



Original Article

Effects of hemoglobin on post-mortem oxidation of bromazepam

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(Received May 4, 2021; Accepted May 6, 2021)

ABSTRACT — Benzodiazepines are widely used psychoactive drugs, and have been detected in several clinical cases of accidental exposure and suicide. It was reported that benzodiazepine concentration was changed in post-mortem blood. However, there is no concrete evidence to reasonably explain why benzodiazepine concentration in post-mortem blood cannot be accurately determined. In this study, we showed that the concentrations of almost all types of benzodiazepines examined were significantly decreased in the presence of hemoglobin (Hb) *in vitro*. In particular, bromazepam was hardly recovered in its intact form. We detected bromazepam metabolites with Hb by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS). The mass spectra showed that bromazepam was metabolized into 3-hydroxybromazepam. Our results suggest that 3-hydroxybromazepam was formed via the Fenton reaction with the divalent iron ion in Hb. Furthermore, 3-hydroxybromazepam was also detected in post-mortem blood of autopsied subjects who intentionally ingested bromazepam, and its concentration increased with time after death. These results suggest that 3-hydroxybromazepam is a potential biomarker of bromazepam poisoning to estimate the amount of bromazepam ingested.

Key words: Bromazepam, 3-hydroxybromazepam, Post-mortem change, Hemoglobin, Fenton reaction

INTRODUCTION

Benzodiazepines are widely used psychoactive drugs that act as minor tranquilizers. Benzodiazepines are structurally characterized by the fusion of a benzene ring and a diazepine ring, and are mechanistically divided into anxiolytic and hypnotic agents on the basis of their clinical effects. Benzodiazepines are relatively safe for 2-4 weeks of use, but their safety has not been characterized beyond the recommended period of clinical use. Addiction develops in approximately half of patients who use benzodiazepines for more than one month (Soyka, 2017). Benzodi-

azepines are frequently detected in fatal cases of suicide and accident (Ida *et al.*, 2019), and are categorized into the frequently detected drug group in dead bodies (Kudo *et al.*, 2010). The accurate determination of blood benzodiazepine concentration is mandatory to prove death by benzodiazepine poisoning in forensic toxicology. A previous report showed that benzodiazepine concentration was changed in post-mortem blood (Dylan *et al.*, 2020). However, there is no concrete evidence to reasonably explain why benzodiazepine concentration in post-mortem blood cannot be accurately determined.

Plasma esterase activity and bacterial contamina-

tion are postulated as factors that induce post-mortem changes of drugs (Peters and Steuer, 2019). A key structure of benzodiazepines does not seem to become a substrate of plasma esterase. Only two nitrobenzodiazepines, nitrazepam and flunitrazepam, were converted to their respective 7-amino metabolites by post-mortem bacterial metabolism (Robertson and Drummer, 1998). At present, changes in benzodiazepine concentration in post-mortem blood cannot be explained by plasma esterase activity and bacterial contamination. Recently, we found that hemoglobin (Hb) participated in the decomposition of drugs in blood after death (Yamagishi *et al.*, 2021). It was reported that Hb is released from blood cells upon hemolysis, and this phenomenon begins 6 hr after death (Hirabayashi, 1953). Hemolysis is one of the most typical post-mortem changes, and results in the efficient release of Hb into blood plasma. As far as we know, there are no reports on the interaction between benzodiazepines and Hb. Hence, we speculated that benzodiazepines interact with Hb to explain changes in benzodiazepine concentration in post-mortem blood.

In the present study, we measured eleven kinds of benzodiazepine *per se* and their metabolites formed in the presence of Hb, to evaluate the interactions between the benzodiazepines and Hb *in vitro*. We used liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS) to analyze benzodiazepines and their metabolites. LC-Q/TOF-MS is a reliable technique to widely screen untargeted small molecules, such as drugs and their metabolites, because it simultaneously gives the accurate molecular mass by TOF-MS and molecular information by tandem MS (Yamagishi *et al.*, 2021).

