CASE REPORT

Determination of cyanide by microdiffusion technique coupled to spectrophotometry and GC/NPD and propofol by fast GC/MS-TOF and in a case of poisoning

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Abstract

A man was found dead in a hotel located near Rome (Italy). The man was still holding a syringe attached to a butterfly needle inserted in his left forearm vein. The syringe contained a cloudy pinkish fluid. In the hotel room the Police found a broken propofol glass vial plus four sealed ones, an opened NaCl plastic vial and six more still sealed, and a number of packed smaller disposable syringes and needles. An opened plastic bottle containing a white crystalline powder labelled as potassium cyanide was also found. Systematic toxicological analysis (STA), carried out on blood, urine and bile, evidenced only the presence of propofol in blood and bile. So the validated L-L extraction protocol and the GC/MS-TOF method for the confirmation of propofol in the biological fluids optimized in our laboratory was applied to blood, urine and bile. The concentration of propofol resulted to be 0.432 µg/mL in blood and 0.786 µg/mL in bile. The quantitative determination of cyanide in blood was carried by microdiffusion technique coupled to spectrophotometric detection obtaining a cyanide concentration of 5.3 µg/mL. The quantitative determination was then confirmed by GC/NPD and the concentration of cyanide resulted to be 5.5 µg/mL in blood and 1.7 µg/mL in bile.

Data emerging from autopsy findings, histopathological exams and the concentrations of cyanide suggested that death might be due to poisoning caused by cyanide, however respiratory depression caused by propofol could not be excluded.

Keywords Cyanide Poisoning · Microdiffusion technique · HS-GC/NPD · Propofol · GC/MS-TOF
Introduction

A man was found dead with a broken propofol glass vial and an opened plastic bottle containing a white crystalline powder labelled as potassium cyanide nearby.

Cyanide is a powerful and fast-acting toxic agent. It has also been used as a poison since ancient times, death occurring very rapidly (1-4). Cyanide salts (in particular sodium or potassium cyanide) are the most used agents for homicide or suicide attempts (5,6). Sodium and potassium cyanide lethal oral doses for adults are 0.15-0.3 g (5). The average endogenous level of cyanide in blood is 0.059 µg/mL for non-smokers and 0.123 µg/mL for smokers. So blood cyanide concentrations lower than 0.25 µg/mL are considered normal, and those above 0.25 µg/mL but below 2.00–3.00 µg/mL as elevated, but not ordinarily causing death. In fire cases, the average blood concentration is reported to be 1.12 µg/mL. On the other hand concentrations found in suicide cases range from 1.00 to 53.00 µg/mL (average of 12.40 µg/mL). (7-9). There is no consensus on the precise toxic threshold for cyanide, but concentrations above 1 µg/mL are generally considered toxic (10).

Propofol (2,6-diisopropylphenol) is one of the most widely used drugs in surgery (11); it is injected intravenously for induction and maintenance of anesthesia and for conscious sedation in intensive care patients (12). The blood concentration required for induction of anesthesia is generally 2-10 mg/L, while a concentration of 2-4 mg/L is sufficient to maintain it (13-15). Propofol is also associated with abuse and dependency, especially among health care professionals (16-18). Fatal cases of poisoning caused by propofol reported in the literature (17-25) show a high variability in the blood concentration of propofol (from 0.08 to 8.7 mg/L) (14).

Systematic and toxicological analysis (STA) (24) applied on blood, urine and bile collected from the autopsy evidenced only the presence of propofol (Fig. 1), excluding other volatile or non-volatile organic compounds. For the quantitative determination of propofol on the three biological fluids, the method optimized in our laboratory was applied (25). The determination of cyanide (26-
was carried out by the microdiffusion technique with spectrophotometric determination previously developed in our laboratory by Froldi-Gambaro (41) which was optimized; in particular attention was devoted to the preparation of the reagents. This method is very important because it is easily applicable when chromatographic techniques are not available. Data were then confirmed by HS-GC coupled with an NPD detector due to the reliability of this technique (41).

