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CASE REPORT

TOXICOLOGY

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Nitrous Oxide Determination in Postmortem Biological Samples: A Case of Serial Fatal Poisoning in a Public Hospital*

ABSTRACT: In a public hospital, eight cases of fatal poisoning by nitrous oxide (N_2O) occurred under oxygen administration, due to an erroneous swapping of the lines in the gas system. The aim of the study was to clarify the factors involved in asphyxia by characterizing gases from different lines and measuring N_2O concentrations in postmortem biological samples from bodies exhumed. Analyses carried out on the gas system confirmed the erroneous substitution of O_2 line with N_2O and air line with O_2. Consequently, high N_2O amounts were revealed in several tissues and gaseous biological samples. All specimens were analyzed by headspace gas chromatography technique. A rigorous quantitative analysis was possible only in blood (11.29–2152.04 mg/L) and urine (95.11 mg/L) and in air samples from stomach and trachea (from 5.28 to 83.63 g/m^3). This study demonstrates that N_2O can be detected in biological samples even 1 month after death.

KEYWORDS: forensic science, forensic toxicology, nitrous oxide, asphyxiation case, accidental death, biological samples

Nitrous oxide (N_2O) is a well-known anesthetic agent that is extensively used in clinics, alone or in combination with other gaseous anesthetics, including halide compounds such as sevoflurane (sevorane).

Anesthetic concentrations are 50–67%, but higher concentrations are asphyxiating. Adverse effects have been documented in animals and humans, such as patients receiving anesthesia and occupational exposed personnel (1,2). In particular, the American Conference of Governmental Industrial Hygienists (ACGIH) has reported adverse effects on the human central nervous, hepatic, hematopoietic, and reproductive systems (3).

Although N_2O is considered a safe analgesic and anesthetic, descriptions of some fatal cases have been published. Accidental deaths are mainly associated with recreational uses due to its pleasant euphoric effects (4–7), but there have been rare reports of accidental inhalation at work or due to its incorrect administration to hospitalized patients during anesthesia.

However, it is difficult to attribute the death of hospitalized patients exclusively to acute N_2O exposure because of the concomitant use of other substances (e.g., other anesthetics) and the potentially fatal complications related to airway management (8,9), and the patient's disease.

Determining the cause of death of N_2O asphyxiation (10–13) may be even more difficult because of the variation in circumstances occurring during the event.

Additionally, even if its determination in biological samples (fluid and tissues) has already been described in ante- and postmortem fluids (14–17), to the best of our knowledge, no information has yet been reported regarding N_2O concentration in samples from exhumed bodies that confirm previous fatal exposure.

Case Report

We here describe a case of serial N_2O poisoning during O_2 administration in a new Cardiovascular Intensive Care Unit (ICU) in a public hospital caused by the erroneous replacement of O_2 with N_2O in the gas system that led to eight patients being fatally exposed to pure N_2O.

The first seven cases were thought to be related to a worsening in the patients' cardiovascular diseases and so the bodies were buried. After the last unexplained death, further investigation revealed the real cause and, subsequently, the exhumed bodies of the buried patients were also analyzed.

The aim of this study was to describe the measures used to clarify the cause of death and identify the factors involved in asphyxia. In particular,

- gases from different lines of the Cardiology Department were characterized in order to confirm the switched lines,

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- N_2O was detected in all postmortem biological samples in order to confirm the previous abnormal exposure.

Materials and Methods

Chemicals

Technical grade N_2O, O_2, N_2, and compressed air were purchased from Sapio (Milan, Italy) and the HPLC-grade dichloromethane (CH_2Cl_2 boiling point, 39.75°C, Lab) used as internal standard (I.S.) was from Sigma Aldrich (Milan, Italy).

Subjects

The eight patients who died (four males and four females with a mean age of 77.75 years, range 67–85) had been admitted to a new Cardiovascular ICU in the Cardiology Department. Five had cardiovascular diseases, two lung diseases, and one gastrointestinal disease. During O_2 administration, an erroneous connection in the gas system meant that all of the patients were fatally exposed to N_2O for a mean period of 58.25 min (range 25–125 min). Gaseous and tissue samples were collected just before and during autopsy 19.12 days (range 6–31) after their death.

Table 1 shows the characteristics of each patient, the duration of N_2O exposure, and the time between death and autopsy.

Sample Preparation

Gas System Characterization—Gaseous samples from the air and O_2 lines in the Cardiovascular IUC Department were drawn directly into sample bags from both the control panel and from the lines supplying each bed. Samples of the technical gases normally used in hospital (N_2O, O_2, N_2, and compressed air) and environmental air were also collected from another Department using the same procedure.

