Chula Med J Vol. 67 No. 3 July - September 2023;231-239 DOI : 10.14456/clmj.2023.29

Original article

The stability of diazepam in authentic blood samples from Thai postmortem cases

Pornpatsorn Siriwattanaskul[a, b], Sunitda Khawthong[b], Peerayuht Phuangphung[b, *]

[a]*Buddhasothorn Hospital, Ministry of Public Health, Chachoengsao, Thailand*
[b]*Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand*

Background: Diazepam is a common drug detected in Thai postmortem cases. The stability of diazepam in blood was previously studied using spiked blood samples. However, there is no available data for stability in authentic postmortem blood samples.
Objectives: This study aimd to determine the stability of diazepam in authentic postmortem blood samples.
Methods: Postmortem blood samples were obtained from the cadavers of Thai people who were sent for medico - legal autopsies at the Department of Forensic Medicine, Siriraj Hospital, Mahidol University. Blood samples were analyzed for diazepam and nordiazepam within one week after sample reception (initial concentrations: day_0). Subsequently, the blood samples were re-analyzed for diazepam and nordiazepam on the 30th, 60th, 90th, 120th, and 180th days. Descriptive statistics and repeated measures analysis of variance were applied for comparison of the diazepam and/or nordiazepam concentrations over the period of 180 days.
Results: There were 24 blood samples recruited for this study. For the statistical analysis, 22 blood samples consisting of diazepam concentrations greater than 10 ng/mL were applied. These 22 samples were classified into low, medium and high concentrations. The mean and median of percent changes from initial diazepam concentrations for low, medium and high groups on the 180th day were - 16.8/ - 12.9, - 24.0/ - 25.9, and - 27.9/ - 22.6, respectively. Diazepam concentrations in the medium and high groups decreased significantly from initial concentrations, particularly on the 120th day and 180th day ($P < 0.01$).
Conclusion: Diazepam concentrations in authentic postmortem blood samples decreased significantly on the 180th day, particularly for medium and high concentrations.

Keywords: Diazepam, postmortem blood, stability, Thai.

Diazepam is a widely prescribed drug that belongs to a class of benzodiazepines and is commonly detected in medico-legal cases. It has been frequently identified in cases of suicide and drug intoxication.[1] Its mechanism of action is the enhancement of $GABA_A$ receptors in the brain, producing anticonvulsant, sedative and hypnotic as well as anxiolytic properties.[2] Thus, the analysis of blood diazepam concentration in dead bodies will assist in diagnoses concerning the cause and manner of death in medico-legal cases. In general, postmortem blood samples are collected from both femoral venous blood and heart blood in the form of unpreserved blood samples except for blood for alcohol, cocaine, heroin and gamma-hydroxybutyric acid (GHB).[3] These blood samples are then stored at 4°C, 0°C, - 20°C, or - 80°C before analysis depending on the analytical objectives and laboratory facilities.[3] At this viewpoint, drug stability data are important for proper interpretation of laboratory results in medico-legal cases.

There are several situations that lead to an interval between sample collection time and time of drug analysis. For example, confirmatory tests can sometimes be halted until the related cases have progressed to the court procedure.[4] In Thailand, specimens are sometimes stored in a refrigerator or freezer for a certain period until police officers can send them for drug analysis and this interval may range

*Correspondence to: Peerayuht Phuangphung, Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
E - mail: peerayuht.phu@mahidol.ac.th
Received: February 9, 2023
Revised: April 11, 2023
Accepted: May 3, 2023

from weeks to months. In addition, the re-analyses of specimens for certain legal purposes after a period of time are not uncommon in forensic cases.[5] Drug stability data are crucial for these situations.

The stability of diazepam has been investigated in previous studies. Mata DC conducted a stability experiment using pooled postmortem blood spiked with multiple drugs including diazepam stored under various conditions.[4] This study showed that diazepam concentrations in postmortem blood stored at 4°C presented a 20.0% concentration decrease after 2 months; the maximal percentage decrease over 8 months could be up to 47.0%.[4] Skopp G, *et al*. performed experiments by spiking benzodiazepines into unpreserved fresh whole blood and plasma collected from living volunteers and storing it at 4°C.[6] They found that diazepam and nordiazepam decreased by at least 40.0% from initial concentrations after a period of 8 months.[6] Atanasov VN, *et al*. conducted a study of diazepam stability using spiked whole blood and plasma collected from living volunteers kept under different experimental conditions.[7] They found that diazepam concentrations in whole blood without preservatives stored at 4°C were reduced by more than 80.0% in the 12th week whereas diazepam concentrations in plasma without preservatives stored at 4°C decreased by more than 70.0% in the 12th week.[7] However, these studies were based on spiked samples and there may have been some limitations for application to authentic blood samples.

