Michael D. Robertson,[1] *Ph.D. and Olaf H. Drummer,*[1] *Ph.D.*

Stability of Nitrobenzodiazepines in Postmortem Blood

REFERENCE: Robertson MD, Drummer OH. Stability of nitrobenzodiazepines in postmortem blood. J Forensic Sci 1998;43(1):5–8.

ABSTRACT: Studies were undertaken to determine the stability of nitrobenzodiazepines and their 7-amino metabolites in water and blood. At 22°C nitrazepam and clonazepam were stable in sterile fresh blood containing preservative over 28 days, whereas 25% of flunitrazepam was degraded. At 37°C all three drugs were substantially lost over 9 h (29–51%). There was only a small loss observed for the 7-amino metabolites and no substantial amounts of parent drug and 7-amino metabolite were degraded in water under these conditions. In the absence of preservative substantial amounts (25–50%) of parent drugs were lost in fresh blood over 10 days at 22°C. In bacterially-contaminated postmortem blood all three drugs were completely degraded over 8 h at 22°C with almost all drug completely converted to the respective 7-amino metabolite. These metabolites were also partially degraded (10–20%) over 45 h at 22°C. All 3 nitrobenzodiazepines were stable in blood stored for up to 24 months at −20°C, or 4°C over 10 months. Their respective 7-amino metabolites were, however, relatively unstable at −20°C with a significant loss (29%) after 2 months. At 4°C a 21% loss occurred after 1 month. Freeze/thawing was found not to affect the concentration of nitrobenzodiazepine and 7-amino metabolites. These results show that the nitrobenzodiazepines and their metabolites are unstable chemically and metabolically in blood. We advise that blood collected for the purpose of nitrobenzodiazepine determinations should be preserved with sodium fluoride, stored at −20°C and assayed as soon as practicable, preferably within a week of collection.

KEYWORDS: forensic science, forensic toxicology, benzodiazepines, stability, postmortem blood, human toxicology

The concentrations of some drugs change significantly after death. These changes may reflect processes such as postmortem redistribution (1,2). Loss of drug may also occur as a result of chemical instability (3,4) and metabolic activity (5).

Lloyd and Parry (6) had shown that nitrazepam was lost and replaced by 7-amino-nitrazepam over several days storage at room temperature. This loss was suppressed by sodium fluoride or low temperatures. The corresponding 7-amino metabolite was found to be stable under these conditions. Another study found that clonazepam was almost totally lost after 6 weeks storage at 20°C (7). It was also shown that chlordiazepoxide, norchlordiazepoxide, and nordiazepam were rapidly lost over 90 days at 25°C (3). The rate of decay was reduced when stored at 4°C. They also found that diazepam and flurazepam were stable over 5 months at room temperature. Al-Hadidi and Oliver (8) showed temazepam was stable when stored at −20°C, but when specimens were stored at 5°C and 25°C a time dependant decrease in concentration occurred over 12 months.

The causes of these losses were not investigated and no distinction was made between chemical decomposition and putrefactive degradation of these drugs. Rapid conversion of the nitrobenzodiazepines to their respective 7-amino metabolites by bacteria has been demonstrated and has been attributed to the labile nature of these drugs under anaerobic conditions (4,5). The stability of these drugs in specimens free of bacteria or under conditions devoid of significant bacterial activity have not been reported.

It was the objective of this study to investigate the short and long term stability of the nitrobenzodiazepines in blood in comparison to water. The effects of collection procedures and storage conditions on the concentration of nitrobenzodiazepines were also examined in an attempt to further understand the fate of nitrobenzodiazepines postmortem and therefore to assist in the toxicological interpretation of deaths involving these drugs.

Materials and Methods

Reagents and Glassware

All drugs and metabolites were obtained from the curator of standards at the Australian Government Analytical Laboratories. Sodium carbonate (Na_2CO_3) and potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical reagent grade (Ajax Chemicals, Australia). Acetonitrile (Mallinckrodt, Australia), and butyl chloride (Fisons, UK) were of HPLC grade.

Extraction tubes were silanized by immersing the glassware in a 5% solution of Surfasil (Pierce Chemical Company, U.S.A.) in toluene for 1 h, followed by rinsing in methanol. These were then dried before use.