Biological Samples—Before autopsy, gaseous samples were drawn from stomach of the first seven patients who died using 50 mL syringe; in the case of the eighth patient (who had not been buried), gas was drawn from the trachea.

During autopsy, blood, liver, bile, kidney, fat, and brain samples were collected from almost all patients; a urine sample was also taken from the eighth deceased patient. All of the tissue samples were immediately weighed and then transferred to 10-mL headspace (HS) vials with airtight plugs. The gaseous samples from the sample bags and syringe were also transferred to HS vials, which were completely filled.

TABLE 1—*Characteristics of the patients admitted to the Cardiovascular ICU at Castellaneta Hospital (Taranto, Italy).*

Subject	Years	Reason for Admission	Exposure Time (min)	Time Lapse from Death to Autopsy (days)
1	82	Cardiovascular disease	51	6
2	73	Cardiovascular disease	25	6
3	80	Cardiovascular disease	90	15
4	84	Cardiovascular disease	50	19
5	67	Cardiovascular disease	35	20
6	85	Suspected gastrointestinal disease	125	25
7	76	Suspected lung disease	45	31
8	75	Suspected lung disease	45	31
Average	77.65		58.25 min	19.12 days

The vials were stored at −20°C and equilibrated for about 1 h at room temperature before analysis; 50 μL of CH_2Cl_2 gaseous solution were added as I.S.

For purposes of comparison, we also analyzed postmortem biological tissue samples taken from subjects not exposed to N_2O, and stored at −20°C until analysis or at room temperature for 2 weeks. Further experiments showed that N_2O (pure or in gaseous solution) is stable for more than 4 months if it is collected in an airtight system such as a gas sample bag or HS vial, and stored at 5°C (data not shown) as well as N_2O in biological samples.

Calibration Standard

N_2O and CH_2Cl_2 standard gaseous solutions were prepared in 10-L sample bag as described in our previous work (18) by filling them with N_2, whose volume was measured accurately by using a flowmeter, and adding known amounts of pure N_2O and CH_2Cl_2.

CH_2Cl_2 was used as I.S. Final concentrations were 7.2, 72.0, and 3600 g/m^3 for N_2O and 13.0 mg/m^3 for CH_2Cl_2. Standards were stabilized for at least 8 h to obtain a homogeneous solution.

In order to evaluate N_2O concentrations in biological gases withdrawn from trachea and stomach, calibrations were performed in empty vials by adding known amounts of N_2O from standard gaseous solution and 50 μL of the I.S. gaseous solution. Two different calibration curves were created depending on the amount detected in the samples: lower concentrations were tested in splitless mode, and higher concentrations in split mode (50/1) (Table 2*a*).

The same calibrations were used to estimate the amount of N_2O released by tissues under HS extraction conditions.

TABLE 2—*Validation of the HS-GC/ECD method applied to (a) gas, and (b) blood and urine. Calibration in air was carried both in splitless and in split (50/1) mode according to N_2O concentration measured in the samples. Calibration in matrix (blood and urine) was performed always in splitless mode.*

(a) Assay Validation in Air

	Splitless Injection	Split 50/1 Injection
Linear range	0.0044–4.4 g/m^3	0.44–440 g/m^3
*b**	0.991 ± 0.046	0.3037 ± 0.0092
*a**	0.0959 ± 0.4740	44.22 ± 7.54
r^2	0.993	0.995
LOD†	0.00044 g/m^3	0.0044 g/m^3
Accuracy (%)‡	101.1 ± 0.5	100.6 ± 0.3
Precision (%RSD)§		
Intra-day	0.5–2.5%	1.3–2.8%
Inter-day	2.2–4.9%	3.2–5.2%

(b) Assay Validation in Matrix

	Urine Splitless Injection	Blood Splitless Injection
Linear range	1–200 mg/L	1–3000 mg/L
*b**	0.253 ± 0.005	0.0914 ± 0.012
*a**	0.03 ± 0.01	0.05 ± 1.2
r^2	0.998	0.995
LOD†	0.001 mg/L	0.005
Accuracy (%)‡	99.5 ± 0.7	99.0 ± 1.2
Precision (%RSD)§		
Intra-day	2.8–4.0	3.5–4.2
Inter-day	3.1–7.0	3.5–7.5

*Calibration fitting: $y = bx + a$ ($n = 8$ analyzed in duplicate) ± 95% CI; linear regression analysis using the least-square method.

†Limit of detection (Signal/Noise = 3).

‡Accuracy ($n = 3$) calculated on samples.

§Intra- and inter-day precision ($n = 3$) calculated on samples.

To evaluate N_2O concentration in blood and urine, calibrations were performed in matrix by adding known amounts of N_2O from standard gaseous solution (Table 2*b*).

