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Postmortem Drug Metabolism by Bacteria

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ABSTRACT: Studies were undertaken to determine the possible role of enteric bacteria in the postmortem bioconversion of the nitrobenzodiazepines flunitrazepam, clonazepam, and nitrazepam. Flunitrazepam, clonazepam, and nitrazepam were completely metabolized in blood in the presence of eight species of enteric bacteria to their respective 7-amino-metabolites. The rates of metabolism, at 37°C, ranged from 0.1 ng/mL/min for *Streptococcus faecalis* to 8.8 ng/mL/min for *Clostridium perfringens.* The rate of conversion was reduced to 87% by a combination of 0.7% (w/v) sodium fluoride and potassium oxalate, and almost completely inhibited (96%) by 1% (w/v) sodium fluoride. pH had variable effects on the rate of metabolic bioconversion of nitrobenzodiazepines, while increasing temperatures were found to generally increase the rate of nitrobenzodiazepine bioconversion. These data support the proposal that bacteria may mediate postmortem bioconversion of the nitrobenzodiazepines.

KEYWORDS: toxicology, drug metabolism, postmortem bioconversion, bacteria, nitrobenzodiazepines

Accurate interpretation of postmortem toxicologic results is very much dependent on the understanding of the processes that take place after death. Postmortem degradation of drugs and poisons is one process that is little understood but that may significantly affect our interpretation of post-mortem toxicological results.

Postmortem degradation may either occur as a result of metabolic processes or derive from chemical decomposition of labile molecules [*1*]. Metabolism of nitrobenzodiazepines such as nitrazepam to 7-amino-nitrazepam has been shown to occur in both liver microsomal and cytosolic preparations [*2*]. Nitrazepam was also found to be reduced in the kidney, heart, lung, skeletal muscle, and spleen preparations [*2*].

The role of intestinal bacteria in nitrobenzodiazepine bioconversion has also been studied. Bacteria have been implicated as being responsible for the postmortem metabolic changes of many drugs including the nitrobenzodiazepines clonazepam, nitrazepam, and flunitrazepam [*3,4*]. Nitrazepam and clonazepam have been shown to be degraded at an increased rate when exposed to fly-borne bacteria [*3*]. Bacteria in the gastrointestinal tract was quantitatively the most important site of nitrobenzene reduction in rats [*5*] and humans [*6*]. Takeno and Sakai [*4*] demonstrated the reduction of nitrazepam to 7-amino-nitrazepam by anaerobic microbial action of cecal contents at rates seven times those for liver microsomal enzymes and Kuroiwa et al. [*7*] showed that flunitrazepam is rapidly converted to 7-amino-flunitrazepam when exposed to human stools.

Bacteria are known to transmigrate throughout the body from the oral cavity, lungs and the gastrointestinal tract following death [*8*]. After death bacteria are known to penetrate the intestinal wall, enter the blood and lymph vessels and migrate further throughout the body [*9*]. This transmigration can occur as soon as 5 h after death at 25°C [*9*]. The most likely bacteria involved in postmortem metabolism of drugs in femoral blood are those originating in the gastrointestinal tract such as *Bacillus spp., Escherichia coli, Proteus miribalis, Clostridium perfringens, Staphylococcus aureus, Staphylococcus epidermis, Streptococcus faecalis* and *Bacteroides fragilis* [*10–12*]. Bacteria, including *Clostridium perfringens, Bacteroides fragilis* and *Escherichia coli* have all been shown to contain an oxygen-sensitive nitroreductase enzyme capable of reducing nitroaromatic compounds, [*6,13,14*].

It was the aim of this study to investigate the ability of various species of enteric bacteria to metabolize nitrobenzodiazepines.

Materials and Methods

Reagents and Chemicals

Sodium dihydrogen orthophosphate (NaH_2PO_4), sodium carbonate (Na_2CO_3), potassium dihydrogen orthophosphate (KH_2PO_4), sodium fluoride, sodium hydroxide and hydrochloric acid were of analytical reagent grade (Ajax Chemicals, Aust.). Acetonitrile (Mallinckrodt Aust.), butyl chloride (Fisons UK) were of HPLC grade. A combination of sodium fluoride/potassium oxalate was removed from blood preservative tubes purchased from Drager Aust. Horse blood (Amadeus Int. Aust.), Columbia agar base (Unipath Ltd. UK) were obtained from the stated suppliers.