Standards and Controls

Stock drug solutions were prepared in methanol at a concentration of 1 mg/mL. Working standard solutions were prepared by adding dilutions of the stock solutions in methanol to the relevant blank blood to give final concentrations ranging from 0.01–0.60 mg/L.

Blood specimens prepared with known concentrations of nitrobenzodiazepines and metabolites were assayed in duplicate in each experiment to provide a measure of quality assurance. These were prepared in-house by QC officer. Target concentrations were 0.10 and 0.40 mg/L with an acceptance range of ±20%. Negative controls were also run with each assay.

Microbiological Techniques

Blood specimens were tested for a large range of skin- and gastrointestinal-derived organisms. Horse blood agar plates were

[1]Victorian Institute of Forensic Medicine, Department of Forensic Medicine, Monash University, Southbank, Australia.

Received 1 July 1996; and in revised form 26 Feb, 25 April 1997; accepted 25 April 1997.

inoculated with blood for 48 h under both anaerobic and aerobic conditions. When growth was present, bacteria were subcultured onto appropriate solid agar for growth and isolation of colonies. A range of standard identification procedures were used including Gram stain, Catalase and Coagulase tests. When growth was not observed, blood was considered bacteria-free.

Stability Studies in Water and Fresh Whole Blood

To either sterile fresh whole blood or sterile distilled water (pH 6.9), the following drugs were added in separate experiments: (a) flunitrazepam, clonazepam and nitrazepam were added together at a concentration of 0.1 mg/L, to 4 mL of sterile distilled water and sterile fresh whole blood, (b) separately, 7-amino nitrazepam, 7-amino clonazepam and 7-amino flunitrazepam were added together at a concentration of 0.2 mg/L each, to 4 mL of sterile distilled water and fresh whole blood, and (c) separately, the minor metabolites 7-acetamido nitrazepam, 7-acetamido-clonazepam, 7-amino-desmethyl-flunitrazepam and desmethyl-flunitrazepam were added at a concentration of 0.2 mg/L each, to 4 mL of sterile distilled water and sterile fresh whole blood. Additional specimens containing the minor metabolites were also stored at −20°C for 28 days.

All specimens were incubated at 4°C, 22°C, and 37°C for 28 days in commercially prepared sterile 10 mL plastic tubes containing a 1% (w/v) combination of sodium fluoride and potassium oxalate as a preservative. Aliquots (500 μL) were removed and analyzed at zero time and after 28 days. After collection, the aliquots were rapidly frozen and stored at −20°C until analyzed.

To determine if the preservative affected the stabilities of the parent nitrobenzodiazepines, a separate experiment was performed. To 4 mL of sterile, fresh whole blood without preservative, flunitrazepam, clonazepam and nitrazepam were added at a concentration of 0.2 mg/L each, and stored in sterile 10 mL plastic tubes at 22°C for 10 days. Aliquots (500 μL) were removed at zero time and after 10 days. After collection aliquots were rapidly frozen and stored at −20°C until analyzed.

Stability Studies in Postmortem Blood

Blood used in the stability studies was removed from the femoral vein of cadavers when the body arrived at the mortuary, prior to autopsy, and stored at −20°C. This "admission specimen" was stored in tubes containing 1% sodium fluoride/potassium oxalate as preservative. Duplicate femoral blood specimens from the same region as above were collected by syringe at the time of autopsy and stored in tubes containing 1% sodium fluoride/potassium oxalate preservative and stored at −20°C and 4°C. Initial concentrations were determined within two weeks of specimen collection and reexamined over time for 10 months when stored at 4°C and over 24 months when stored at −20°C. Storage temperatures were monitored regularly throughout the course of the experiment. Asceptic techniques were used where appropriate to withdraw blood.

Stability of Nitrobenzodiazepines Following Freezing and Thawing

To assess the stability of the nitrobenzodiazepines during a freezing and thawing process, aliquots (4 mL) of fresh whole blood were spiked with nitrazepam, clonazepam and flunitrazepam (0.1 mg/L) and rapidly frozen in a −30°C alcohol bath, then thawed in cold water. This cycle was repeated 5 times, and was followed by the analysis of nitrobenzodiazepine concentrations. Each freeze/thaw cycle took approximately 2 min.

