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

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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 urino-cultures 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.

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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.¹⁻⁵

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,⁶ happened despite the close adherence to the rules to prevent all possible causes of preanalytical errors,⁷—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 reported⁸ 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.⁹

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; *Streptococcus sanguis*; *Escherichia coli*, strain No 1; *Escherichia coli*, strain No 2; *Escherichia coli*,

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strain No 3; *Klebsiella pneumoniae*; *Klebsiella ozaenae*; *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 N_2O concentration of 0.3, 1.0, and 78.5 $\mu\text{g/l}$ 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 $1-5 \times 10^6$ cfu/ml. Vials were tightly closed and incubated at 37°C for 24 hours.

N_2O PRODUCTION KINETICS

To study the kinetics of production of N_2O , six vials were filled with 3 ml of a sample of urine, negative for bacteria, and were inoculated with *Enterobacter aerogenes* (bacterial count 1×10^5 cfu/ml). The suspensions were incubated at 37°C and the amount of N_2O produced was measured at 0, 2, 5, 8, 24, and 48 hours after inoculation.

N_2O 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 N_2O content. For four patients the N_2O measurement was carried out immediately after harvesting the sample.

N_2O MEASUREMENT

The urinary N_2O content was measured by head space gas chromatography.³ The vials were set in a thermostatic, automatic sampler (model FISIONS HS800) and 250 ml aliquots of head space air were automatically transferred to the gas chromatograph (model FISIONS 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 N_2O cylinders with certified concentrations from 100 to 500 ppm.

Figure 1 shows, as examples, three chromatograms with N_2O concentrations of 0.3, 1.0, and 78.5 mg/l.

The calibration curves were constructed by adding known amounts of N_2O with a microsyringe to closed vials containing 3 ml of urine to make 1, 10, and 100 mg/l. The aliquots of N_2O were extracted from vials in which the diluted N_2O came from cylinders with a known concentration of 100 ppm. Concentrations of urinary N_2O 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 1%.

Table 1 Urinary N₂O with various infections

Bacterial strain	N ₂ O in urine (µg/l) *
<i>Staphylococcus aureus</i> , strain 1	0.1
<i>Staphylococcus aureus</i> , strain 2	3.4
<i>Staphylococcus aureus</i> , strain 3	0.9
<i>Streptococcus agalactiae</i> (group B), strain 1	0.8
<i>Streptococcus agalactiae</i> (group B), strain 2	4.2
<i>Enterococcus faecalis</i> (group D), strain 1	0.6
<i>Enterococcus faecalis</i> (group D), strain 2	2.3
<i>Streptococcus sanguis</i>	0.1
<i>Escherichia coli</i> , strain 1	132.8
<i>Escherichia coli</i> , strain 2	169.2
<i>Escherichia coli</i> , strain 3	464.7
<i>Klebsiella pneumoniae</i>	181.9
<i>Klebsiella ozaenae</i>	210.2
<i>Enterobacter cloacae</i>	134.0
<i>Enterobacter aerogenes</i>	381.2
<i>Proteus mirabilis</i>	78.5
<i>Proteus vulgaris</i>	87.2
<i>Serratia marcescens</i>	373.4
<i>Pseudomonas aeruginosa</i> , strain 1	284.5
<i>Pseudomonas aeruginosa</i> , strain 2	245.7
<i>Pseudomonas aeruginosa</i> , strain 3	218.3
<i>Candida albicans</i>	1.0
<i>Candida tropicalis</i>	0.1
<i>Torulopsis glabrata</i>	1.5

*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 (1×10^5 and 1×10^6 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.^{2,3,10} 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.¹¹ 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

Table 2 Correlation between N₂O measurement and urinoculture results

Sample number	N ₂ O production in urine (µg/l) *	Urinoculture	
		Micro-organism	cfu/ml
1	0.10	Negative	
2	415.00	<i>Escherichia coli</i>	2×10^6
3	5.73	<i>Citrobacter freundii</i>	1×10^6
4	2.81	<i>Enterococcus faecalis</i>	2×10^6
5	1.04	Negative	
6	5.59	<i>Escherichia coli</i>	2×10^6
7	54.55	<i>Escherichia coli</i>	1×10^5
8	12.56	<i>Escherichia coli</i>	2×10^5
9	11.10	<i>Proteus mirabilis</i>	2×10^6
10	0.45	Negative	
11	1.98	<i>Enterococcus faecalis</i>	5×10^5
12	0.73	Negative	
13	50.00	<i>Escherichia coli</i>	2×10^5
14	1.01	Negative	
15	13.12	<i>Escherichia coli</i>	2×10^6
16	1.56	<i>Pseudomonas aeruginosa</i>	1×10^6

* Production of N₂O in urine as measured by headspace gas chromatography.

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.¹²

Our results showed that several micro-organisms 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 anaesthetic gas. We think that there are two probable explanations for this finding. Firstly, is that some 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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