Thus, this study aimed to investigate the stability of diazepam in authentic postmortem blood samples stored in a refrigerator (4°C), which is a common storage condition in hospitals. The findings provide fundamental information for the stability data of diazepam and would be useful for laboratory interpretation of drug analysis after a period of time. In addition, this information would be applicable as guidance when a request for drug re-analysis is made for forensic purposes.

Materials and methods

Study design and subjects

This study was carried out using an *in vitro* laboratory-based approach. The postmortem blood samples used in this study were repository blood samples collected from femoral veins in medico-legal cases that were sent for forensic autopsy at the Department of Forensic Medicine, Siriraj Hospital, Mahidol University between 1st November 2021 and 30th June 2022. First, the blood samples were analyzed for drug screening panel within one week after specimen collection. Then, the blood samples were routinely stored in a refrigerator (4°C) which is the normal storage condition in the department for approximately 1 year after drug analysis (which is the routine period in the department) in case of the request for re-analysis from the police or court. The inclusion criteria for blood samples recruited in this study were blood samples collected from Thai people aged 18 years old or older who had no previous history of medical treatment with benzodiazepines before death. All subjects had died from traffic accidents and had the medical history of receiving diazepam administration during hospitalization. All blood samples were tested for diazepam with or without nordiazepam and composed of at least 15 mL of blood volume.

The exclusion criteria were blood samples obtained from Thai people with causes of death other than traffic accident and blood samples positive for ethanol and any drugs of abuse including stimulants (amphetamine,methamphetamine, 3, 4-methylenedioxy-methamphetamine (MDMA), 3, 4-methylenedioxy-amphetamine (MDA), 3, 4-methylenedioxy-N-ethyl-amphetamine (MDEA)), cocaine and its metabolites (ecgonine methyl ester, benzoylecgonine, and cocaethylene), opiates and opioids (morphine, codeine, 6-acetylmorphine, methadone, 2-ethylidene-1, 5 -dimethyl-3, 3-diphenylpyrrolidine (EDDP), fentanyl, and tramadol), cannabis and its metabolites (delta-9-tetrahydrocannabinol (THC), and 11-nor-9-carboxy- delta-9-tetrahydrocannabinol (THC-COOH)), ketamine, and mitragynine. All of these inclusion and exclusion criteria were employed to ensure that all blood samples had a very low risk for re-analysis because approximately 3 mL of each blood sample would remain after the experiment.

Blood samples fit for criteria were analyzed for diazepam and nordiazepam within one week after specimen collection. Diazepam with or without nordiazepam concentrations at this period were defined as the initial day (day_0). Then, all blood samples were stored at 4°C and each blood sample was taken for the re-analysis on 30th, 60th, 90th, 120th, and 180th days, respectively. Every re-analysis was performed in duplicate, and the average concentrations of diazepam and/or nordiazepam were calculated. Case data including gender, age and postmortem

interval (PMI) which was defined as the time interval between time of death and time of blood collection were recorded. These data and average diazepam and/or nordiazepam concentrations at each time period were employed for statistical analysis.

This study was approved by the Siriraj Institutional Review Board, Faculty of Medicine, Siriraj Hospital, Mahidol University (Certificate of Approval no. Si 681/2021, Research Project no. 727/2564).

Chemicals and reagents

Diazepam, nordiazepam at concentrations of 1 mg/mL and diazepam-d_5 at a concentration of 0.1 mg/mL were purchased from Sigma-Aldrich. Acetonitrile and methanol LC/MS grade were sourced from Duksan Pure Chemicals. Ammonium acetate and formic acid was obtained from Merck. Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Daejung Chemicals. All standards and chemicals were supplied by U&V Holding (Thailand) Co., Ltd. Deionized water (dH_2O) was generated from Merck Millipore Direct-Q® 3 UV-R Water Purification System.

