Interferences of urinary tract infection in the measurement of urinary nitrous oxide

P Apostoli, M Gelmi, L Alessio, A Turano

Abstract

Objectives—To investigate the effective role of micro-organisms in producing N₂O.

Methods—The N₂O in either urine samples inoculated with 24 microbial strains or urine samples from patients with urinary tract infections were measured.

Results—Gram negative bacilli generally produced high amounts of nitrous oxide (N₂O), whereas Gram positive cocci and yeasts did not. The production of N₂O depends on the incubation time and follows exponential kinetics, reaching a plateau at 48 hours. Furthermore, the results of urinocultures agreed well with N₂O concentrations found in urine samples: samples negative for bacteria were found to contain very low concentrations of N₂O whereas those positive—for example, for Enterobacteriaceae—gave highest N₂O values.

Conclusion—The urinary tract infections caused by Gram negative bacilli are important confounding factors in biological monitoring practices of exposure to inhalation anaesthetics. The current methods adopted to avoid these factors (urine acidification, storage of samples at 4°C) are not good enough because of the relative acid tolerance of some strains and the production of N₂O directly into the bladder.

Keywords: nitrous oxide; urinary tract infection; biological monitoring; confounding factors

The biological monitoring of exposure to anaesthetic gas is more and more frequently carried out by measuring the concentration of nitrous oxide (N₂O) in urine. It has been reported, however, that urinary tract infections can significantly interfere with the concentration of N₂O in urine samples due to the capability of a variety of microbial species to produce this gas by different mechanisms.

The measurement of urinary nitrous oxide (N₂O) as a reliable biological indicator of exposure to this anaesthetic and, possibly, also to mixtures containing halogenated compounds, has increased in recent years. In fact, considering the low solubility of the anaesthetic in the blood and assuming a constant production of urine during exposure and no exchanges at the bladder wall level, the urinary concentration of N₂O can be considered to be directly proportional to the air concentration evaluated by a time weighted environmental sample.

During our routine monitoring of surgery room staff, we found very high values of urinary N₂O in about 2% of the subjects, that could not be attributed solely to the gas exposure: in their urine samples N₂O concentrations ranged from 150 to 750 mg/l, but in samples from other workers operating in the same rooms the values ranged from 5 to 70 mg/l. This evidence, recently confirmed by other authors, happened despite the close adherence to the rules to prevent all possible causes of preanalytical errors, that is, collection of samples in non-contaminated areas, acidification of urine to avoid endogenous N₂O production, storage at 4°C, and measurement within 24 hours of sample collection.

It has been reported that urinary tract infection can significantly interfere with the concentration of N₂O in urine samples. In fact, a variety of microbial species, including those most frequently responsible for urinary infection such as Enterobacteriaceae, are capable of producing N₂O by different mechanisms.

To gain further insight into the effective role of microorganisms as sources of N₂O, we investigated N₂O formation in urine samples inoculated with different microbial strains and the correlation between the amount of N₂O detected and the result of urine cultures from patients with urinary tract infections.

Materials and methods

PRODUCTION OF N₂O BY DIFFERENT MICROBIAL SPECIES

Micro-organisms

The following organisms, all isolated from clinical materials (sputum, urine, and blood), were cultured on agar plates and identified at the Laboratory of Microbiology of the Spedali Civili of Brescia, on the basis of their morphology and metabolic activities: Staphylococcus aureus, strain No 1; Staphylococcus aureus, strain No 2; Staphylococcus aureus, strain No 3; Streptococcus agalactiae (group B), strain No 1; Streptococcus agalactiae (group B), strain No 2; Enterococcus faecalis (group D), strain No 1; Enterococcus faecalis (group D), strain No 2; Escherichia coli, strain No 1; Escherichia coli, strain No 2; Escherichia coli,
strains No 3; *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Proteus vulgaris*; *Serratia marcescens*; *Pseudomonas aeruginosa*, strain No 1; *Pseudomonas aeruginosa*, strain No 2; *Pseudomonas aeruginosa*, strain No 3; *Candida albicans*; *Candida tropicalis*, and *Torulopsis glabrata*.

Urine—A urine sample from a healthy donor, negative for the presence of bacteria, leucocytes, and for the nitrate test, was used as substrate for the microbial growth.

**Figure 1** Typical gas chromatograms: peaks (arrows) in A, B, and C, correspond to N₂O concentration of 0.3, 1.0, and 7.85 μg/l respectively.

Procedure—10 ml gas proof glass vials with teflon caps were filled with 3 ml of urine; each vial was inoculated with 100 μl of suspension of a single strain in sterile saline solution to give a final bacterial count corresponding to 1–5 × 10⁶ cfu/ml. Vials were tightly closed and incubated at 37°C for 24 hours.