All drugs were obtained from curator of standards from the Australian Government Analytical Laboratories. Stock solutions were prepared in methanol and deionized water was used for all further dilutions. Fresh whole blood was supplied by the Victorian branch of the Red Cross Blood Bank.

Postmortem blood was removed from the femoral region of deceased persons by forensic technicians and stored in plain collection tubes that were refrigerated at 4°C until required.

Microbiology

Blood collected at autopsy was plated on horse blood agar and incubated at 37°C for 24 h both aerobically and anaerobically in an anaerobic jar containing a gas-generating kit (Unipath Ltd. UK). Horse blood agar (11.7 g/300 mL Columbia agar base with 8% horse blood) was used for the aerobic and anaerobic detection and isolation of enteric bacteria from postmortem blood. Pure isolates

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were stored at −20°C. Identification was done by Gram stain and API 20E identification system (bioMerieux Vitek-Aust. Pty. Ltd.).

At the conclusion of all experiments, microbial sterility or purity was reassessed.

Stability Studies

The inherent chemical stabilities of nitrobenzodiazepines in buffer and blood were determined. Flunitrazepam and clonazepam were added at a concentration of 500 ng/mL each to 18 mL of sterile 0.1 M sodium phosphate buffer (pH 5.5). Flunitrazepam, clonazepam, and nitrazepam were added together at a concentration of 500 ng/mL each to 18 mL of whole blood. These were stored in sterile McCartney bottles at 22°C or −20°C for 21 days. Aliquots (500 μL) were removed at zero time and after 21 days. Two types of blood were used, sterile fresh whole blood (blood bank) and blood collected at autopsy from a 32-year-old male who had committed suicide, found to contain *Streptococcus faecalis, Escherichia coli, Bacteroides fragilis* and *Proteus vulgaris.*

Femoral postmortem blood, found to be sterile, was also collected from six cadavers. Nitrazepam and clonazepam were added to this blood (500 ng/mL each) and stored at 22°C. Aliquots (500 μL) were removed at various times over 540 min.

Aliquots taken in this experiment and all further experiments were stored at −70°C for quantification of parent drug and the respective 7-amino-metabolites by HPLC.

Examination of Postmortem Blood

Blood collected at autopsy from another cadaver (61-year-old male), who died naturally, contained *Staphylococcus aureus, Bacteroides fragilis, Escherichia coli* and was used to determine the metabolic activity of bacteria. Flunitrazepam, clonazepam and nitrazepam were added to 18 mL of blood (500 ng/mL) with or without 1% (w/v) sodium fluoride and stored air tight in McCartney bottles at 22°C. Aliquots (500 μL) were removed at intervals over 360 min. A parallel study was conducted with the addition of 1% (w/v) of sodium fluoride which was added immediately prior to the addition of the drugs.

Examination of Bacterial Species and Rates of Bioconversion

The ability of individual bacterial species to metabolize the nitrobenzodiazepines were examined using 18 mL of sterile fresh whole blood stored in sterile McCartney bottles and inoculated with 1 pure colony of either *Bacteroides fragilis, Clostridium perfringens, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, Streptococcus faecalis, Staphylococcus epidermis, Escherichia coli* or *Klebsiella pneumoniae.* These bacteria were isolated from postmortem specimens by the method described earlier. These bottles were then stored at 37°C for 48 h to allow bacterial growth. After this time nitrazepam, clonazepam and flunitrazepam were then added together (500 ng/mL each). Aliquots (500 μL) were taken over 480 min. Temazepam was added (500 ng/mL) as a nonmetabolized internal control to account for poor mixing and volume variation between vials at the time of nitrazepam, clonazepam, and flunitrazepam addition.