In detail, 5 mL of urine or blood was collected in 10-mL glass vials containing 0.5 g of NaCl and sealed with Teflon-lined septa and hole caps. Salt was added with the aim to drive compounds into the HS according to "salting-out" effect (19) and to normalize natural salt concentrations and the ionic strength of the different samples, mainly in the case of urine.

Then, known amounts of N_2O from standard gaseous solution were added to each vial in order to obtain two different calibration samples with concentration of N_2O in the range of 1–200 mg/L and 1–3000 mg/L (n = 8, analyzed in duplicate).

Fifty microliters of I.S. gaseous solution was added to each sample (gaseous and biological specimens) before analysis.

Finally, they were stabilized for at least 30 min at room temperature before analysis to have a homogeneous solution.

Assay Validation

Assay validation was performed both in air and in matrix (blood and urine) by studying, beyond the linearity, the limit of detection (LOD), and intra- and inter-day analytical precision (calculated as % RSD) and accuracy for all determinations (Table 2*a,b*).

Assay validation in air was performed in empty vials sealed with Teflon-lined septa and hole caps containing known amounts of N_2O from standard gaseous solution and 50 μL of the I.S. gaseous solution.

Assay validations in matrix were performed on 5 mL of urine or blood collected in 10-mL glass vials containing 0.5 g of NaCl and sealed with Teflon-lined septa and hole caps by adding known amounts of N_2O from standard gaseous solution and 50 μL of I.S. gaseous solution.

LOD was calculated on three spiked samples as the standard concentration at which signal to noise ratio was equal to 3.

Intra- and inter-day analytical precision (n = 3) was assessed spiking each matrix with a specific amount of N_2O.

Accuracy was performed on three spiked samples by means of a recovery study on the basis of the added and found concentrations (20).

HS-GC/ECD Analysis

N_2O was extracted from the samples by pre-heating at 80°C for 15 min (equilibration time) using a Agilent 7694 static HS sampler (Agilent, Palo Alto, CA) equipped with a 3 mL sample loop. According to the gaseous nature of N_2O together with the low boiling point of CH_2Cl_2 (39.8°C), at this temperature after 15 min the equilibrium distribution of both compounds was achieved between biological matrix and gas phase.

At the end of equilibrium time, helium entered vial through needle at 18 psi for 0.2 min, after which the vent valve opened and the HS gas filled the sample loop for 0.2 min. Thus the compounds in gas phase equilibrated to the higher loop temperature for 0.05 min. Finally, the gases into sample loop were injected into the GC through a transfer line.

The loop and transfer line temperatures were respectively 80 and 90°C as preliminary experiments had shown that N_2O is stable under these condition (data not shown).

The analyses were made using a Agilent 6890 gas chromatograph with ECD as detector. The compounds were separated on a 30 m RT-QPLOT column with an internal diameter of 0.32 mm (Restek, Bellefonte, PA) using helium as the carrier gas (1 mL/min). The GC conditions were: 40°C hold for 3 min, 15°C/min to 130°C, hold for 1 min; and then 10°C/min to 180°C, and hold for 2 min. The injector and ECD temperatures were respectively 280 and 300°C. N_2 was used as make-up gas.

Results

Gas System Characterization

The results of the analyses of the gaseous samples taken from the control panel and the lines supplying each bed are shown in Fig. 1.

All of the samples taken from the O_2 lines contained pure N_2O and a negligible amount of O_2, confirming the erroneous connection of the O_2 line to N_2O cylinder. In particular, the median percentage of N_2O was 111.9 ± 24.62% at the control panel, and 104.4 ± 15.66% and 110.5 ± 22.1% at the bed outlets.

On the contrary, the air line contained almost pure O_2 with traces of N_2O (from 0% to 0.06%) thus demonstrating its connection to the O_2 cylinder. The median O_2 percentage at the control panel was 112.81 ± 33.84% while at the two bed outlets it was 94.41 ± 26.25% and 67.87 ± 16.96%. This lower O_2 concentration might have been due to a leakage in the plumbing supplying bed 2 or a mistake during the taking of the sample.

Figure 2 shows the N_2O and O_2 percentages in the technical gases normally used in the hospital (N_2O, O_2, and N_2) and compressed air drawn from another hospital Department. The environmental air was also sampled and analyzed. The results showed the purity of the gases and the absence of N_2O in N_2, compressed air and environmental air. The percentages of O_2 in the compressed and environmental air were similar (19.57% and 20.42%).

Figure 3 shows the chromatogram profiles of the gas samples taken from the O_2 lines (a) and air line (b) in the Cardiovascular ICU, and pure O_2 (c) and standard 5% N_2O (d).