The procedure was also repeated using the respective 7-amino metabolites at a concentration of 0.1 mg/L.

Quantification of Benzodiazepines

Nitrobenzodiazepines and the 7-amino-metabolites were quantified in blood following extraction with butyl chloride and analyzed by isocratic HPLC (9).

Statistics

Mean ± standard deviation are shown in the text. Statistical evaluation of these data was conducted using the InStat 2.01 program run on an Apple Macintosh personal computer. Paired specimens were analyzed by the non-parametric Wilcoxon test and non-paired specimens using the unpaired alternate Welch t-test. Data were also analyzed by ANOVA when appropriate.

Results

Stability Studies in Water and Fresh Whole Blood

Nitrazepam and clonazepam were stable in sterile whole blood, containing preservative, over 28 days at both 4°C and 22°C (Table 1). However, at 37°C there was a 51% loss of nitrazepam and 29% loss of clonazepam ($p < 0.05$). There was also a significant loss of flunitrazepam at 4°C, 22°C and at 37°C, with only 53% remaining when incubated at 37°C ($p < 0.05$).

There were relatively small losses of all the parent nitrobenzodiazepines when incubated in water. The loss of parent drugs in water was variable and did not increase with rising temperatures (Table 1).

7-Amino nitrazepam was stable in sterile whole fresh blood for 28 days at 4°C, however, there was a significant loss at 22°C and 37°C ($p < 0.05$) (Table 1). 7-Amino clonazepam was stable when incubated at all 3 temperatures. In contrast, the concentrations of 7-amino flunitrazepam declined by ~10% at all temperatures. Incubation in water produced a small increase in concentration of all three 7-amino metabolites at all three temperatures, however these were generally within the experimental error of ±20%.

TABLE 1—*Percentage remaining of the nitrobenzodiazepines and their respective 7-amino metabolites following incubation in both whole preserved fresh blood and water at various temperatures over 28 days.*

Drug	Temperature		
	4°C	22°C	37°C
	Blood		
Nitrazepam	106 ± 15	95 ± 4.1	49 ± 4.1*
7-Amino nitrazepam	101 ± 2.5	79 ± 5.9*	76 ± 5.2*
Clonazepam	104 ± 11	95 ± 3.9	71 ± 5.6*
7-Amino clonazepam	119 ± 9.4	126 ± 15	92 ± 8.7
Flunitrazepam	92 ± 4.1*	75 ± 1.5*	53 ± 9.1*
7-Amino flunitrazepam	90 ± 3.6*	88 ± 4.8*	91 ± 13*
	Water		
Nitrazepam	89 ± 9.2*	99 ± 7.7	92 ± 1.3†
7-Amino nitrazepam	115 ± 4.6*	119 ± 4.9*	127 ± 8.1*
Clonazepam	86 ± 3.6*	95 ± 14	88 ± 6.5*†
7-Amino clonazepam	107 ± 3.8	112 ± 5.2*	123 ± 1.9*
Flunitrazepam	90 ± 3.9*	90 ± 10*	98 ± 1.5†
7-Amino flunitrazepam	112 ± 4.2*	116 ± 6.6*	123 ± 4.0*

Values are expressed as the mean (% remaining) ± SD of 7 experiments.
*$p < 0.05$ compared to pre-incubation.
†$p < 0.05$ compared to pre-incubation.

In the absence of preservative, the concentration of all three parent nitrobenzodiazepines were found to significantly decrease in fresh blood over 10 days, when incubated at 22°C ($p < 0.05$, Fig. 1). Loss of drug was highest for flunitrazepam (50%) followed by nitrazepam (25%) and clonazepam (15%). The concentration of the 7-amino metabolites produced did not reflect the amount of parent drug lost. No other metabolites were detected in any of these incubations.

7-Amino-desmethyl-flunitrazepam, desmethyl-flunitrazepam, 7-acetamido-clonazepam and 7-acetamido-nitrazepam were all stable when incubated at −20°C, in bacteria-free fresh blood for 28 days. 7-Amino-desmethyl-flunitrazepam, desmethyl-flunitrazepam and 7-acetamido-nitrazepam were rapidly lost on incubation at higher temperatures. At 37°C, the greatest loss was for desmethyl-flunitrazepam (92%), followed by 7-acetamido-nitrazepam (66%) and 7-amino-desmethyl-flunitrazepam (38%). The rate of loss increased with increasing temperatures. In contrast, there was no loss of 7-acetamido-clonazepam at 22°C and at 37°C.