Effects of Preservatives on the Rate of Bioconversion

The effects of common blood preservatives were examined using 18 mL of sterile fresh whole blood in sterile McCartney bottles and inoculated with 1 pure colony of either *Bacteroides fragilis, Clostridium perfringens, Staphylococcus aureus* or *Bacillus cereus.* These bottles were then incubated at 37°C for 48 h to allow bacterial growth. The blood was then cooled to 22°C prior to the aseptic addition of flunitrazepam (500 ng/mL). Temazepam was added (500 ng/mL) as a nonmetabolized internal control to account for poor mixing and volume variation between vials at the time of flunitrazepam addition. Sodium fluoride was added at either 1 or 2% (w/v) concentration after completion of the 48 h incubation. To other bottles the extract from two tubes containing a commercially produced combination of sodium fluoride and potassium oxalate 0.7% (w/v) was added to blood containing bacteria either prior to or following incubation. Aliquots were taken over 480 min.

Effects of pH on the Rate of Bioconversion

To study the effects of pH on bacterial activity, 18 mL of sterile fresh whole blood stored in sterile McCartney bottles was inoculated with 1 pure colony of either *Bacteroides fragilis, Clostridium perfringens, Staphylococcus aureus, Bacillus cereus* or *Staphylococcus epidermis.* These bottles were then stored at 37°C for 48 h to allow bacterial growth. The pH of the blood was then adjusted to between 4.4 and 10.7 with either dilute hydrochloric acid or sodium hydroxide solution. The bioconversion reaction was started by the aseptic addition of a solution containing nitrazepam, clonazepam and flunitrazepam (500 ng/mL each) at 37°C. Temazepam was added (500 ng/mL) as a nonmetabolized internal control to account for poor mixing and volume variation between vials at the time of nitrazepam, clonazepam and flunitrazepam addition. Aliquots (500 μL) were taken at regular intervals for drug analysis. The pH of blood was recorded at the conclusion of each experiment.

Effects of Temperature on the Rate of Bioconversion

The effect of temperature on bacterial activity was examined as for the rate experiments described earlier, however after the initial inoculation and incubation blood was maintained at either 4°C, 22°C or 37°C after the addition of nitrobenzodiazepines.

Quantification of Benzodiazepines

Benzodiazepines and the 7-amino-metabolites were quantified in blood following extraction with butyl chloride and isocratic HPLC [*15*].

Statistics

Mean ± standard deviation are shown in the text. Statistical evaluation of these data was conducted by a Mann-Whitney test using the In Stat 2.01 program run on an Apple Macintosh personal computer.

Results

Stability Studies in Sterile Media

There was no loss of flunitrazepam or clonazepam over 21 days when incubated at 22°C in sterile 0.1 M sodium phosphate buffer, or when sterile whole blood was frozen at −20°C. There was minimal loss of nitrazepam (6 ± 1%), clonazepam (6 ± 1%) or flunitrazepam (21 ± 2%) over 21 days when incubated at 22°C in sterile fresh whole blood to their respective 7-amino-metabolites. There was no loss of nitrazepam and clonazepam incubated in sterile post-mortem blood over 540 min at 22°C ($n = 6$).

Stability Studies in Bacterially Contaminated Postmortem Blood

In blood taken from one cadaver 28 h after death, containing *Streptococcus faecalis, Escherichia coli, Bacteroides fragilis* and *Proteus vulgaris,* complete conversion of flunitrazepam and clonazepam to their respective 7-amino-metabolites occurred within 540 min at 22°C. There was no loss of flunitrazepam or clonazepam over 21 days when this blood was frozen at −20°C.

The rate of flunitrazepam, clonazepam and nitrazepam bioconversion in postmortem blood from a cadaver 48 h after death, containing *Staphylococcus aureus, Bacteroides fragilis,* and *Escherichia coli* was rapid at 22°C with a corresponding production of the respective 7-amino-metabolite. By 240 min parent drug concentrations had fallen to less than detectable concentrations (Fig. 1). The addition of 1% sodium fluoride inhibited this conversion with 24 ± 13% flunitrazepam, 60 ± 14% clonazepam and 46 ± 15% nitrazepam still remaining after 360 min (Fig. 1).