Biological Samples Analysis

Despite the time interval between death and autopsy, abnormal amounts of N_2O were found in all of the biological specimens.

The air samples collected from the stomachs of the patients during autopsy revealed N_2O concentrations ranging from 5.28 to

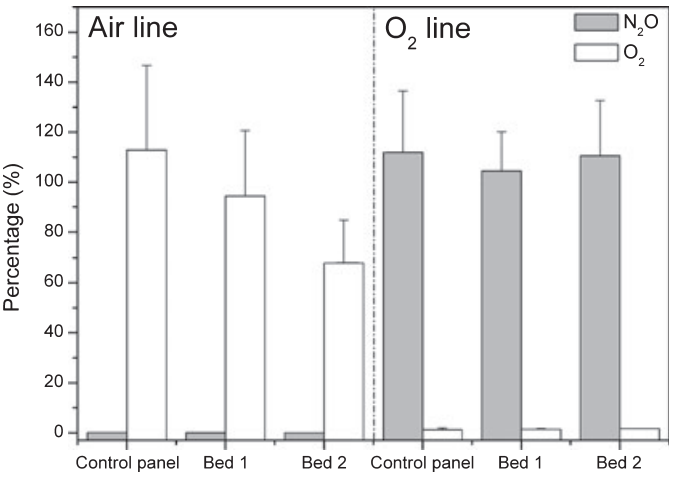

FIG. 1—*N_2O and O_2 percentages in the O_2 and air lines in the Cardiovascular ICU. Gaseous samples were taken from the control panel and from the lines supplying the beds.*

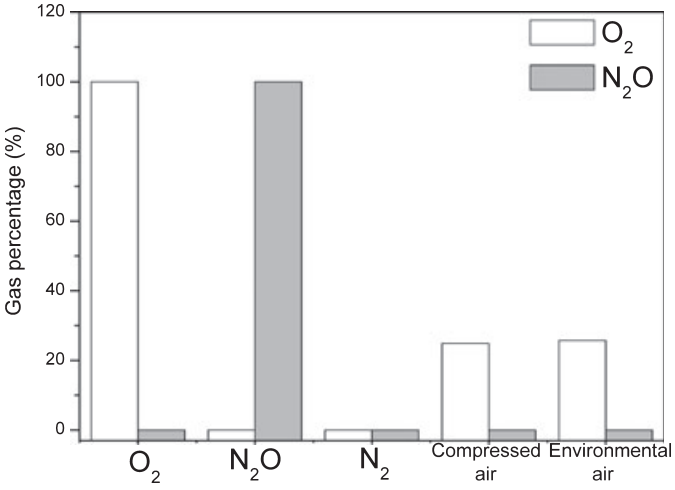

FIG. 2—*N_2O and O_2 percentages in the gases normally used in hospital (N_2O, O_2, N_2, compressed air); the environmental air in an operating theater was also analyzed.*