Instrumentation

The analysis of diazepam and nordiazepam in blood was performed using an electrospray ionization liquid chromatography Quadrupole Time-of-Flight (QTOF) mass spectrometry (ESI - LC - QTOF - MS). A thermo scientific dionex ultimate 3,000 model high performance liquid chromatography (HPLC) was coupled with a Maxis Impact Bruker Daltonics QTOF-MS instrument. Chromatographic separation was conducted using a Phenomenex Luna C18 column (10 cm x 2 mm x 3 µm, 100 A) equipped with a guard column (4 cm x 2 mm x 3 µm, 100 A). The mobile phase was composed of 0.1% formic acid and 5 mm ammonium acetate in dH_2O (A) and Acetonitrile (B). The analysis was performed using gradient elution with a flow rate of 0.3 mL/min. The gradient program started with mobile phase 75.0% A: 25.0% B, then increasing mobile phase B to 75.0% in 10 minutes, increasing mobile phase B to 90.0% in 0.1 minute and holding 10.0% A: 90.0% B for 3 minutes, back to 75.0% A: 25.0% B within 0.1 minutes and continuing with this condition for 5 minutes. Total run time was 18 minutes. The injection volume was 5 µL. The column temperature was set at 40°C.

Mass spectrometry was operated in positive ESI mode. ESI parameters were set as capillary voltage 2,900 V, Nebulizer gas 2.0 bar, drying gas 8.0 L/min and drying temperature 180°C. Broad band collision induced dissociation (bbCID) mode was performed with a mass range of 50 - 1,500 m/z. Collision energy for MS and MS/MS (bbCID) mode were set at 4.0 and 35.0 eV, respectively. Multiple reaction monitoring (MRM) transitions for diazepam were 285 > 193 and 285 > 154 whereas MRM transitions for nordiazepam were 271 > 140 and 271 > 208, respectively (quantitation ion was defined as underscored ion). The retention times for diazepam and nordiazepam were 7.7 and 6.5 minutes, respectively.

Sample preparation

After 1 mL of postmortem blood sample was pipetted into a test tube, 1.5 mL of cold acetonitrile was added. The blood sample was vortexed and centrifuged at 4,000 rpm/min. The supernatant part was taken and 2 mL of 0.1M phosphate buffer pH6.0 was added into the supernatant. Then, the sample was extracted by solid phase extraction (SPE) using SPE cartridge Oasis HLB® 60 mg 3mL. The SPE cartridge was conditioned with methanol and dH_2O. Subsequently, the sample was loaded into the SPE cartridge followed by washing with 10.0% methanol in dH_2O. Finally, the elution step was achieved by methanol. The eluent solution was evaporated under nitrogen stream at 40°C. The sample was then re-constituted with 75.0%: 25.0% mobile phase A: B and injected into LC-QTOF-MS.

Method validation

Method validation was performed following Standard Practices for Method Validation in Forensic Toxicology issued by the Scientific Working Group for Forensic Toxicology (SWGTOX) 2013.[8] Method validation was conducted using expired whole blood from the Department of Transfusion Medicine, Siriraj Hospital, Mahidol University. Selectivity and interference studies were performed to achieve complete separation between diazepam/ nordiazepam and baseline noise. In addition, other benzodiazepine drugs were tested to ensure that they did not interfere with diazepam/nordiazepam analysis. Limit of detection (LOD) and lower limit of quantitation (LLOQ) were evaluated by spiking decreasing concentrations of diazepam and nordiazepam into blood samples. LOD and LLOQ were determined by the lowest concentrations that generated a signal-to-noise (S/N) greater than 3 and 10 times, respectively.

In this study, LOD and LLOQ for both diazepam and nordiazepam were determined as 5 and 10 ng/mL, respectively.

The matrix effect (ionization suppression/ enhancement experiment) was assessed because the method was involved in using LC-QTOF/MS. Average peak areas for three concentrations (30, 80 and 400 ng/mL) of diazepam and nordiazepam in standards prepared in the mobile phase were compared with standards spiked in extracted blank blood samples. The matrix effect for all analytes should not exceed ± 25.0%. The matrix effects for the three concentrations of diazepam and nordiazepam in this study were - 2.3 - 13.5% and - 17.6 - 12.9%, respectively.

Linearity ranges were assessed using six diazepam and nordiazepam calibrators at 10, 20, 50, 100, 200 and 500 ng/mL prepared as five replicates and run on five separate days. Calibration curves for diazepam and nordiazepam were generated by Bruker Daltonics Compass for OTOF Series 1.7 Software®. Criteria of $r^2 \geq 0.99$, and accuracy of each calibrator within ± 15.0% (LLOQ ± 20.0%) and % coefficient of variation (%CV) ≤ 15.0% should be achieved. The accuracy and %CV of the diazepam calibrator at 10 ng/mL (LLOQ) were - 6.8 - 16.0% and ≤ 10.6%, respectively, whereas the accuracy and %CV of other diazepam calibrators were - 12.9 - 13.9% and ≤ 8.8%. The accuracy and %CV of the nordiazepam calibrator at 10 ng/mL (LLOQ) were - 7.9 - 6.4% and ≤ 9.8%, respectively, whereas the accuracy and %CV of other nordiazepam calibrators were -14.2 - 13.7% and ≤ 12.9%.