Examination of Bacterial Species and Rate of Bioconversion

The rate of nitrobenzodiazepine reduction to their respective 7-amino-metabolites by individual bacteria in blood was found to be species dependent (Table 1). The obligate anaerobic species (those requiring strict anaerobic conditions) *Bacteroides fragilis* and *Clostridium perfringens* exhibited higher conversion rates than the faculative anaerobic species (those able to survive in either aerobic or anaerobic conditions) *Bacillus cereus, Staphylococcus epidermis, Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, Proteus miribalis* and *Klebsiella pneumoniae* ($P < 0.05$). There was little difference among the species for their ability to preferentially metabolize nitrazepam, clonazepam and flunitrazepam (Table 1).

Effects of Preservatives on the Rate of Bioconversion

The addition of 1% sodium fluoride after inoculation and incubation, produced a statistically significant inhibition of metabolism in all species examined. 1% sodium fluoride produced a 95 ± 2% reduction of metabolism with *Clostridium perfringens,* a 92 ± 3% reduction with *Staphylococcus aureus* and a 65 ± 9% reduction with *Bacillus cereus* (Table 2, $P < 0.05$). There was no statistical difference in the inhibitory effects of 1 or 2% sodium fluoride ($P > 0.05$). Metabolic inhibition was increased from 95 ± 2 to 98 ± 3% for *Clostridium perfringens* and from 65 ± 9 to 82 ± 12% for *Bacillus cereus* when sodium fluoride/potassium oxalate was added to blood at a concentration of 0.7% prior to the addition of bacteria. *Staphylococcus aureus* was less sensitive to sodium fluoride/potassium oxalate, with inhibition decreasing from 92 ± 3 to 77 ± 7% ($P < 0.05$). When sodium fluoride/potassium oxalate was added post incubation, inhibition was significant only for the *Clostridium* and *Staphylococcus* spp. but not for *Bacillus* spp. The inhibition observed when sodium fluoride/potassium oxalate was added post incubation was less effective than when added pre incubation in all species examined ($P < 0.05$). Similarly, 1% sodium fluoride was a more effective inhibiter of *Bacillus* and *Clostridium* than 0.7% sodium fluoride/potassium oxalate (Table 2, $P < 0.05$).

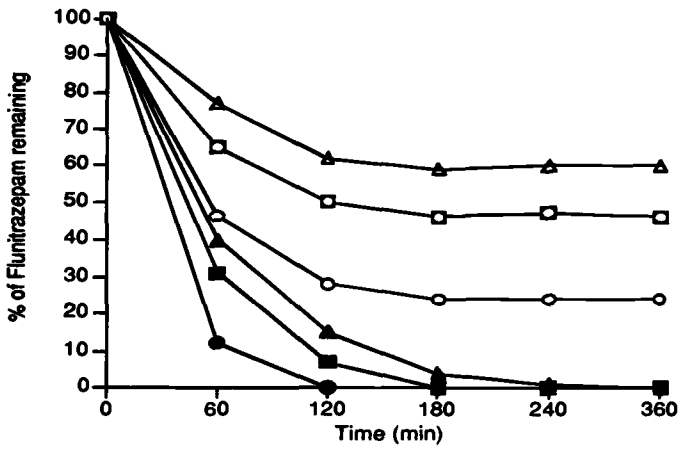

FIG. 1—*Graph showing conversion in bacterially contaminated postmortem blood of flunitrazepam without preservative* (closed circles) *and with preservative* (open circles), *clonazepam without preservative* (closed triangles) *and with preservative* (open triangles), *and nitrazepam without preservative* (closed squares) *and with preservative* (open squares). *Values are expressed as a mean of 4 experiments at 22°C.*

TABLE 1—*Rates of 7-amino-nitrazepam (7AN), 7-amino-clonazepam (7AC) and 7-amino-flunitrazepam (7AF) formation from parent drug (ng/mL/min) for different bacterial species at 37°C in blood.*