MATERIALS AND METHODS

Chemicals

Bromazepam, Hb, and Amicon ultra-0.5 centrifugal filter units with the molecular weight cut-off of 10 kDa were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brotizolam, clonazepam, diazepam, ethyl loflazepate, etizolam, flunitrazepam, midazolam, nitrazepam, oxazepam, triazolam, dimethyltrisulfide (DMTS), catalase from bovine (CAT), glutathione (GSH), and phosphate buffer (0.1 mol/L) were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan). Acetonitrile (ACN, LC/MS grade), distilled water (LC/MS grade), formic acid (FA, 98-100%), and 1 mol/L ammonium formate solution (HPLC grade) were purchased from Kanto Chemical (Tokyo, Japan). Diazepam-d5 was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka,

Japan).

Instrumentation

A liquid chromatograph hyphenated with quadrupole/time-of-flight mass spectrometers was used. 5600+QTOF (AB Sciex, Foster City, CA, USA) was equipped with Nexera X2 (Shimadzu, Kyoto, Japan) as the liquid chromatograph. The electrospray ionization (ESI) source parameters were as follows: ionspray voltage floating 5500 V, temperature 500°C, ion source gas 1 (GS1) 50 psi, ion source gas 2 (GS2) 50 psi, curtain gas (CUR) 25 psi, declustering potential (DP) 50 V, collision energy (CE) 10 V, and range m/z 100-1000. Chromatographic separation was accomplished with an Atlantis T3 C18 column (50 × 2.0 mm I.D., 3 μm) maintained at 40°C. The mobile phase was composed of 0.1% formic acid and 10 mM ammonium formate (A) and acetonitrile (B), and was eluted in the following gradient: 0 to 12 min: 10-60% B; 12 to 18 min: 60-100% B; 18 to 23 min: 100% B; and 23 to 30 min: 10% B. The flow rate was 0.3 mL/min, and the injection volume was 2 μL.

Recovery of benzodiazepines from reaction mixture containing Hb

A benzodiazepine mixture composed of bromazepam, Brotizolam, clonazepam, diazepam, ethyl loflazepate, etizolam, flunitrazepam, midazolam, nitrazepam, oxazepam, and triazolam was used. A 0.1 mL aliquot of a reaction mixture that contains Hb (final concentration, 100 mg/mL) and the benzodiazepine mixture (final concentration, 1 μg/mL) was incubated at 37°C for 0 and 24 hr. After the incubation, diazepam-d5 (50 ng/mL) as the internal standard in 1.9 mL of ACN was added to the reaction mixture. The reaction mixture was vortexed, sonicated, and centrifuged at 9,982 × g for 10 min, and the supernatant was subjected to LC-Q/TOF-MS to determine benzodiazepine concentrations. Extracted ions of bromazepam, Brotizolam, clonazepam, diazepam, ethyl loflazepate, etizolam, flunitrazepam, midazolam, nitrazepam, oxazepam, and triazolam were detected at m/z 316.0080, m/z 392.9570, m/z 316.0483, m/z 286.0789, m/z 361.0750, m/z 343.0779, m/z 314.0936, m/z 326.0855, m/z 282.0873, m/z 287.0582, and m/z 343.0512, respectively.

Detection and identification of bromazepam metabolite in the presence of Hb

A 0.1 mL aliquot of a reaction mixture containing 100 mg/mL Hb and 10 μg/mL bromazepam in 0.1 M phosphate buffer was incubated at 37°C for 24 hr. After the incubation, diazepam-d5 (50 ng/mL) as the inter-

nal standard in 1.9 mL of ACN was added to the reaction mixture. The reaction mixture was vortexed, sonicated, and centrifuged at $9,982 \times g$ for 10 min, and the supernatant was subjected to LC-Q/TOF-MS.