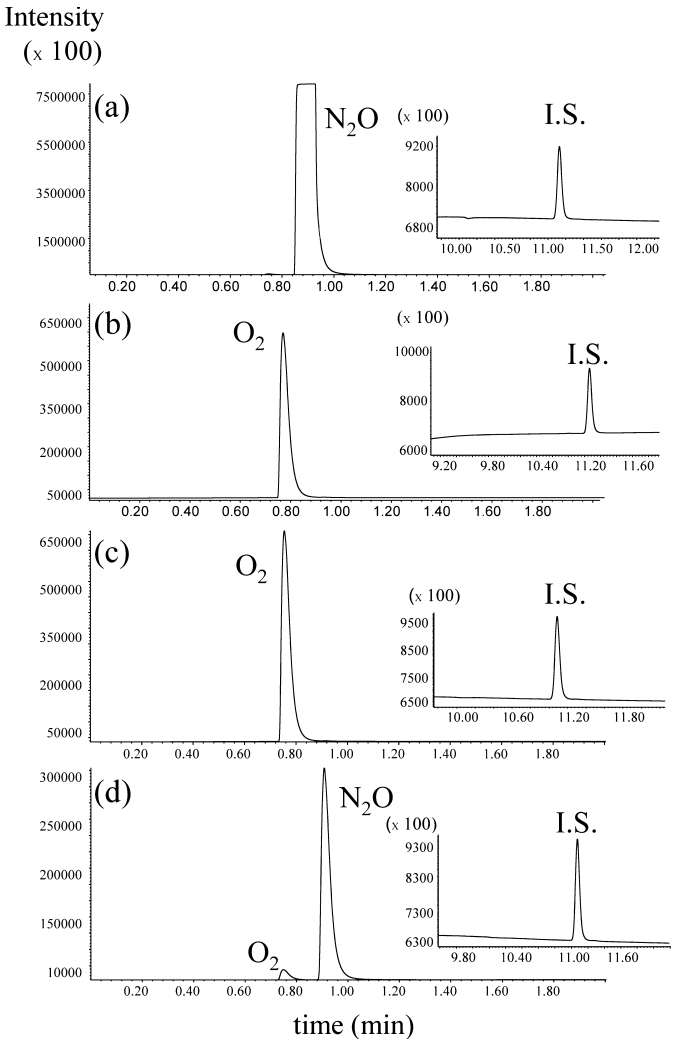

FIG. 3—*Chromatogram profiles of the gas samples taken from the O_2 lines (a) and air line (b) in the Cardiovascular ICU, and of pure O_2 (c) and standard 5% N_2O (d).*

83.63 g/m^3 (respectively 0.30% and 4.55%). All of the samples were collected in duplicate and stored in 50 mL syringes until analysis. The N_2O concentration in the trachea of the eighth patient who died was lower than in the stomach and equal to 1.188 g/m^3 (0.06%). In particular, Fig. 4 (8 and 9) shows the N_2O concentrations in gaseous samples collected from stomach (subject 2) and trachea (subject 1), respectively.

Tissue samples were all also positive to N_2O determination as described by chromatogram profiles as obtained from exposed patients and from controls (Fig. 4).

However, a quantitative analysis was possible only with blood and urine samples by performing calibrations in matrix under the same operative conditions already described in our study (18). In the case of other tissues, since a homogeneous addition to the sample was not possible, the amount of N_2O released in gas phase after HS extraction was evaluated using calibration carried out in air (empty vials).

Figure 5*a* summarizes N_2O blood and urine concentrations, while Fig. 5*b* describes the amount of N_2O released in gas phase by tissues after the first HS extraction.

The high amount of N_2O detected in all samples from exposed subjects together with the fact that N_2O was always below the LOD of the method in tissue samples from unexposed ones, make the specificity and the sensitivity of the assay 100%, taking all the samples tested into account.

According to the multiple HS extraction theory (21–24), a possible approach to extrapolate real N_2O tissue concentrations is to perform continuous HS extractions on the same sample until an exhaustive extraction has been obtained. The total amount of N_2O can be calculated by summarizing all individual peak areas obtained after each extraction. However, this method was possible only in the case of kidney samples since these specimens had been collected in duplicate. In this case, 17 successive extractions from the kidney samples were made in all subjects.

As an example, Fig. 6 shows the results relating to the kidney samples of subject 3.

Figure 6*a,b* show the total amount of N_2O extracted from kidney and the percentage of recovery as a function of the number of HS extractions, respectively.

Data in Fig. 6*a* were fitted with the function $f(x) = V_{max}*\{1-\exp(-n°.\ \text{HS extraction}/\tau)\}$. Since $v_{max} = \lim_{n\to\infty} f(x)$, such a value can be considered as the total amount of N_2O extractable from the sample and the ratio between it and the total wet weight of the sample could be considered as the real N_2O concentration in tissue. The τ value was 7.52 ± 0.52 extractions and thus $t_{1/2}$ (the extraction at which the recovery was 50%) was equal to 5.61 extractions. Therefore, after 17 extractions, the percentage of recovery calculated from the amplitude of the fitting curve normalized to 100 (Fig. 6*b*) was equal to 85.9%.

Figure 7 shows that the amount of N_2O extracted during the first extraction was highly correlated ($R^2 = 0.81$, $p < 0.01$) on a log-log scale with the total amount of N_2O (extrapolated by the fitting curves) released by the tissue. The total amount extracted during the first extraction was equal to 32.1 ± 9.2% (media ± SD) of the total extrapolated one.

Discussion

The main finding of this study is that high amounts of N_2O (a gas that is heavier than air) can be detected in postmortem biological samples as late as 1 month after death. Furthermore, almost all