Case history

A 33-year old male was found dead by the chambermaid of a hotel located in a small town by the sea, South of Rome (Italy).

The man was lying on the bed; he was fully dressed except for the belt and the shoes, no trauma was evidenced. He was still holding in his right hand a 20 cc syringe attached to a butterfly needle inserted in his left forearm vein. The syringe contained about 10 cc of a cloudy pinkish fluid, identified as propofol. The man was an orthoptist from a Northern Italian region who worked in an eye clinic in Milan. In the hotel room the Police found a broken propofol glass vial – 10 mg/mL, 20 cc - plus four sealed ones, an opened NaCl 0.9% plastic vial and six more still sealed, and a number of packed smaller disposable syringes and needles. On the tabletop there was an opened plastic bottle containing a white crystalline powder labelled as potassium cyanide.

Due to the unclear circumstances of the death, the body was made available to the judiciary. The autopsy was performed 72 hours after.

According to further investigative results, the man worked in Milan as an orthoptist in an eye clinic. He leaved home (Scapaccino near Asti town in northern Italy) at 8.00 p.m. on the 21st of October 2015, he drove for 711 km to reach Anzio near Rome. The man had ordered on the Internet
potassium cyanide. Analyses confirmed that the crystalline powder was effectively potassium cyanide.

**Autopsy Findings**

External examination of the body showed fixed pink lividity of the face, chest, scrotum and a venipuncture sign at the lower third of the left forearm.

Nothing relevant at the autopsy, except for signs of parenchymal congestion and edema of the brain, liver and lungs revealed when cut.

The pericardial sac was undamaged and the heart had a normal volume; the endocardium was thin; the myocardium was red and his consistency was not increased. The coronary arteries were of normal size and elastic. The blood was fluid.

Arterial blood (3 mL), bile (3 mL), urine (2 mL) were collected from the autopsy and stored as indicated in ref 41.

**Materials and methods**

Reagents, chemicals and standards

All reagents were of analytical grade and were stored as indicated by the supplier. pH 6 buffer, barbituric acid for spectrophotometric determination of cyanide > 99.5% were purchased from Sigma-Aldrich (St. Louis, MO, USA);

Potassium cyanide (KCN) standard was purchased from Carlo Erba Reagents (Rodano, Italy). Acetonitrile (IS), chloramine T trihydrate, H₂SO₄, NaOH, NaH₂PO₄ and HCl were purchased from J.T. Backer (Deventer, Holland). Pyridine, glacial acetic acid were purchased from Fluka
Water (18.2 MΩ·cm⁻¹) was prepared by a Milli-Q System (Millipore, Darmstadt, Germany); other common chemicals were of the highest purity commercially available.

Pyridine-barbituric acid reagent was prepared as follows: 2.0 g barbituric acid were placed in a flask, 25 mL of water were added, the mixture was stirred until dissolution at 80 °C (5 min). 2 mL of concentrated HCl were dropped into the mixture and then 10 mL of pyridine were added. The solution is stable for one month at 4 °C.

Sample preparation and extraction procedure for the analysis of propofol

Blood (1 mL), urine (1 mL) or bile (250 µL) was added with IS (100 µL, 10 µg/mL), physiological solution (until a volume of 2 mL was reached), 50 mg of a mixture of sodium bicarbonate and carbonate (2/1 w/w) and extracted with ethyl acetate (4 mL). The mixture was put on a rotary shaker (20 min, 15 rpm) and then centrifuged (5 min, 5000 rpm). The organic phase was separated, dried with sodium sulfate and after centrifugation (5 min, 5000 rpm) the supernatant was withdrawn and the solvent evaporated with a gentle stream of nitrogen (40 °C). The residue was dissolved in ethyl acetate (100 µL) before the analysis.