**N₂O Production Kinetics**

To study the kinetics of production of N₂O, six vials were filled with 3 ml of a sample of urine, negative for bacteria, and were inoculated with *Enterobacter aerogenes* (bacterial count 1 × 10⁶ cfu/ml). The suspensions were incubated at 37°C and the amount of N₂O produced was measured at 0, 2, 5, 9, 24, and 48 hours after inoculation.

**N₂O Measurement in Urine Cultures**

Urine samples were collected from 16 patients admitted in various units of Spedali Civili of Brescia and 10 ml of each sample was inoculated by a sterile calibrated loop on the following solid media (all from Biomerieux): CLED agar, suitable for the isolation of all common bacteria and yeasts; blood agar containing the antimicrobial drug aztreonam (150 mg/l) for the selection of Gram positive species; McConkey agar for the selection of *Enterobacteriaceae* and *Pseudomonadaceae*. The urine samples, stored overnight at 4°C, were then transferred in vials and analysed for N₂O content. For four patients the N₂O measurement was carried out immediately after harvesting the sample.

**N₂O Measurement**

The urinary N₂O content was measured by head space gas chromatography.³ The vials were set in a thermostatic, automatic sampler (model FISON S HS800) and 250 ml aliquots of head space air were automatically transferred to the gas chromatograph (model FISON S HS8560 HRGC Mega 2). The analytical conditions were as follows:

- Steel column, 2 m in length, 0.3 cm in diameter, filled with Poropack Q 80/100 mesh resin; injection temperature, 200°C; bake temperature, 40°C; carrier gas, for ECD nitrogen, carrier, at 20 ml/min, make up 30 ml/min; working conditions, ECD temperature detector 260–285°C.

The calibration curve was constructed by use of N₂O cylinders with certified concentrations from 100 to 500 ppm.

Figure 1 shows, as examples, three chromatograms with N₂O concentrations of 0.3, 1.0, and 7.85 μg/l.

The calibration curves were constructed by adding known amounts of N₂O with a micro syringe to closed vials containing 3 ml of urine to make 1, 10, and 100 μg/ml. The aliquots of N₂O were extracted from vials in which the diluted N₂O came from cylinders with a known concentration of 100 ppm. Concentrations of urinary N₂O were calculated by the method of Imbriani et al.³ The detection limit was assessed at 0.2 mg/l and the coefficient of variation varied from 0.5% to 10%.
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Table 1  Urinary N₂O with various infections

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>N₂O in urine (μg/l)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus, strain 1</td>
<td>0-1</td>
</tr>
<tr>
<td>Staphylococcus aureus, strain 2</td>
<td>3-4</td>
</tr>
<tr>
<td>Staphylococcus aureus, strain 3</td>
<td>0-9</td>
</tr>
<tr>
<td>Streptococcus agalactiae (group B), strain 1</td>
<td>0-8</td>
</tr>
<tr>
<td>Streptococcus agalactiae (group B), strain 2</td>
<td>4-2</td>
</tr>
<tr>
<td>Enterococcus faecalis (group D), strain 1</td>
<td>0-6</td>
</tr>
<tr>
<td>Enterococcus faecalis (group D), strain 2</td>
<td>2-3</td>
</tr>
<tr>
<td>Streptococcus sanguis</td>
<td>0-1</td>
</tr>
<tr>
<td>Escherichia coli, strain 1</td>
<td>132-8</td>
</tr>
<tr>
<td>Escherichia coli, strain 2</td>
<td>169-2</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>181-9</td>
</tr>
<tr>
<td>Klebsiella oxacina</td>
<td>210-2</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>134-0</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>381-2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>78-5</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>87-2</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>373-4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa, strain 1</td>
<td>284-5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa, strain 2</td>
<td>245-7</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa, strain 3</td>
<td>218-3</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>1-0</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>0-1</td>
</tr>
<tr>
<td>Torulaopsis glabrata</td>
<td>1-5</td>
</tr>
</tbody>
</table>

*Urinary N₂O concentration measured by headspace gas chromatography after incubation of samples at 37°C for 24h.

Figure 2  Kinetics of N₂O production by Enterobacter aerogenes.

Results

N₂O FORMATION IN URINE SAMPLES INOCULATED WITH DIFFERENT MICROBIAL SPECIES

Table 1 shows results of samples incubated for 24 hours at 37°C. It can be seen that micro-organisms behave differently in their production of N₂O. Gram negative bacilli generally produce high amounts of N₂O, whereas Gram positive cocci and yeasts produce only small amounts of N₂O, 0-5 mg/l, not significantly higher than blank controls.

MONITORING OF N₂O PRODUCTION DURING BACTERIAL GROWTH

Figure 2 shows the amount of N₂O produced by Enterobacter aerogenes at different times. The N₂O values in fig 2 seem to be lower than those in table 1 probably because of the difference in the initial inoculum (1 × 10⁴ and 1 × 10⁶ cfu/ml, respectively).