Accuracy and precision were evaluated by the injection of five replicates of spiked blood samples at low, medium and high quality control (QC) concentrations (30, 80 and 400 ng/mL) on five separate days. Accuracy for each QC concentrations should be within ± 15.0% and precision that was evaluated by %CV should also be ≤ 15.0%. The accuracy of the three concentrations of diazepam ranged from - 13.1% to 12.0%, and %CV was ≤ 8.6%. The accuracy of the three concentrations of nordiazepam ranged from - 9.1 to 13.7%, and %CV was ≤ 10.8%.

Statistical analysis

The statistical analysis was performed using SPSS for Window® version 25. Descriptive statistics including mean, median, and standard deviation were analyzed for gender, age and PMI. Repeated measures analysis of variance (ANOVA) with multiple comparisons were performed for the comparisons of diazepam and nordiazepam concentrations which were greater than 10 ng/mL at the initial day over the period of 180 days. Multiple comparisons were analyzed using Mauchly's test of Sphericity, and Bonferroni correction was applied for statistical analysis with an adjusted *P* - value at $P < 0.01$ (due to the number of comparisons between each period and day_0). Bonferroni correction with an adjusted *P* - value at $P < 0.01$ was employed because there were five comparisons between initial day and the other five dates. Thus, conventional *P* - value at $P < 0.05$ should be divided by five due to five comparisons. In addition, standard deviations for data in this study were large and Bonferroni correction which was a conservative correction was applied to ensure genuine statistical significance.

Results

There were 24 blood samples recruited for this study comprising 6 female and 18 male subjects with an overall mean age of 43.7 years old (age range = 25 - 70 years old). The mean ages of the female and male subjects were 49.0 and 41.9 years old, respectively (female and male age ranges = 29 - 68 and 25 - 70 years old). The mean PMI was 19.5 hours (range of time interval = 4.5 - 29.0 hours). The concentrations of diazepam and nordiazepam over the period of 180 days in each blood sample are shown in Table 1, which shows that diazepam in blood samples 3 and 8 were less than 10 ng/mL at the initial time. Thus, they were not included in the statistical analysis for the stability of diazepam. Accordingly, 22 blood samples were included for the stability study of diazepam. These 22 blood samples presented with a wide range of diazepam concentrations. Thus, diazepam concentrations were categorized into three levels based on the three QC ranges used in this study, including low (< 30 ng/mL), medium (30 - 80 ng/mL) and high (80 - 400 ng/mL), to reduce the effect of wide standard deviation. Unfortunately, nordiazepam was presented in only 5 blood samples. Thus, these nordiazepam concentrations were applied for statistical analysis without categorization.

After categorization, there were 5, 9, and 8 blood samples consisting of diazepam in low, medium, and high concentrations, respectively. Diazepam concentrations on the 30^{th}, 60^{th}, 90^{th}, 120^{th}, and 180^{th} days were compared with initial time and calculated

as the percent change from the initial concentration. According to the SWGTOX guidelines for method validation, the stability of a drug is focused on ± 15.0% change from the initial drug concentration. [8] Thus, the percent changes from initial concentrations of diazepam are shown in Table 2 and Figure 1. In addition, repeated measures ANOVA with multiple comparisons were performed for the comparison of diazepam between the initial period and all following days over the period of 180 days and the statistical significance levels at $P < 0.01$ are also shown in Table 2.

According to Table 2 and Figure 1, diazepam concentrations tended to decrease over the period of 180 days. When the cut-point of ± 15.0 % change from the initial concentration was considered, two groups of medium and high diazepam concentrations decreased beyond - 15.0% particularly on the 120th day and the 180th day. In addition, these reductions also produced significant differences with $P < 0.01$ on the 120th day and the 180th day. However, the median percent change from the initial concentration for the group of low diazepam concentration remained within ± 15.0% and this change did not produce a significant difference over the period of 180 days.

The percent change from the initial concentration of nordiazepam is shown in Table 3 and Figure 2. Nordiazepam concentrations also decreased over the period of 180 days. When the percent change was considered, all nordiazepam concentrations decreased beyond - 15.0% from the 30th day to the 180th day. However, the significant difference for nordiazepam was developed only on the 180th day. This result was caused by the small number of positive nordiazepam samples and the wide range of nordiazepam concentrations that produced high standard deviation.