	7AN	7AC	7AF
Sterile	NA (<0.01)	NA (<0.01)	NA (<0.01)
Obligate anaerobes			
B. fragilis	5.5 ± 1.6[a]	5.3 ± 1.0[a]	4.0 ± 0.6[a]
C. perfringens	7.6 ± 0.9[a]	8.8 ± 0.9[a]	8.7 ± 0.2[a]
Faculative anaerobes			
B. cereus	0.3 ± 0.1[b]	0.4 ± 0.1[b]	0.4 ± 0.03[b]
S. epidermis	0.8 ± 0.1[b]	0.7 ± 0.04[b]	1.0 ± 0.1[b]
S. aureus	0.3 ± 0.03[b]	0.2 ± 0.1	0.3 ± 0.04[b]
K. pneumoniae	0.2 ± 0.04	0.2 ± 0.03	0.2 ± 0.03
E. coli	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.02
S. faecalis	0.1 ± 0.04	0.2 ± 0.1	0.2 ± 0.04

All experiments were conducted at 37°C and are the mean ± SD of 4 experiments.
[a] $P < 0.05$ when compared to faculative species.
[b] $P < 0.05$ when compared to *S. faecalis.*
All rates were significant when compared to the sterile control.
NA = No activity detected.

Effects of pH on the Rate of Bioconversion

The effects of pH variation on the metabolic activity of bacteria was variable (Table 3). After adjusting the pH of blood to a more basic pH than the control blood, the rate of production of 7-amino-nitrazepam (7AN), 7-amino-clonazepam (7AC) and 7-amino-flunitrazepam (7AF) was significantly different for the *Bacillus cereus* species only ($P < 0.05$, Table 3).

The acidification of blood decreased the rate of *Bacteroides fragilis, Clostridium perfringens* and *Staphylococcus epidermis* mediated 7AN, 7AC and 7AF production, whilst the activity of *Staphylococcus aureus* and *Bacillus cereus* remained largely unchanged (Table 3).

Effects of Temperature on the Rate of Bioconversion

At 4°C, only *Clostridium perfringens* exhibited any detectable metabolic activity (Table 4). At 22°C a significant rise in metabolism occurred for all species except *Clostridium perfringens,* which

TABLE 2—*Rates of flunitrazepam bioconversion to 7-amino-flunitrazepam (ng/mL/min) by different bacterial species in blood exposed to the preservatives: 1% sodium fluoride (NaF), 2% NaF, NaF/Kox (post)—(0.7% combination of sodium fluoride/potassium oxalate added post-incubation), NaF/Kox (pre)—(0.7% added prior to incubation).*

	Control	1% NaF	2% NaF	NaF/Kox (post)	NaF/Kox (pre)
Obligate anaerobes					
B. fragilis	1.2 ± 0.3	ND	ND	ND	0.17 ± 0.06[a]
C. perfringens	1.9 ± 0.1	0.1 ± 0.03[a,c]	0.1 ± 0.1[a]	0.3 ± 0.1[a,b]	0.1 ± 0.1[a]
Faculative anaerobes					
B. cereus	0.2 ± 0.03	0.1 ± 0.04[a,c]	0.1 ± 0.01[a]	0.20 ± 0.1[b]	0.03 ± 0.02[a]
S. aureus	0.5 ± 0.2	0.04 ± 0.01[a]	0.03 ± 0.01[a]	0.2 ± 0.1[a,b]	0.1 ± 0.03[a]

All experiments were conducted at 22°C and are the mean ± SD of 4 experiments.
[a]$P < 0.05$ compared to control.
[b]$P < 0.05$ (post) compared to (pre).
[c]$P < 0.05$ 1% NaF compared to fluoride/oxalate combination (post).
ND—Not determined.

showed no increased metabolism compared to 4°C ($P > 0.05$). At 37°C all species produced metabolites at rates higher than those at 22°C ($P < 0.05$).

Discussion

Flunitrazepam, clonazepam, and nitrazepam are all nitro-containing benzodiazepines that are metabolized during life to the major 7-amino-metabolite at levels similar to those of parent drug. The minor metabolites include the acetamido- and desmethyl-metabolites [*16–18*].