Determination of bromazepam and 3-hydroxybromazepam in reaction mixture treated with FeCl₂ or FeCl₃

Bromazepam (1 mg/mL) in 1 μ L of ACN was added to 99 μ L of FeCl₂ (40 mM) or FeCl₃ (40 mM) in 0.1 M phosphate buffer. The reaction mixture was incubated at 37°C for 24 hr. After the incubation, diazepam-d5 (50 ng/mL) as the internal standard in 1.9 mL of ACN was added to the reaction mixture. The reaction mixture was vortexed, sonicated, and centrifuged at $9,982 \times g$ for 10 min, and the supernatant was subjected to LC-Q/TOF-MS. Extracted ions of bromazepam and 3-hydroxybromazepam were detected at m/z 316.0080 and m/z 332.0029, respectively.

Determination of bromazepam and 3-hydroxybromazepam in reaction mixture treated with Hb or DMTS-induced methemoglobin

As mentioned below, we used the reported experimental conditions for DMTS-induced methemoglobin (metHb) with minor modifications (Dong *et al.*, 2017). A 5 mL aliquot of a reaction mixture consisting of 100 mg/mL Hb and 500 μ g/mL DMTS in 0.1 M phosphate buffer was incubated at 37°C for 24 hr. After the incubation, 0.5 mL of the reaction mixture was transferred into an ultrafiltration device (Amicon ultra-0.5 centrifugal filter unit) and centrifuged at $9,984 \times g$ for 10 min. The aliquot was washed three times with 0.3 mL of phosphate buffer and centrifuged again at $9,984 \times g$ for 10 min. After dilution to 0.5 mL with 0.1 M phosphate buffer, the mixture was centrifuged at $9,984 \times g$ for 10 min. The aliquot was DMTS-induced metHb.

Bromazepam (1 mg/mL) in 1 μ L of ACN was added to 99 μ L of Hb or DMTS-induced metHb in 0.1 M phosphate buffer. The reaction mixture was incubated at 37°C for 24 hr. After the incubation, diazepam-d5 (50 ng/mL) as the internal standard in 1.9 mL of ACN was added to the reaction mixture. The reaction mixture was vortexed, sonicated, and centrifuged at $9,982 \times g$ for 10 min, and the supernatant was subjected to LC-Q/TOF-MS. Extracted ions of bromazepam and 3-hydroxybromazepam were detected at m/z 316.0080 and m/z 332.0029, respectively.

Effect of CAT and GSH on conversion of bromazepam into 3-hydroxybromazepam by Hb

Bromazepam (1 mg/mL) in 1 μ L of ACN was added to 99 μ L of CAT (1 mg/mL), Hb (100 mg/mL), Hb/CAT (100 mg/mL, 1 mg/mL), GSH (10 mM) or Hb/GSH (100 mg/mL, 10 mM) in 0.1 M phosphate buffer. The reaction mixture was incubated at 37°C for 24 hr. After the incubation, diazepam-d5 (50 ng/mL) as the internal standard in 1.9 mL of ACN was added to the reaction mixture. The reaction mixture was vortexed, sonicated, and centrifuged at $9,982 \times g$ for 10 min, and the supernatant was subjected to LC-Q/TOF-MS. Extracted ions of bromazepam and 3-hydroxybromazepam were detected at m/z 316.0080 and m/z 332.0029, respectively.

Detection of bromazepam and 3-hydroxybromazepam in post-mortem blood from bromazepam poisoning subjects

This study was performed with approval from The Research Ethics Committees of the Graduate School of Pharmaceutical Sciences (Approval No. R001) and the School of Medicine (Approval No. 2819), Chiba University. Use of autopsy samples for analyses was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). We have included a disclaimer on our official website stating that we occasionally collect samples from autopsies for research use. Families who do not consent to it can freely opt out by informing us.

Blood samples obtained from seventeen cases were used, in which bromazepam was detected by drug screening performed in our laboratory.