Stability Studies in Postmortem Blood

Incubation of the nitrobenzodiazepines in bacteria-free postmortem blood from 6 cadavers at 22°C for 9 h, showed no significant loss of either nitrazepam or clonazepam, ($p > 0.05$).

In a parallel study, flunitrazepam and clonazepam were rapidly converted to their respective 7-amino metabolites when incubated in bacterially-contaminated postmortem blood for 8 h at 22°C (Fig. 2). The concentration of flunitrazepam had fallen by 96% within the first 8 h, at a rate of approximately 1.2 ng/mL/min. Similarly, clonazepam concentrations had fallen by 89% within the first 8 h also at a rate of 1.2 ng/mL/min. The blood was found to contain 4 bacterial species. These were *Streptococcus faecalis, Clostridium perfringens, Bacteroides sp. and Proteus vulgaris.*

The addition of the 7-amino metabolites to bacterially-contaminated postmortem blood and incubated at 22°C for 45 h, showed a small but significant loss of all the 7-amino metabolites ($p < 0.05$). The loss was greatest for 7-amino nitrazepam (20%) followed by 7-amino clonazepam (15%) and 7-amino flunitrazepam (10%). No parent nitrobenzodiazepine, nor metabolites were detected in any specimens. Incubation of the minor metabolites (7-amino-desmethyl-flunitrazepam, desmethyl-flunitrazepam, 7-acetamido-nitrazepam and 7-acetamido-clonazepam) in bacterially-contaminated blood, showed complete loss over 14 days at 22°C.

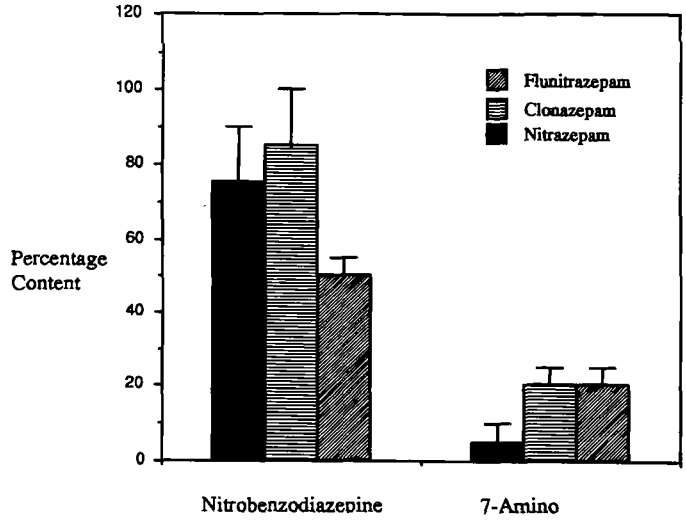

FIG. 1—*Content of parent nitrobenzodiazepine (as percentage of original) following incubation in fresh whole blood over 10 days at 22°C. The 7-amino metabolite values reflect the amount produced over this time (as percentage of original parent drug).*

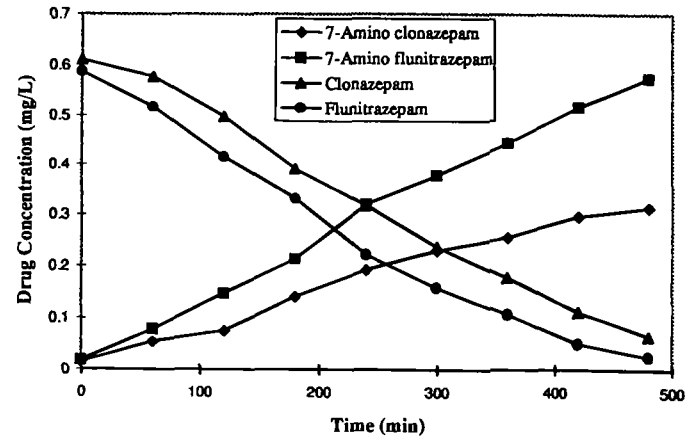

FIG. 2—*Graph showing conversion of bacterially-contaminated postmortem blood of flunitrazepam and clonazepam to their respective 7-amino metabolites, 7-aminoflunitrazepam and 7-aminoclonazepam.*