Table 1. Concentrations of diazepam and nordiazepam over a period of 180 days.

Case	Drug	Gender	Age	PMI (hour)	Concentration (ng/mL)					
					Day 0	Day 30	Day 60	Day 90	Day 120	Day180
1	Diazepam	M	51	15	140.4	119.9	109.5	107.3	102.3	58.7
2	Diazepam	M	41	26	48.5	43.1	39.4	41.6	40.4	37.4
3	Diazepam	M	39	26	< 10	< 10	< 10	< 10	ND	ND
	Nordiazepam				121.3	99.6	86.4	87.4	68.2	51.2
4	Diazepam	M	25	23	19.0	18.2	16.5	13.9	12.6	12.9
	Nordiazepam				98.0	77.3	73.0	63.8	64.9	45.4
5	Diazepam	M	70	29	120.0	108.0	107.0	105.2	111.5	94.5
6	Diazepam	F	29	26	26.9	23.6	30.4	28.0	16.7	20.1
	Nordiazepam				24.6	14.1	19.6	24.2	10.8	ND
7	Diazepam	M	48	4.5	46.9	40.9	43.2	41.7	37.6	36.4
8	Diazepam	M	32	19.5	< 10	< 10	< 10	ND	ND	ND
	Nordiazepam				220.2	177.5	176.9	160.9	165.5	90.7
9	Diazepam	M	48	13	42.8	40.4	39.7	43.7	44.6	37.3
10	Diazepam	F	45	17	49.4	35.6	44.2	47.4	44.1	44.3
11	Diazepam	F	68	25	110.2	97.5	87.7	86.9	87.4	85.8
12	Diazepam	M	40	25.5	28.7	25.1	24.1	26.5	27.4	26.4
13	Diazepam	F	31	24	158.0	141.9	139.5	133.0	140.1	131.7
14	Diazepam	M	45	17.5	70.3	61.0	55.6	56.6	55.1	49.1
15	Diazepam	M	46	17	281.6	280.2	260.2	254.7	237.8	216.8
16	Diazepam	M	28	26	24.8	23.4	25.2	24.7	24.5	23.5
17	Diazepam	M	67	23	48.6	39.8	41.7	42.6	43.5	36.1
18	Diazepam	M	32	22	51.7	46.0	42.9	41.7	43.7	34.7
19	Diazepam	M	32	19	68.0	65.4	59.9	57.4	51.9	50.3
20	Diazepam	M	32	21.5	26.7	25.6	22.9	25.8	24.0	23.3
21	Diazepam	M	37	15.5	119.9	110.7	115.8	101.7	97.2	96.8
22	Diazepam	M	42	8.5	62.9	61.4	51.0	43.7	42.2	42.6
23	Diazepam	F	54	6.5	90.3	80.8	72.3	67.5	63.4	60.9
24	Diazepam	F	67	18.5	83.7	75.7	74.3	63.1	58.6	58.45
	Nordiazepam				50.0	37.7	32.4	24.4	19.5	19.2

F = Female, M = Male, ND = not detected (< 5 ng/mL)

Table 2. Percent change from the initial concentration of diazepam from 30th day to 180th day.

