no data on human organs of the general population or smokers were reported [26]. In our cases, cyanide concentrations were higher than those normally present both after a common environmental and/or food exposure, and in smokers.

In case #1, blood cyanide concentration was high enough to cause lethal poisoning. In case #2, although blood was not available (the corps was exhumated 1 year after death), the examined samples resulted alike useful. Indeed, organs cyanide concentrations were close to those reported in other fatal cases. For example, Musshoff et al. reported the following cyanide concentrations after HCN inhalation for suicidal intent: 5.3 μg/ml in blood, 0.96 μg/g in brain, 2.79 μg/g in lungs, and 0.20 μg/g in gastric content [1]. In fire victims, Htike et al. have observed the following cyanide mean values: 0.06 μg/g in brain, 2.2 μg/g in lung, 0.08 μg/g in liver, and 0.09 μg/g in

![Chromatogram of cyanide analysis on brain sample of case #2](wileyonlinelibrary.com)
kidney [27]. Particularly, in case #2, brain and liver cyanide concentrations resulted higher than those reported in the literature, while cyanide lung concentration was lower. This difference is explainable considering that the cases of poisoning reported by Musshoff et al. [1] and Hike et al. [27] are related to HCN inhalation, while our case has occurred after ingestion.

Cyanide concentration found in gastric content of case #2 resulted lower than those reported in a review by Rhee et al. [28]. In the 21 described cases related to cyanide intoxication by oral ingestion, the authors reported that gastric content cyanide concentrations ranged from 2.0 to 6398.0 mg/kg. Neither the doses taken by the subjects, nor the amounts of gastric content have been described. The authors observed that no correlation was found between gastric content and blood concentrations [28]. As they were all cases of suicide, it is possible to hypothesize that the subjects have ingested a large amount of cyanide to be sure of dying. However, the concentrations that can be found in gastric content could not have toxicological relevance if the taken dose and the total amount of gastric content are not known. In fact, for the same ingested dose, the gastric substance concentration will be higher if the stomach is almost empty, compared to when it is full. It is our opinion that gastric content data are useful to assess that a substance was ingested.

In our case #2, we do not know how much cyanide the woman gave to her husband, but, evidently, it was adequate to cause his death. The data collected by the police, anamnestic and circumstantial, also confirmed the homicide by poisoning. It is important to note that the warfarin concentrations found in organs were too low to be related to the death.

Another consideration concerns the possibility of finding cyanide after some time from death. It has been reported that, over time, cyanide concentration decreases until it disappears completely; it seems that the reduction is greater immediately after death. Based on studies reported in McAllister’s review, the rate of cyanide decreases in blood and tissue specimens is dependent on the initial cyanide concentration at time of death, the length of time between death and sample collection, and storage sample conditions. If cyanide concentrations are lethal at death, these can be detected in corpse up to several months later [29].

Instead, according to a more recent study, blood cyanide concentrations remain stable over time and the decrease in concentrations reported by other studies is mainly due to the past use of low sensitive and accurate methods [30].

The use of a sensitive and specific technique is very important for cyanide determination since postmortem phenomena make the specimens’ matrix more complex. The cyanide ion could continue to react with other substances that are formed or released by post mortem phenomena. As well known, cyanide can react with transition metals [31,32] and with aliphatic and aromatic amines [33]. Our hypothesis is that in postmortem, free cyanide fraction can form coordination complexes, for example, with Fe²⁺ ions released by hemoglobin degradation; or, bind with amines produced by putrefaction, to make cyanohydrins and other compounds. For this reason, cyanide could not be longer detectable by several techniques.

In conclusion, the method reported here showed a good selectivity and sensitivity with no carryover and matrix interference. The extraction by steam distillation in acid environment reduced the interference of the matrices and allowed to perform the analysis with good precision and accuracy.

Finally, the cyanide concentrations found in the two reported cases, much higher than those normally present in the body, must be considered suitable to cause the death by poisoning.

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