Effects of Storage on Stability in Toxicological Specimens

The concentrations of parent nitrobenzodiazepines in postmortem blood over the 24 month storage period at −20°C, or over 10 months at 4°C (Table 2) showed a good deal of variability in these experiments, particularly at 4°C, however, these were not significant given the low rate of detection of the parent drug. However, there was a significant loss observed in the concentrations of the 7-amino metabolites ($p < 0.05$). After 2 months at −20°C, there was a 29% loss and after 24 months a 68% loss was observed. When stored at 4°C for 1 month there was a 21% loss, after 2 months a 27% loss, and after 10 months a 53% loss ($p < 0.05$). In this table data for individual nitrobenzodiazepines were combined to simplify presentation of data. All three benzodiazepines showed similar changes.

Stability of Nitrobenzodiazepines Following Freezing and Thawing

There was no loss of either the parent nitrobenzodiazepine or 7-amino metabolite after 6 cycles of freezing and thawing ($p > 0.05$). Mean concentrations after freeze/thawing ranged from 98–110% of original (SD 8–14%).

Discussion

The stability profiles of the parent nitrobenzodiazepines and their respective metabolites were complex. In water the nitrobenzodiazepines were relatively stable. There were small losses, but these did not increase at higher temperatures. These observations suggest that these substances are stable under these conditions.

When incubated in preserved fresh whole blood, the nitrobenzodiazepines were stable at 4°C, however at 37°C as much as half the parent drugs were lost over 28 days. Flunitrazepam was the most unstable of the nitrobenzodiazepines. Similarly, there were temperature-dependent losses of the 7-amino metabolites in blood.

The presence of bacteria had the greatest effect on the stability

TABLE 2—*Percentage remaining of nitrobenzodiazepines (NB) and 7-amino metabolites (7 AM) when stored at −20°C for 24 months and 4°C for 10 months.*

Months	−20°C		4°C	
	7 AM	NB	7 AM	NB
0.5	94 ± 13 (n = 13)	95 ± 10 (n = 4)	88 ± 25 (n = 4)	71 ± 36 (n = 4)
1	96 ± 12 (n = 18)	94 ± 10 (n = 6)	79 ± 27* (n = 11)	88 ± 25 (n = 4)
2	71 ± 23* (n = 17)	95 ± 10 (n = 4)	73 ± 31* (n = 17)	46 ± 30 (n = 2)
10	42 ± 22* (n = 14)	73 ± 25 (n = 5)	47 ± 30* (n = 9)	67 ± 29 (n = 3)
24	32 ± 26* (n = 22)	100 ± 0 (n = 5)	nr	nr

All values expressed are the mean (% remaining) ± SD.
*$p < 0.05$ when compared to zero time, nr = no result.

of the nitrobenzodiazepines in postmortem blood. In bacteria-free postmortem blood at 22°C, significant loss of drug occurred after 1 to 2 weeks, however in the presence of bacteria, substantial losses occurred in hours. Although the 7-amino derivatives were relatively stable, the non-7-amino metabolites were unstable in the presence of bacteria.

Toseland (10), also found that in blood, at least at therapeutic concentrations, nitrazepam degraded at ambient temperatures. He also found that both nitrazepam and clonazepam were more stable in aqueous solutions than blood, and suggested that decomposition may be mediated by enzymes rather than by chemical instability. These findings may explain the observations made by Iten (11), Drummer et al. (12) and Lloyd and Parry (6), that in postmortem blood specimens, rarely is the parent compound detected, leaving the 7-amino compound the only indication of nitrobenzodiazepine usage. Metabolic activity by enzymes in many bacteria has been recently confirmed (5).

Stevens (4), related the greater stability of the 7-amino derivatives to the strength of bonds between the nitrogen and the hydrogen on the 7-amino compound, which was not present on the parent compound, making the 7-amino metabolites more resistant to bacterial metabolism than the parent nitrobenzodiazepines. The poor stability of 7-acetamido compounds, was also observed by Lloyd and Parry (6).