Diazepam	Concentration D0 (ng/mL)	(D30-D0)/D0 (%)	(D60-D0)/D0 (%)	(D90-D0)/D0 (%)	(D120-D0)/D0 (%)	(D180-D0)/D0 (%)
Low_04	19.0	-4.4	-13.1	-13.1	-33.7	-32.0
Low_06	26.9	-12.5	12.7	4.0	-38.0	-25.4
Low_12	28.7	-12.6	-15.9	-7.5	-4.4	-7.9
Low_16	24.8	-5.9	1.5	-0.5	-1.3	-5.5
Low_20	26.7	-4.0	-14.3	-3.4	-10.3	-12.9
Mean/Median		-7.9 / -5.9	-5.8 / -13.1	-6.8 / -3.4	-17.5 / -10.3	-16.8 / -12.9
***P*-value**		0.03	0.4	0.2	0.1	0.02
Medium_02	48.5	-11.2	-18.8	-14.3	-16.8	-23.0
Medium_07	46.9	-12.7	-7.9	-11.0	-19.9	-22.3
Medium_09	42.8	-5.6	-7.2	2.2	4.3	-12.8
Medium_10	49.4	-27.9	-10.6	-4.1	-10.8	-10.5
Medium_14	70.3	-13.2	-20.9	-19.6	-21.6	-30.2
Medium_17	48.6	-18.2	-14.3	-12.4	-10.6	-25.9
Medium_18	51.7	-11.1	-17.1	-19.4	-15.5	-33.0
Medium_19	68.0	-3.9	-11.8	-15.6	-23.7	-26.1
Medium_22	63.0	-2.4	-19.0	-30.5	-33.0	-32.3
Mean / Median		-11.8 / -11.2	-14.2/ -14.3	-13.9 / -14.3	-16.4 / -16.8	-24.0 / -25.9
***P*-value**		0.02	0.04	0.04	0.003*	<0.001*
High_01	140.4	-14.6	-22.0	-23.5	-27.1	-58.2
High_05	120.0	-9.9	-10.8	-12.4	-7.1	-21.2
High_11	110.2	-11.5	-20.4	-21.1	-20.7	-22.1
High_13	158.0	-10.2	-11.7	-15.8	-11.4	-16.6
High_15	281.6	-0.5	-7.6	-9.6	-15.6	-23.0
High_21	119.9	-7.6	-3.4	-15.2	-18.9	-19.3
High_23	90.3	-10.5	-19.9	-25.3	-29.7	-32.9
High_24	83.7	-9.6	-11.2	-24.7	-30.1	-30.2
Mean/Median		-9.3 /- 10.0	-13.4/-11.5	-18.4/- 18.5	-20.1/- 19.8	-27.9 / -22.6
***P*-value**		0.048	0.01	<0.001*	<0.001*	0.002*

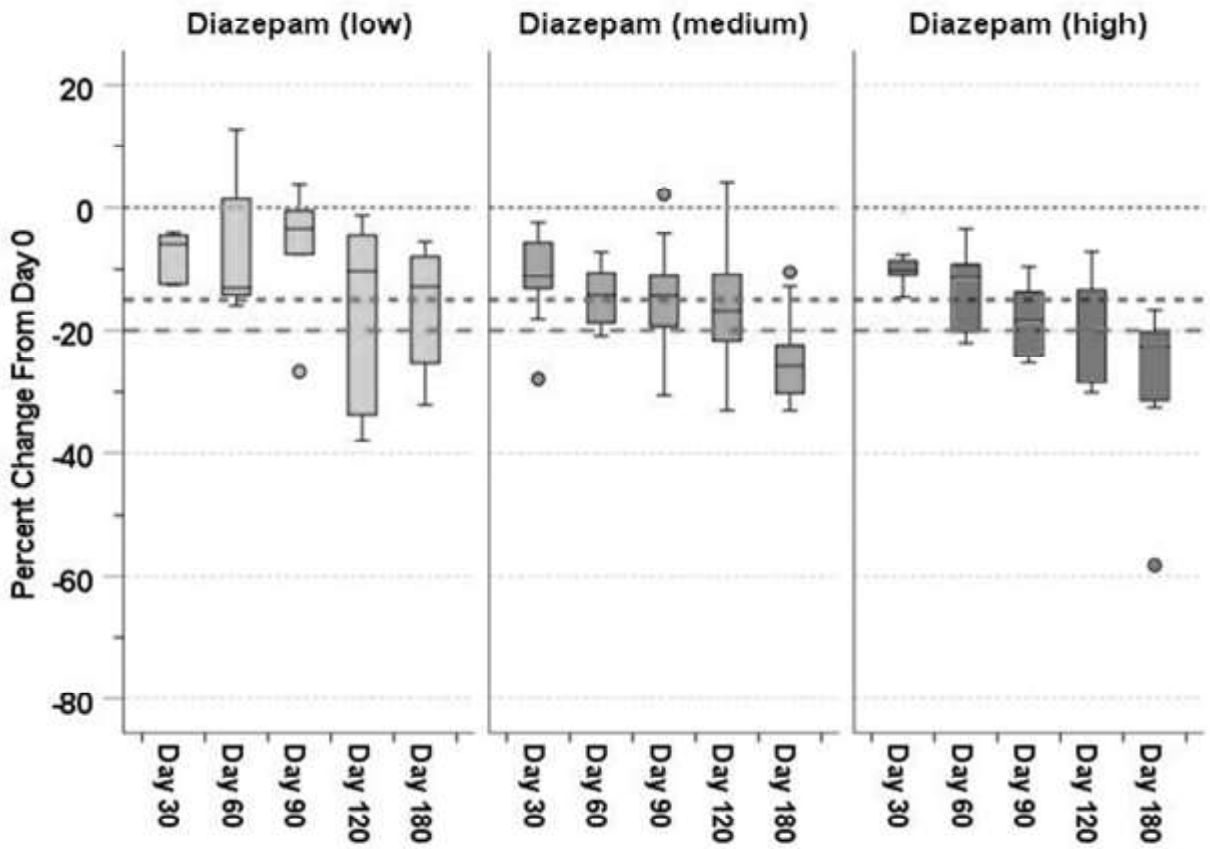

Figure 1. Percent change from initial concentration on day_0 of diazepam between 30th day and 180th day.