Diazepam-d5 (50 ng/mL) as the internal standard in 0.4 mL of ACN was added to 0.1 mL of blood sample. The reaction mixture was vortexed, sonicated, and centrifuged at $9,982 \times g$ for 10 min, and the supernatant was subjected to LC-Q/TOF-MS. Extracted ions of bromazepam and 3-hydroxybromazepam were detected at m/z 316.0080 and m/z 332.0029, respectively.

Statistics

Data are expressed as means \pm standard deviation (SD). The Student's *t*-test and the Tukey test were performed for comparisons between two groups and among all groups, respectively. Asterisks (* and **) denote significance at $p < 0.05$ and $p < 0.01$, respectively.

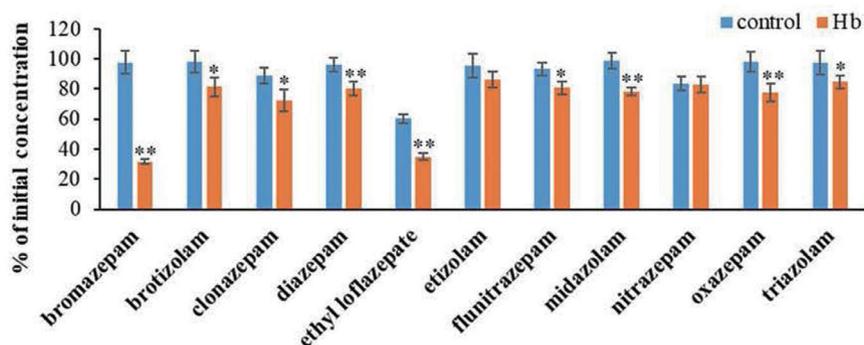


Fig. 1. Effect of Hb treatment on benzodiazepine concentrations. One $\mu\text{g/mL}$ of benzodiazepine was incubated without or with Hb for 24 hr. The Student's *t*-test was performed to compare control and Hb-treated benzodiazepine solutions. Significant difference from control is indicated by * and ** at $p < 0.05$ and $p < 0.01$, respectively.

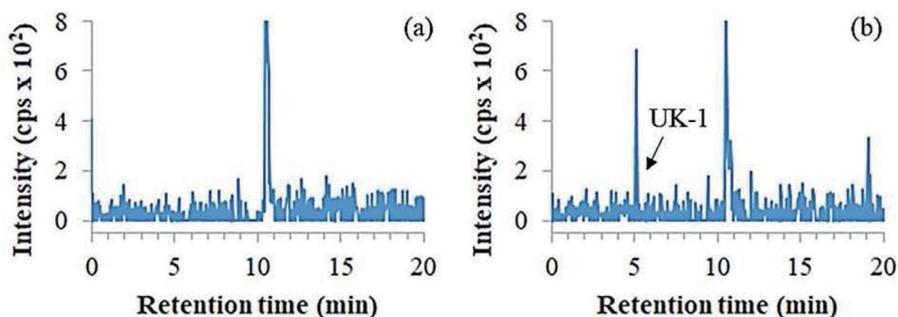


Fig. 2. Elution profiles at m/z 332.0033 (a and b) without (a) or with (b) Hb treatment. Retention time of UK-1 was 5.1 min.

RESULTS

Stability of benzodiazepines in Hb solution

The Hb treatment significantly reduced the concentrations of benzodiazepines except etizolam and nitrazepam at 24 hr after incubation (Fig. 1). The reduction rates of bromazepam, brotizolam, clonazepam, diazepam, ethyl loflazepate, flunitrazepam, midazolam, oxazepam, and triazolam were 68.4, 18.6, 27.7, 19.8, 65.1, 19.2, 21.6, 22.4, and 15.3%, respectively. The recovery of ethyl loflazepate in control was lower than that of the other benzodiazepines, although the mechanism underlying the low recovery was unclear.