Flunitrazepam, clonazepam, nitrazepam and their minor metabolites are also thought to be metabolized postmortem to their respective 7-amino-metabolites, [*16–18*]. Degradation or decomposition may occur not only within the decaying body but also after specimens are taken [*1,18*]. Drummer et al. [*19*] reported a series of deaths involving flunitrazepam in which no parent drug was detected postmortem in femoral blood, however large amounts of flunitrazepam were found in the stomach contents of many of the cases.

Our stability studies demonstrated that nitrobenzodiazepines are rapidly converted to their respective 7-amino-metabolites *in-vitro* in the presence of bacteria. In contrast, sterile postmortem blood did not produce any metabolism of benzodiazepines over 540 min, nor was bioconversion detected in sterile buffer over three weeks, while less than 21% of nitrobenzodiazepines were converted in sterile fresh whole blood over a three week period.

While all the bacteria examined possess the ability to metabolize flunitrazepam, clonazepam and nitrazepam to their 7-amino-metabolites in blood, they were found to have differing abilities to metabolize the nitrobenzodiazepines. The fastest rate of flunitrazepam metabolism was found for *Clostridium perfringens* which was some 87 times faster than for *Escherichia coli* (0.1 ng/mL/min at 37°C). Overall the rates for the obligate anaerobes *Bacteroides* and *Clostridium* spp. were significantly higher than the facu-

TABLE 3—*Rates of 7-amino-nitrazepam (7AN), 7-amino-clonazepam (7AC) and 7-amino-flunitrazepam (7AF) formation (ng/mL/min) by various species of bacteria in control blood, blood made more basic and blood made more acidic. pH values are in parentheses.*

	Control	More Basic	More Acidic
B. fragilis	(7.3)	(10.4)	(4.7)
7AN	1.1 ± 0.2	ND	0.4 ± 0.1[a]
7AC	1.3 ± 0.7	ND	0.2 ± 0.1[a]
7AF	1.3 ± 0.1	ND	0.5 ± 0.1
C. perfringens	(7.0)	(7.9)	(5.6)
7AN	3.3 ± 1.5	1.2 ± 0.2	0.5 ± 0.2[a]
7AC	2.4 ± 1.3	2.5 ± 0.4	0.5 ± 0.2[a]
7AF	2.8 ± 1.0	3.0 ± 0.4	0.7 ± 0.1[a]
B. cereus	(7.7)	(9.0)	(4.4)
7AN	0.1 ± 0.02	0.3 ± 0.1[a]	0.1 ± 0.01
7AC	0.1 ± 0.1	0.4 ± 0.04[a]	0.0 ± 0.01[a]
7AF	0.1 ± 0.03	0.6 ± 0.1[a]	0.1 ± 0.03
S. epidermis	(6.7)	(9.7)	(4.4)
7AN	0.5 ± 0.1	0.6 ± 0.04	0.2 ± 0.1[a]
7AC	0.5 ± 0.1	0.5 ± 0.02	0.1 ± 0.1[a]
7AF	0.6 ± 0.04	0.6 ± 0.01	0.4 ± 0.1[a]
S. aureus	(6.6)	(10.7)	(4.8)
7AN	0.03 ± 0.01	NA	0.04 ± 0.03
7AC	0.04 ± 0.01	0.01 ± 0.01	0.03 ± 0.03
7AF	0.03 ± 0.01	0.04 ± 0.03	0.1 ± 0.1

All experiments were conducted at 22°C and are the mean ± SD of 4 experiments.
[a]$P < 0.05$ compared to control.
ND—Not determined.
NA—No activity.

TABLE 4—*Rates of 7-amino-nitrazepam (7AN), 7-amino-clonazepam (7AC) and 7-amino-flunitrazepam (7AF) formation (ng/mL/min) by various species of bacteria in fresh whole blood at 4°C, 22°C and 37°C.*