Table 3. Percent change from the initial concentration of nordiazepam on day_0 between 30th day and 180th day.

Nordiazepam	**Concentration D0 (ng/mL)**	**(D30 - D0) /D0 (%)**	**(D60 - D0) /D0 (%)**	**(D90 - D0) /D0 (%)**	**(D120 - D0) /D0 (%)**	**(D180 - D0) /D0 (%)**
< 10_03	121.3	- 17.9	- 28.8	- 28.0	- 43.8	- 57.8
Low_04	98.0	- 21.1	- 25.4	- 34.8	- 33.8	- 53.7
Low_06	24.6	- 42.5	- 20.3	- 1.6	- 56.1	- 100.0
<10_08	220.2	- 19.4	- 19.6	- 26.9	- 24.8	- 58.8
High_24	50.0	- 24.6	- 35.1	- 51.2	- 61.1	- 61.6
Mean/Median		**- 25.1/- 21.1**	**- 25.8/- 25.4**	**- 28.5/- 27.9**	**- 43.9/- 43.8**	**- 66.4/- 58.8**
P - value		0.02	0.02	0.03	0.027	< 0.001*

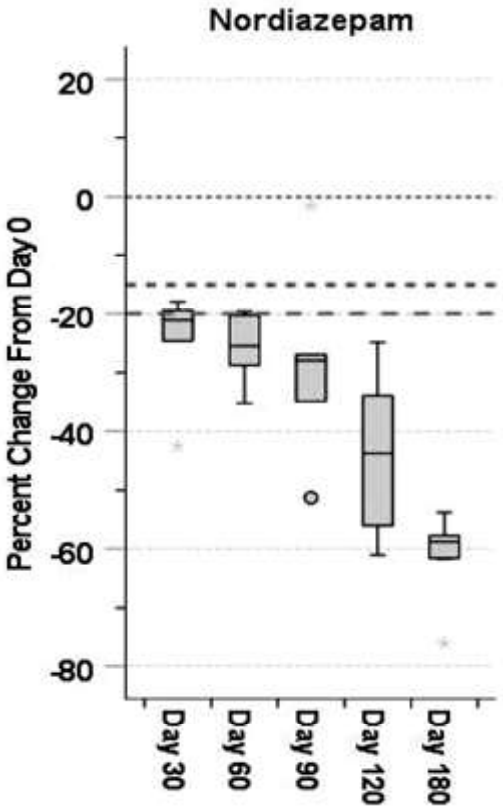

Figure 2. Percent change from initial concentration on day_0 of nordiazepam between 30th day and 180th day

Discussion

This study showed that diazepam concentrations in postmortem blood samples stored at 4°C had a tendency to decrease over the period of 180 days, particularly in medium and high concentrations, with mean percent changes for all three categories between - 16.8% and - 27.9% and a maximal percent decrease of - 58.2%. In detail, the mean percent changes of diazepam for all three categories on the 60th day ranged from - 5.8% to - 14.2% with a maximal percent change of - 22.0%. This result was consistent with a previous study that indicated a 20.0% reduction of diazepam concentration in spiked postmortem blood samples after 2 months.[4] However, the mean percent changes of diazepam of all three categories on the 90th day ranged from - 6.8% to - 18.4% with a maximal percent change of - 25.3%. This result contrasted with a previous study that produced an approximately 80.0% decrease of diazepam concentration in spiked blood samples obtained from living people in the 12th week.[7] This study provided comparable results to the previous study using spiked postmortem blood. This finding indicated that ante-mortem and postmortem blood may have an effect on the stability of diazepam. Further study should be conducted to demonstrate this impact. In addition, this study showed that the stability of diazepam in low concentration group was different from medium and high concentration groups. However, there were a small number of low diazepam concentrations compared with medium and high diazepam concentrations. Increased sample size in low diazepam concentrations should be considered in further research.