Tissue samples were homogenized with a blender or ball mill, depending on the quantity of material. The deproteinization of the biological matrix was performed by means of an ultrasonic bath: 100 mg of tissue (brain, liver or kidney) previously added with 4 mL of physiological solution, 50 mg of a mixture of sodium bicarbonate and carbonate (2/1 w/w) and 100 µL of IS (10 µg/mL) were sonicated for 15 minutes at room temperature. After 5 min centrifugation, a clear supernatant was separated and extracted with ethyl acetate (4 mL). The mixture was placed on a rotary shaker (20 min, 15 rpm) and then centrifuged (5 min, 5000 rpm). The organic phase was separated, dried with anhydrous sodium sulfate and after centrifugation (5 min, 5000 rpm) the
supernatant was withdrawn and the solvent evaporated with a gentle stream of nitrogen (40 °C). The residue was dissolved in ethyl acetate (100 μL) before the analysis.

Hydrolysis of propofol glucuronide and sulfate in urine and bile samples

The sample of urine (1 mL) or bile (250 μL) was added with saline solution until a volume of 2 mL and 1 mL of 6N hydrochloric acid was added. The mixture was heated at 105 °C for 1 h. After cooling, IS (100 μL, 10 μg/mL) and 50 mg of a mixture of sodium bicarbonate and carbonate (2/1 w/w) were added. Then the mixtures were extracted as described before.

GC/MS-TOF analysis of propofol

The analyses were performed on a Dani Master GC system, with a split-splitless injection system and a Dani Master TOF Plus detector (Dani Instruments, Cologno Monzese, Italy) operated in electron ionization (EI) mode (70 eV). The GC was equipped with a Rxi®-5ms (Crossbond®, 5% diphenyl/95% dimethyl polysiloxane, 10 m x 0.10 mm i.d., film thickness 0.15 μm) capillary column (Restek, Bellefonte, PA, USA).

The GC/MS conditions: split ratio 100:1; injector temperature, 250°C; interface transfer line, 280°C; ion source, 200°C; oven temperature program, initial 70°C, 20°C/min up to 200°C, then 30°C/min up to 300°C (17 s). Helium was used as the carrier gas at a flow rate of 0.5 mL/min. The MS detector was operated in the scan mode, acquiring ions from m/z 50 to 550. The total analysis time was 8 min. The selected ions for quantitative analysis were 163 and 178 for propofol and 135 and 150 for the IS (Fig. 1).
Microdiffusion

Glass Conway microdiffusion cells were used (18 • 70 mm o.d.; 8–10 • 41 mm o.d., inner chamber). Adsorbing solution (2 mL, 0.1 M NaOH) was added to the inner compartment of each Conway cell, and the liberating solution (2 mL, 50% H₂SO₄) was added to the outer compartment. Blood (100 µL blood + 900 µL of 0.1 N NaOH), standard (100 µL of standard solution + 900 µL of 0.1 N NaOH) or blank samples (100 µL of physiological solution + 900 µL of 0.1 N NaOH) were added to the opposite part of the outer chamber, as mixing had to be avoided. The cell was then quickly closed by a Teflon-lined screw cap and gently rotated to mix blood and liberating solution. After 30 min contact at 38 °C, 1 mL of the inner chamber contents from each cell was taken up and transferred into a 10 mL volumetric flask. To each flask 3 mL of 1 M KH₂PO₄ and 1 mL of Chloramine-T (2.5 g/L, freshly prepared) were added, mixed, and allowed to stand for 2–3 min. Pyridine-barbituric acid reagent (3 mL) was then added and the solution diluted to 10 mL with H₂O. Absorbance was determined at 585 nm against a blank.

Spectrophotometric Analysis

UV-Visible determinations were performed on a Varian CARY50 (Torino, Italy) Spectrophotometer. Standard cyanide solution was prepared by placing 25.0 mg of KCN in a 100 mL volumetric flask, to yield a solution with a concentration of 100 µg/mL of cyanide; 0.1 N NaOH was used as diluent. The standard cyanide solution was further diluted to yield six working solutions at concentrations in the range of 0.2–5.0 µg/mL of cyanide.
HS-GC/NPD analysis of cyanide