It was of interest that in postmortem specimens, the 7-amino metabolites were lost with prolonged storage. After only 1 month at 4°C, 21% of the original concentration of 7-amino metabolites had been lost. After 2 years 68% had been lost during storage frozen at −20°C. In contrast, the parent nitrobenzodiazepines, were stable under these conditions, although little parent drug was present in these specimens initially because of metabolic degradation (5). The stability of the nitrobenzodiazepines has been confirmed by Knop et al. (7), who found that the conversion of clonazepam in plasma at 20°C was decreased by approximately 80% when the temperature was reduced to 1°C and further decreased by approximately 95% at −20°C after 20 weeks.

Interestingly, the process of freezing and thawing did not effect the concentrations of the parent nitrobenzodiazepines and their metabolites. This would suggest that time and temperature rather than any mechanical process are the most important factors in the changes observed.

In conclusion, under sterile conditions, the nitrobenzodiazepines were relatively stable in sterile blood; however, when bacteria were present, there was rapid conversion of the parent nitrobenzodiazepines to their respective 7-amino metabolites. In contrast, the 7-amino metabolites were quite stable under these conditions. However, the non-7-amino metabolites were also unstable under these conditions. Of particular significance, was the substantial loss in the concentration of 7-amino metabolites during long-term storage. The loss, even at −20°C has important implications in the interpretation of the toxicology findings in cases where prolonged storage has occurred. We therefore advise that concentrations of nitrobenzodiazepines and metabolites should be determined as soon as possible after specimen collection.

Acknowledgments

We would like to thank the forensic technical staff for supplying blood specimens of deceased persons and for the assistance and support provided by the microbiology and toxicology laboratories. This work formed, in part, research towards a Ph.D. (MDR).

References

1. Pounder DJ, Jones GR. Postmortem drug redistribution—A toxicological nightmare. Forensic Sci Int 1990;45:253–63.
2. Prouty WR, Anderson WH. The forensic science implications of site and temporal influences on postmortem blood-drug concentrations. J Forensic Sci 1990;35:243–70.
3. Levine B, Blanke RV, Valentour JC. Postmortem stability of benzodiazepines in blood and tissues. J Forensic Sci 1983;28:102–15.
4. Stevens, HM. The stability of some drugs and poisons in putrefying human liver tissues. J Forensic Sci Soc 1984;24:577–89.
5. Robertson MD, Drummer OH. Postmortem drug metabolism by bacteria. J Forensic Sci 1995;40:282–6.
6. Lloyd JBF, Parry DA. Forensic applications of the determination of benzodiazepine in blood specimens by microcolumn cleanup and high-performance liquid chromatography with reductive mode electrochemical detection. J Anal Toxicol 1989;132:163–8.
7. Knop HJ, van der Kleun E, Edmunds LC. The determination of clonazepam in plasma by gas-liquid chromatography. Pharmaceutisch Weekblad, 1975;110:297–309.
8. Al-Hadidi KA, Oliver JS. Stability of temazepam in blood. Science and Justice 1995;35:105–8.
9. Robertson MD, Drummer OH. An HPLC procedure for the measurement of nitrobenzodiazepines and their 7-amino-metabolites in blood. J Chromatog 1995;667:179–84.
10. Toseland PA. Samples and sampling. In: Moffat AC, Editor, Clarke's isolation and identification of drugs (2nd edition), The Pharmaceutical Press, 1986.
11. Iten PX. No flunitrazepam in postmortem blood in fatal flunitrazepam intoxications (No. 83). In: Nagata T, Editor, Proceedings of the 30th International Meeting TIAFT, Fukuoka, Japan, 1992;343–6.
12. Drummer OH, Syrjanen ML, Cordner SM. Deaths involving the benzodiazepine flunitrazepam. Am J Forensic Med Pathol 1993;14:238–43.

Additional information and reprint requests:
Olaf H. Drummer, Ph.D.
Victorian Institute of Forensic Medicine
Department of Forensic Medicine
Monash University
57-83 Kavanagh St.
Southbank, Australia 3006