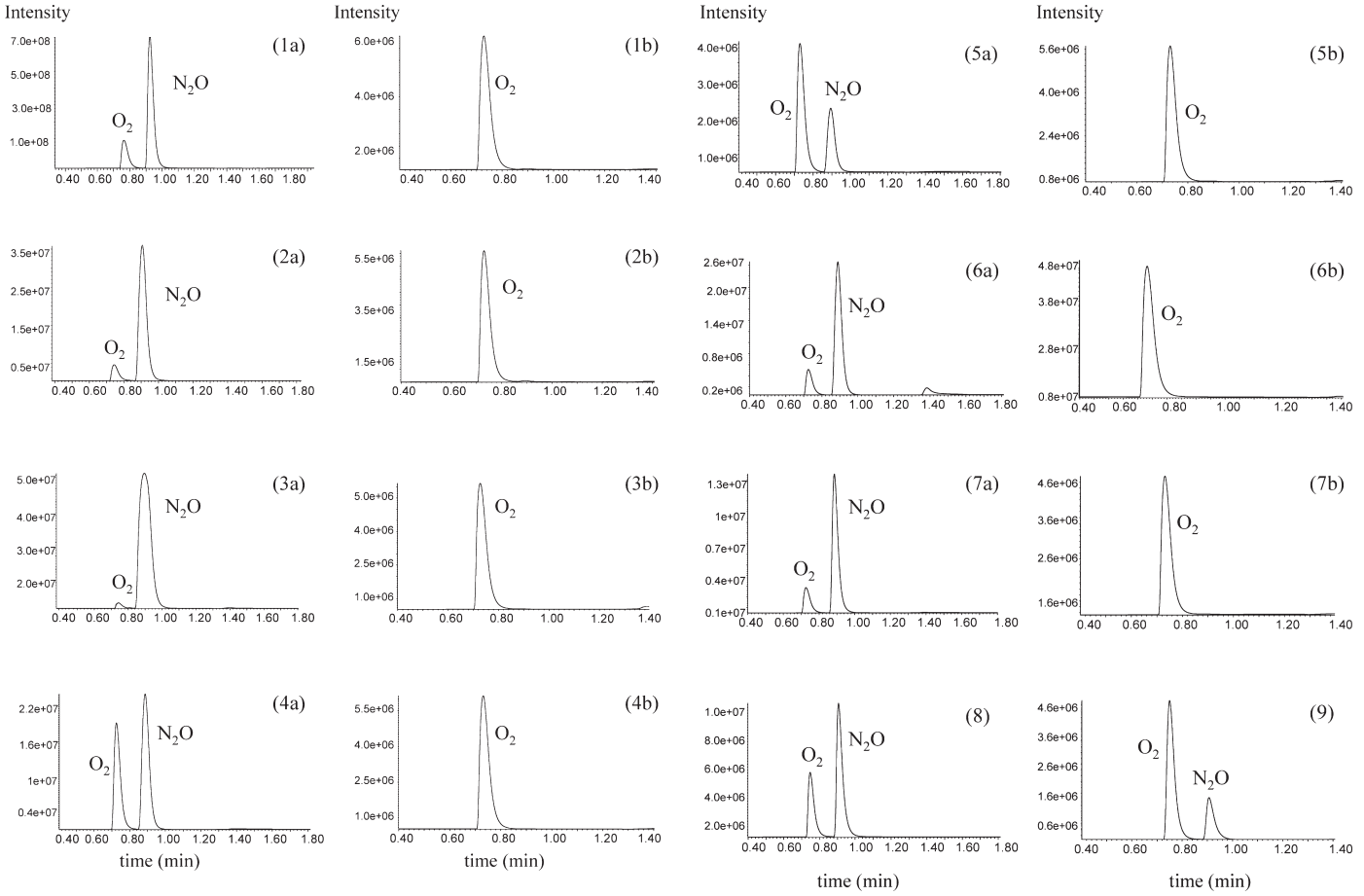

FIG. 4—*Chromatogram profiles of representative specimen types from different patients and controls. (1) Blood samples: (a): 2152.04 mg/L—subject 1 (b) unexposed subject. (2) Urine samples: (a): 95.11 mg/L—subject 2 (b) unexposed subject. (3) Liver samples: (a): 1.35 mg—subject 6 (b) unexposed subject. (4) Bile samples: (a): 0.388 mg—subject 7 (b) unexposed subject. (5) Kidney samples: (a): 0.027 mg—subject 8 (b) unexposed subject. (6) Brain samples: (a): 0.74 mg—subject 5 (b) unexposed subject. (7) Fat samples: (a): 0.44 mg—subject 1 (b) unexposed subject. (8) Air sample from stomach: 5.28 g/m^3—subject 2. (9) Air sample from trachea: 1.188 g/m^3—subject 1. I.S. peak (retention time = 11.18 min) is not shown.*

of the patients had been buried, which may have led to the partial loss of N_2O between death and the time of autopsy.

It is surprising that seven deaths were attributed to the patients' disease before the real reason was discovered, but this was mainly due to the fact that the Department was brand new and the situation was very unusual. After the accident, the entire Department was carefully checked in order to identify how such unreasonable deaths occurred and the analyses of the gas samples from the O_2 and air lines revealed that they had been mistakenly switched.