Apparatus—Automated headspace GC analysis was carried out on a ThermoFinningan Trace TG, equipped with an NP detector 850, and interfaced with an autosampler (all from ThF, Rodano, Italy). A 10 mL vial and a 2.5 mL Hamilton 1002 NTL headspace syringe (Hamilton Co, Reno, NV, U.S.A.) were used. After addition of acetic acid to the sample vial, the autosampler moved the vial from the vial tray holder to the sample heater, where the vial was heated at 60 °C with continuous agitation for 40 min. Using a thermostated syringe, 750 µL of headspace vapor was injected into the GC inlet at a split rate of 40:1. Injector temperature was 100 °C. Gas chromatographic separation took place in a PoraBOND U fused-silica capillary column (30 m · 0.32 mm i.d., 7 µm film thickness) (Varian, Torino, Italy). Purified helium was used as carrier gas, at constant pressure to assure a steady column flow rate of 2.0 mL/min. Detector gas flow rate was set at 60 and 3 mL/min for hydrogen and air, respectively. Column temperature was programmed at 90 °C for 5 min and increased by 10 °C/min to a final temperature of 140 °C. Detector temperature was 300 °C. In these chromatographic conditions, retention times (tR) were about 1.62 and 3.57 min for cyanide and IS, respectively. (Fig. 2).

Standard cyanide solution was prepared by placing 25.0 mg of KCN in a 100 mL volumetric flask to yield a solution with a concentration of 100 µg/mL of CN; 0.1 N NaOH was used as diluent. In another volumetric flask, 5 mL of this was transferred and added with 95 mL of 0.1 N NaOH to yield a solution with a concentration of 5 µg/mL. Standard cyanide solution was further diluted to yield six working solutions at a concentration in the range of 5.0–0.1 µg/mL. Approximately, 0.5 mL of each solution was transferred into a 10 mL vial together with 0.5 mL of IS and 0.5 mL of blank blood. Lastly, 50 µL of glacial acetic acid was then introduced through the silicone rubber
with a microsyringe. Internal standard solution was prepared by placing 1 mL acetonitrile in a 100 mL volumetric flask and diluting with water. This solution was then further diluted to yield a solution with a final concentration of 0.005 µg/mL. Preliminary analysis of samples revealed high concentrations of cyanide, so that the blood samples had to be suitably diluted to yield concentrations in a calibration range. 1mL of each blood samples was diluted in a 10 mL volumetric flask with water. Therefore, 0.5 mL each of diluted sample, IS, and 0.1 N NaOH were placed in a 10 mL glass vial, which was then capped with teflon-coated silicone rubber and sealed by crimping an aluminium cap. An aliquot (50 µL) of glacial acetic acid was introduced through the silicone rubber with a microsyringe. After each injection, the syringe was flushed many times with air to ensure no carry-over of residual cyanide. Blank air injections were also routinely run between samples to avoid this problem completely.

Results

Linearity of the microdiffusion method was assessed in the range 0.2 – 5.0 µg/mL (0.2, 0.4, 0.8, 2.0, 3.0, 5.0 µg/mL) of cyanide. The linearity equation was $y = 0.3256x + 0.0032$ ($R^2 = 0.998$). The concentration of cyanide calculated with the microdiffusion method was 5.3 µg/mL.

Analyses carried out by HS-GC/NPD (41) confirmed the presence of cyanide giving a blood concentration of 5.5 µg/mL, value comparable with that obtained by microdiffusion. With this method the presence of cyanide in bile was also evidenced (1.7 µg/mL). On the contrary cyanide was not evidenced in urine.

Analysis of propofol by GC/MS-TOF (25) evidenced a concentration of 0.432 and 0.786 µg/mL respectively in blood and bile, while urine resulted negative.
Discussion

The microdiffusion method, previously developed by Froldi-Gambaro (41), was optimized to improve accuracy and sensitivity. Particular attention had to be paid to the barbituric acid used for this determination. 2.0 g instead of 3.0 g were used in the optimized method, dissolved in 25 mL of water instead of 7 mL of water, thus improving the dissolution of the reagent. The reagent must be dissolved under stirring at 80 °C. Working with more diluted solutions is very important because the absorbance of the standard solutions follows in the range of the linearity of the instrument. With the more concentrated solutions the response of the instrument was slightly outside the range and therefore the spectrophotometric determinations were less accurate. The purity of this reagent is crucial. The best reagent was that labelled as “barbituric acid for spectrophotometric determination of cyanide > 99.5%”.