The production of N₂O depends on incubation time: it is low during the first five hours of incubation, then increases exponentially up to 24-36 hours. Prolonged incubation does not lead to further increase of N₂O production: the plateau is reached at 48 hours.

CORRELATION BETWEEN N₂O MEASUREMENT AND RESULTS OF URINE CULTURE

Table 2 shows the N₂O measurements and results of the urine culture.

We found a good correlation between the results of urine cultures and the measurement of N₂O in urine. Urine samples negative for bacteria, considered as controls, were found to contain very low concentrations of N₂O (samples numbers 1, 5, 10, 12, and 14). Samples positive for Enterobacteriaceae (samples numbers 2, 3, 6, 7, 8, 9, 13, and 15) gave significantly higher N₂O values. Samples infected with Gram positive cocci (numbers 4 and 11) had slightly higher N₂O measurements than controls. Surprisingly, the sample positive for Pseudomonas (number 16) was negative for N₂O.

Thus, as expected, different microorganisms can produce different amounts of N₂O as a consequence of their growth in urine and metabolic activities.

There is no clear relation between the bacterial count in urine and the amount of N₂O measured: probably other factors—for example, the time since infection occurred, presence of bacteriostatic substances, or low pH—can interfere with such measurements.

Discussion

The urinary tract is the main site of excretion of anaesthetics, either in metabolised or unmodified form. The fluctuations in environmental concentrations are rapidly reflected in the urine because the concentration of anaesthetics in urine newly formed in the kidney is in equilibrium with the blood concentration. Assuming a constant production of urine over time, the concentration of anaesthetics in urine collected after the end of exposure is correlated with the weighted average value of a sample of air breathed during the exposure time. The urinary concentration of anaesthetics eliminated in the unmetabolised form is further weighted as the bladder acts as a collection and mixing vessel for the urine coming from the kidney. During the passage along the excretion system and, especially, during the stay in the bladder, it is possible that N₂O produced by micro-organisms adds to that introduced by inhalation. Moreover, if the
urine sample is not correctly stored (for example at room temperature or for long periods of time) further \( N_2O \) production could occur due to the microbial proliferation in the vials used for analysis.

Bacteria produce \( N_2O \) as a consequence of their metabolism of nitrogen. Several mechanisms are possibly involved, including the oxidation of ammonia and nitrite to nitrate (nitrification) and conversely the reduction of nitrate. If molecular nitrogen is the main product of nitrate reduction, the process is called denitrification. Denitrifier micro-organisms, which encompass species of various genera, reduce nitrate to nitrite, then to \( N_2O \) and finally to nitrogen. Other bacteria—for instance mostly enterobacteria and staphylococci—reduce nitrate only to the level of nitrite and that is excreted (nitrate/nitrite respiration). Several organisms reduce nitrite further to ammonia (nitrate ammonification). Members of Enterobacteriaceae produce small amounts of \( N_2O \) although they are not true denitrifiers.

Our results showed that several microorganisms frequently responsible for urinary tract infections can actually produce \( N_2O \). We noted that the amount of \( N_2O \) production varies. Generally speaking, Gram negative bacilli are high producers, whereas Gram positive cocci are not. The amount of \( N_2O \) produced depends not only on the species of micro-organism but also on the initial bacterial load in the sample and on the delay between sample harvesting and \( N_2O \) measurement. We noted that the steady state for the bacterial growth in urine is reached after 45–50 hours.

Normal conditions of sample storage—that is, addition of acetic acid to the urine maintained at 4°C—do not seem to be sufficient to prevent the preanalytical error linked to the presence of bacteria during the routine monitoring of exposure to anesthetical gas. We think that there are two probable explanations for this finding. Firstly, it is a well-known fact that species of bacteria are relatively acid tolerant and can survive and reproduce at a low pH. Escherichia coli, for instance, can survive at pH 4.5. Secondly, and probably the more plausible explanation is that \( N_2O \) is produced in the bladder and is already present in freshly harvested urine. To investigate this possibility we examined four patients with positive urine cultures, whose samples were collected and immediately processed and analysed for \( N_2O \) content. We found \( N_2O \) concentrations of 4, 27, 91, and 110 mg/l (data not reported on table 2). The \( N_2O \) production agreed well with the result of the urine cultures and the bacterial count. Our findings suggest that the different concentrations of \( N_2O \) detected could also be linked to the period of onset of the infection.

**Conclusion**

In conclusion, the confounding effect connected with the presence of bacteria, seems to be the difficulty in control by means of current antimicrobial strategies. Therefore, the possible outliers should be identified, mainly on the basis of the information concerning exposure (\( N_2O \) in air, urinary \( N_2O \) concentration in other workers operating in the same room, etc) and verifying the clinical history of urinary tract infections. Additional validation of the bacterial source of \( N_2O \) in urine may be gained by finding blood \( N_2O \) concentrations which are lower than expected.

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doi: 10.1136/oem.53.9.591

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