The samples from the O_2 lines showed the presence of pure N_2O, and those from the air lines contained pure O_2 with a small percentage of N_2O. The same results were obtained analyzing the samples from the lines supplying each bed. In particular, N_2O concentrations in the O_2 line were similar at the central control panel and the bed outlets, thus confirming the exposure of the patients. N_2O and O_2 values obtained from the O_2 and air lines were more than 100% because of the difficulty in precisely measuring the pure gasses, as shown by their high standard deviation.

For purposes of comparison, we also analyzed the technical gases normally used in hospitals (N_2O, O_2, N_2, compressed air) and environmental air in order to measure O_2 and N_2O concentrations. Figure 2 shows the purity of the normally used gases. It is interesting to note that it was possible to measure O_2 precisely using ECD as the detector instead of the normally used μTCD. In fact, about 20% of O_2 was found in both the compressed and environmental air, a value similar to the percentage of O_2 in air.

In order to demonstrate that the deaths were caused by asphyxia due to the inhalation of N_2O alone, biological specimens (gaseous samples and tissues) were also analyzed and found to contain abnormal amounts of N_2O. This result is particularly interesting because seven of the eight bodies had been buried and the sample gases were taken an average of 19 days after death. It was possible to draw gas from the trachea (which might better represent previous N_2O exposure) only in the case of the eighth patient who had not been buried whereas, in the other subjects, the sample gases were taken from the stomach and analyses showed that N_2O can persist free and unabsorbed by tissues for a long time after death.

Additionally, to exclude N_2O production due to bacterial activity after death, we also analyzed postmortem tissue samples taken from an unexposed subject. It has been reported that urinary tract infections can significantly interfere with the concentration of N_2O in urine samples because it can be produced by a variety of microbial species (18,25) but, to the best of our knowledge, there are no published descriptions of the production of N_2O in tissues (or urine) after death. Furthermore, it seems unlikely that bacterial activity alone would be enough to produce the abnormal amount of N_2O measured in all of the biological tissues.

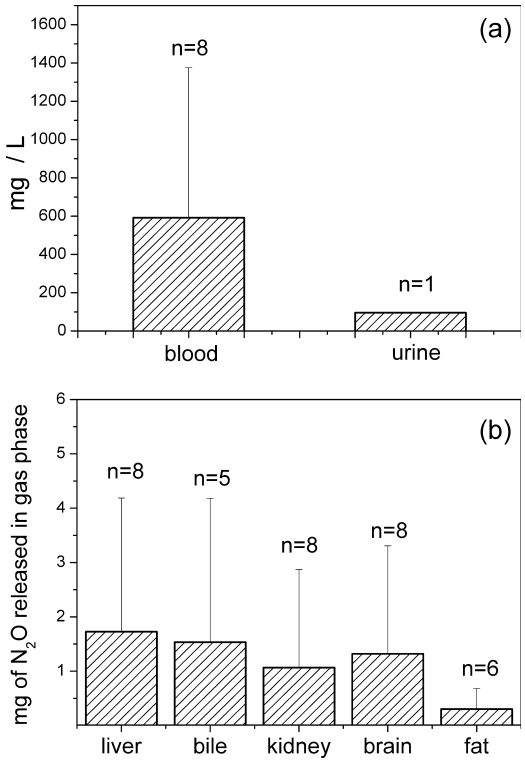

FIG. 5—*Exposed subjects: (a) N_2O concentrations (mg/L, mean, and SD) in blood and urine samples, (b) N_2O amount (mg, mean, and SD) released by the tissues in gas phase after the first HS extraction.*

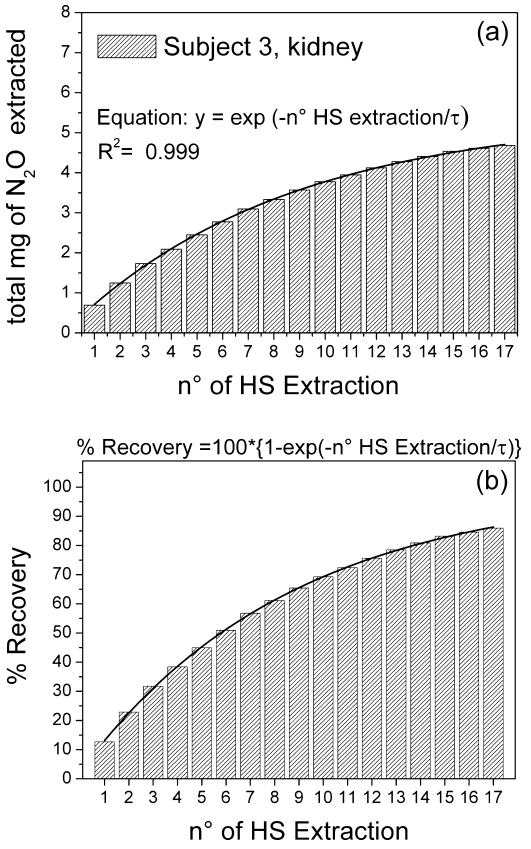

FIG. 6—*Results relating to the serial HS extractions performed on the same kidney sample from subject 3. (a) Total mg of N_2O extracted as a function of the number of HS extractions. Fitting function: $f(x) = V_{max}*\{1-exp(-n°.\ HS\ extraction/\tau)\}$. (b) Percentage (%) of recovery as a function of the number of HS extractions and its fitting curve.*