The sensitivity of the optimized method was improved respect to the previous one. In fact the LOQ of the previous method was 0.5 µg/mL working with 1 mL of sample, in the optimized method the LOQ is 0.2 µg/mL, working with 100 µL of sample. In the new method LOD was established to be 0.1 µg/mL working with 100 µL of sample, lower than the LOD of the previous method (0.2 µg/mL, working with 1 mL of sample). Working with 1 mL of blood the LOD and LOQ of the optimized method are respectively 0.01 and 0.02 µg/mL. Thus the increase of sensitivity was meaningful. Moreover with the new LOD and LOQ it is possible to work with 100 µL of blood, this was not possible with the previous method. This feature is very important because in forensic analysis 1 mL of blood is not always available.

The optimized microdiffusion method was applied to the determination of cyanide in blood collected from the autopsy of the case reported. The results were confirmed applying the HS-GC/NPD (41) method (Fig. 2). As regard as blood, the two techniques gave comparable results (Table 1). Due to its high sensitivity the HS-GC/NPD method allowed the determination of cyanide in bile. As it is
possible to note propofol concentration in blood (Fig. 3) was quite low (0.432 μg/mL) and likely it was not responsible for death (11-23). On the other hand the blood concentration of cyanide was very high (5.3-5.5 μg/mL). Taking into account literature data (1-10) it was possible to assess that death was caused by cyanide, however respiratory depression caused by propofol without respiratory support could not be excluded.

Probably the man had planned suicide, in fact he left a farewell letter and a fair amount of money. Because working in the medical field he knew the properties of propofol. Likely he ingested cyanide and then injected himself propofol, withdrawing it from the glass vial labelled as “propofol kabi 10 mg/mL”. As cyanide poisoning causes nausea, confusion, restlessness and anxiety followed by loss of consciousness, convulsions and fatal shock, probably the man assumed propofol to avoid cyanide symptoms. In fact propofol causes sedation, loss of consciousness and anesthesia in a very short period of time.

**Conclusions**

The microdiffusion method for the determination of cyanide in blood previously developed in our laboratory was optimized and applied to a case of suspected cyanide/propofol poisoning. The presence of propofol in the biological fluids was confirmed by a GC/MS-TOF method. The quantitative determination of cyanide in blood carried by the microdiffusion technique coupled to spectrophotometric analysis was then confirmed by GC/NPD analysis.

Data emerging from autopsy findings, histopathological exams and the high concentration of cyanide (5.3-5.5 μg/mL) suggested that death might be due to poisoning caused by cyanide, however propofol could have had a role in death due to its toxicity without respiratory support.
Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study
References


**Figure legends**

**Fig. 1** SCAN analysis of blood (a); Mass spectrum of propofol-TMS (b)

**Fig. 2** HS-GC/NPD of blood.

**Fig. 3** (A) GC/MS-TOF chromatogram for the determination of propofol in blood; $t_R=2.830$ min IS; $t_R=3.273$ min Propofol-ME B) Mass spectrum of methylated thymol (IS); C) Mass spectrum of methylated propofol.
a)

Fig. 1

b)
Fig. 3

PROPOFOL

A

IS

B

C

m/z

Int.
Table 1. Results of the quantitative determination of cyanide and propofol in the specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Cyanide (µg/mL)</th>
<th>Propofol (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microdiffusion</td>
<td>HS-GC/NPD</td>
</tr>
<tr>
<td>Blood</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Bile</td>
<td>n.e.</td>
<td>1.7</td>
</tr>
<tr>
<td>Urine</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
</tbody>
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n.e. = not examined; n.d. = not detectable.