One hypothesis for the instability of diazepam in authentic postmortem blood samples could result from pH changes in postmortem blood. A previous study showed that postmortem human blood pH declined from 7.5 (a physiologic state) to acidic condition.[9] Diazepam is a 1, 4 - benzodiazepine drug susceptible to hydrolysis under both aqueous acidic and alkaline conditions.[10, 11] Under aqueous acidic condition which is closed to postmortem human blood condition, diazepam is subject to hydrolysis of azomethine bond followed by amide bond hydrolysis.[10, 11] Benzophenone degradation products were identified in the stability study of diazepam under aqueous acidic condition.[10] Further research should focus on degradation products from diazepam in authentic postmortem blood and the relationship with pH changes in postmortem blood.

This study showed that nordiazepam concentrations decreased beyond - 20.0% on the 30th day to the 180th day. This finding contrasted with a previous study that indicated a - 20.0% concentration reduction in spiked postmortem blood in the 3rd month.[4] However, there were only a small number of positive nordiazepam blood samples in this study. Thus, further study should be conducted on nordiazepam to ensure the instability of nordiazepam in postmortem blood. In addition, this study showed that nordiazepam in all postmortem blood samples did not increase while diazepam in blood samples decreased. This finding indicated that there was no conversion from diazepam to nordiazepam in any studied postmortem blood sample and this finding was consistent with the previous study.[4]

This study had some limitations. First, only a small number of postmortem blood samples with low diazepam concentrations and nordiazepam were recruited. This may have produced a wide range of standard deviation in percent change and affected the power of the statistical analysis. Second, this study did not measure some factors such as hospitalization days, the number of administered doses of diazepam, pH of blood samples and the amount of albumin that may be important factors for the stability of diazepam. Lastly, this study did not identify degradation products generated over the period of 180 days to prove the conversion of diazepam to other products.

Conclusion

The mean and median percent changes in diazepam in postmortem blood on the 180th day ranged from - 12.9% to - 27.9%. Diazepam concentrations in authentic postmortem blood samples decreased significantly on the 180th day (6 months), particularly for medium and high concentrations.

Acknowledgements

The authors gratefully acknowledge Asst. Prof. Dr. Chulaluk Komoltri for her invaluable assistance and advice with the statistical analysis.

Conflicts of interest statement

The author have each completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

The present review is based on the references cited. Further details, opinions, and interpretation are available from the corresponding authors on reasonable request.

References

1. Wang LL, Ren XX, He Y, Cui GF, Wei ZW, Jia J, et al. Study on the pharmacokinetics of diazepam and its metabolites in blood of Chinese people. Eur J Drug Metab Pharmacokinet 2020;45:477-85.
2. Skov L, Holm KM, Johansen SS, Linnet K. Postmortem brain and blood reference concentrations of alprazolam, bromazepam, chlordiazepoxide, diazepam, and their metabolites and a review of the literature. J Anal Toxicol 2016;40:529-36.
3. Dinis-Oliveira RJ, Vieira DN, Magalhães T. Guidelines for Collection of biological samples for clinical and forensic toxicological analysis. Forensic Sci Res 2017;1:42-51.
4. Mata DC. Stability of 26 sedative hypnotics in six toxicological matrices at different storage conditions. J Anal Toxicol 2016;40:663-8.
5. Moretti M, Freni F, Tomaciello I, Vignali C, Groppi A, Visonà SD, et al. Determination of benzodiazepines in blood and in dried blood spots collected from post-mortem samples and evaluation of the stability over a three-month period. Drug Test Anal 2019;11:1403-11.
6. Skopp G, Pötsch L, König I, Mattern R. A preliminary study on the stability of benzodia-zepines in blood and plasma stored at 4 degrees C. Int J Legal Med 1998;111:1-5.
7. Atanasov VN, Stoykova S, Runiov A, Dimitrova T, Aleksandrova D, Tsakovski S, et al. Stability of diazepam in blood samples at different storage conditions and in the presence of alcohol. Forensic Sci Int 2012;215:159-63.
8. Scientific Working Group for Forensic Toxicology. Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. J Anal Toxicol 2013;37:452-74.
9. Donaldson AE, Lamont IL. Biochemistry changes that occur after death: potential markers for determining post-mortem interval. PLoS One 2013;8:e82011.
10 Maślanka A, Krzek J, Stolarczyk M, Walczak M, Głogowska A. Stability studies of clonazepam, diazepam, haloperidol, and doxepin with diverse polarities in an acidic environment. J AOAC Int 2011;94:1791-9.
11. Loftsson T. 1,4-Benzodiazepines: Chemical stability and cyclodextrin solubilization. J Drug Deliv Sci Technol 2021;66:102936.