However, some N_2O was certainly lost during the time between death and autopsy, as well as during the autopsy itself. This could at least partially explain why the N_2O concentrations in the gaseous samples taken from the trachea and stomach were lower than those measured in blood and urine and to the amount released by the other tissues after HS analysis.

The abnormal amount of N_2O which patients had been exposed to, is confirmed by its high concentrations measured both in blood (11.29–2152.04 mg/L) and urine (95.11 mg/L) (Fig. 5*a*). The urinary value was about four thousand times higher than 25 μg/L, the concentration proposed as a biological index of exposure. This value is equivalent to an environmental concentration of 50 ppm (92 mg/m^3) (26,27), the environmental threshold values for occupational exposure proposed by the ACGIH (28).

In the case of the tissues, it was not possible to perform a direct quantitative analysis because of the difficulty of obtaining a homogeneous N_2O addition to the sample. Therefore, the values reported in Fig. 5*b* refer to the amount of N_2O released by tissues after the first HS extraction, and not to the whole amount absorbed by the tissues. In fact, it is known that HS extraction is not an exhaustive sampling technique since only a portion of the HS is removed and replaced by inert gas (e.g., N_2 or He). Thus, the equilibrium of the distribution of the substances is modified and, in this situation, samples continue to release N_2O until the partition coefficient has been regained.

However, to overcome this limit, a stepwise HS extraction, better known as "multiple headspace extraction" or "discontinuous gas extraction" (21–24), can be used. This method, first described in 1977 by Kolb (21), allows for the direct determination of compounds in both solid and complex liquid samples by removing the matrix effects. The authors demonstrated that the amount of

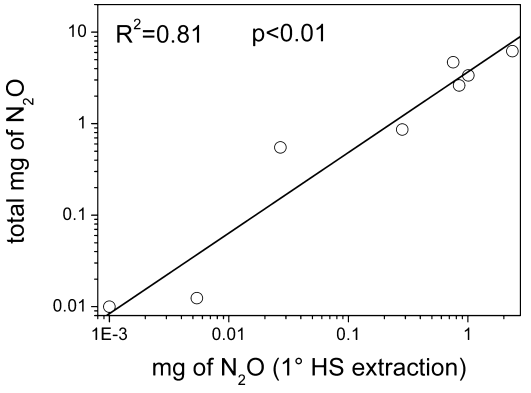

FIG. 7—*Relationship between the mg of N_2O at the first HS extraction and the total mg of N_2O (extrapolated by the fitting curves) released by the kidney samples, with the relative regression straight line on a log-log scale. The squared Pearson correlation coefficient with the relative significance are also reported.*

compounds extracted after first HS analysis is correlated with their total amount. In all cases this relationship follows a logarithmic function despite the physical/chemical properties of the compounds or the matrix involved as also confirmed in our work (Fig. 7).

Despite the methodological limits when performing homogeneous addition in tissues, the recovery study made on kidney

samples confirms that it is possible to quantify the real N_2O tissue concentrations. In fact, such a value can be extrapolated from the fitting curve of the function obtained after subsequent extractions on these same specimens.

In an ideal world, this type of analysis should also be confirmed separately in all the other tissues, taking into account their different characteristics. For this reason, further ex vivo studies on rats are in progress to evaluate toxicokinetics and tissue distribution of N_2O after fatal acute exposure.

However, while one of the aims of this case report was to demonstrate that all tissues were capable of releasing N_2O even several days after death, in order to confirm an acute systemic exposure of patients to the anesthetic, a rigorous quantification of N_2O in the different tissues, as performed for the kidney, is beyond the aim of this study.

Conclusions

Analyses carried out on the gas system confirmed the exchange of the lines (O_2 into N_2O and air flow into O_2) which explains the cause of the accident. In line with this result, biological analyses showed abnormal amounts of N_2O in all samples, demonstrating the high concentration which all patients were exposed to. Additionally, this study highlights that N_2O can be revealed in biological samples even 31 days after death in the case of fatal abnormal exposure.

In conclusion, our findings allowed us to obtain valuable evidence to clarify the true nature of the cause of death.

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