	4°C	22°C	37°C
B. fragilis			
7AN	NA	1.1 ± 0.2[a]	5.5 ± 1.6[b]
7AC	NA	1.3 ± 0.7[a]	5.3 ± 1.0[b]
7AF	NA	1.3 ± 0.1[a]	4.0 ± 0.6[b]
C. perfringens			
7AN	5.8 ± 1.7	3.3 ± 1.5	7.6 ± 0.9[b]
7AC	5.1 ± 1.6	2.4 ± 1.3	8.8 ± 0.9[b]
7AF	6.2 ± 2.4	2.8 ± 1.0	8.7 ± 0.2[b]
B. cereus			
7AN	NA	0.1 ± 0.1[a]	0.3 ± 0.1[b]
7AC	NA	0.1 ± 0.1[a]	0.4 ± 0.1[b]
7AF	NA	0.1 ± 0.03[a]	0.4 ± 0.03[b]
S. epidermis			
7AN	NA	0.5 ± 0.1[a]	0.8 ± 0.1[b]
7AC	NA	0.5 ± 0.1[a]	0.7 ± 0.04[b]
7AF	NA	0.6 ± 0.04[a]	1.0 ± 0.1[b]
S. aureus			
7AN	NA	0.03 ± 0.01[a]	0.3 ± 0.03[b]
7AC	NA	0.04 ± 0.01[a]	0.2 ± 0.1[b]
7AF	NA	0.03 ± 0.01[a]	0.3 ± 0.04[b]

All experiments are the mean ± SD of 4 experiments.
[a]$P < 0.05$ when compared to 4°C.
[b]$P < 0.05$ when 37° compared to 22°C.
NA—No activity < 0.01 ng/mL/min.

lative anaerobes including *Bacillus* and *Staphylococcus* spp. It has been suggested by others that nitroreductases, including those found in bacteria, are oxygen sensitive and hence are inhibited by the presence of molecular oxygen [*5,14,20,21*]. It may be possible that the obligate anaerobes, requiring strict anaerobic conditions, contain more efficient nitroreductases and are therefore able to reduce the nitrobenzodiazepines at a faster rate. The oxygen sensitivity of obligate anaerobes may also account for the large variations in rates highlighted by the large standard deviations in the rate experiments.

Sodium fluoride, an inhibitor of enzyme systems involved in glycolysis [*22*], when added to blood after it was inoculated with bacteria and incubated for 48 h inhibited the rate of nitrobenzodiazepine metabolism significantly. Higher concentrations of sodium fluoride (2%) did not further inhibit metabolism suggesting a 1% concentration was probably optimal for sodium fluoride. When 0.7% sodium fluoride/potassium oxalate (potassium oxalate forms insoluble complexes with calcium ions and inhibits several enzymes [*22*]) was added to blood, prior to bacterial inoculation and incubation, significant inhibition occurred. When added after bacterial inoculation and incubation the amount of inhibition was less than that achieved when added prior to inoculation and incubation. It was also found that sodium fluoride was a significantly better inhibitor of *Clostridium perfringens* and *Bacillus cereus* than sodium fluoride/potassium oxalate when either are added after inoculation and incubation. These results suggest that metabolism of nitrobenzodiazepines may still occur postmortem in collection tubes if significant bacterial contamination of blood has already occurred or if preservatives have not been used to store blood. The choice of preservative, the extent of bacterial contamination and the species of bacteria present will therefore also affect the rate of nitrobenzodiazepine bioconversion.

The pH of blood is known to vary over the postmortem interval [*23*]. pH demonstrated a variable effect on rate, with all bacteria still active at pH between 4.4 and 10.4. Acidification of blood only had a significant effect on *Bacillus cereus,* however alkalinization significantly reduced the rates of bioconversion by the *Bacteroides, Clostridium* and *Staphylococcus* species. Rafii et al. [*13*] showed that the nitroreductases in *Clostridium perfringens* were most active between pH 5.5 and 9.

Higher temperatures were generally found to increase the rate of metabolism. While most species showed no activity at 4°C, it was of interest that *Clostridium* showed significant activity. This bacteria is found in about 15% of cases [*24*]. Total inhibition can however be achieved if specimens are stored at −20°C.

These data would suggest that bacteria deriving from the intestine have the ability to metabolize nitrobenzodiazepines. Since these bacteria are often found in postmortem specimens bacteria may be responsible for the metabolism of nitrobenzodiazepines to 7-amino-metabolites postmortem. The possibility of competing processes occurring in bodies however cannot be ruled out completely.

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