Detection and identification of bromazepam metabolites produced by Hb treatment

Difference analysis in mass spectra was performed between Hb-treated and untreated bromazepam solution. Because the peak at the retention time of 10.6 min was

detected in both spectra, it was considered a non-specific peak (Fig. 2a). Specific peak UK-1 was detected at the retention time of 5.1 min, and was assigned to the precursor ion at m/z 332.0033 (Fig. 2b).

Figure 3 shows the results of MS/MS analyses of UK-1. One precursor (M1) and three fragment (M2-M4) ions were detected. The m/z values of M1 to M4 were 332.0023, 314.9758, 302.9996, and 286.9810, respectively. The assumed elemental compositions for the product ions of UK-1 are summarized in Table 1, and the product ions were assignable to the fragment ions depicted in Fig. 3. These results unambiguously indicate that UK-1 is 3-hydroxybromazepam.

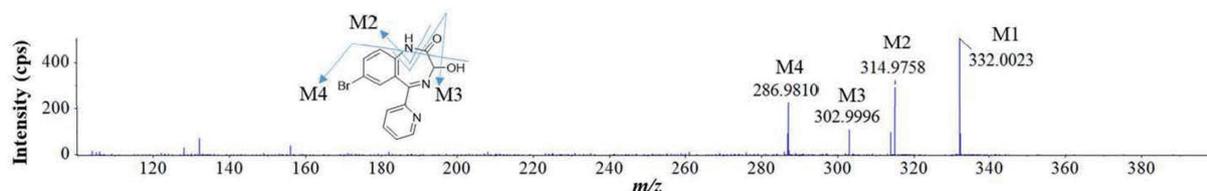
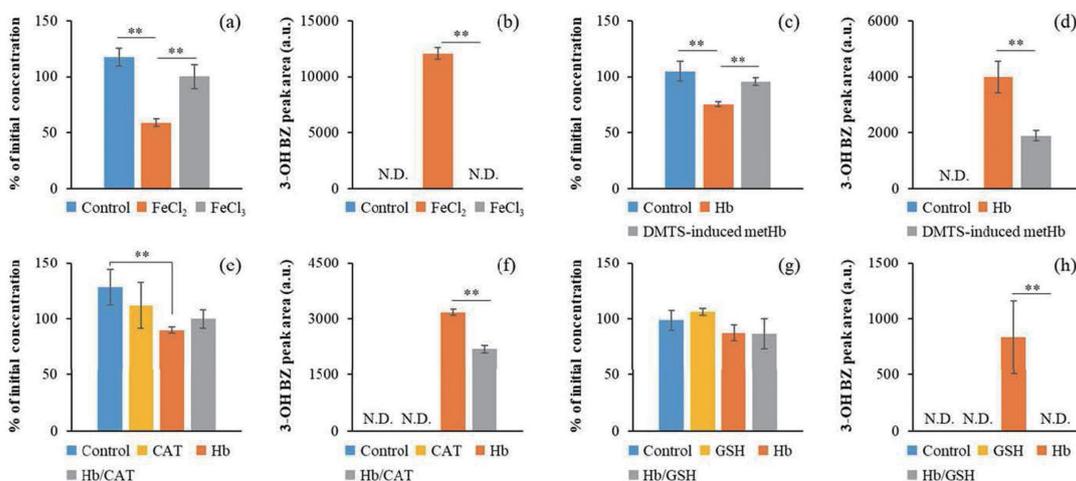
Effects of pro- and antioxidants on 3-hydroxybromazepam production

FeCl_2 treatment significantly reduced the concentration of bromazepam compared with control and FeCl_3 treatment at 24 hr (Fig. 4a), the reduction rate being 41.1%.

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Table 1. Assignment of precursor and product ions of UK-1.

| Peak label | Elemental composition | m/z theoretical | m/z measured | $\Delta m/z$ (ppm) |
|------------|-----------------------------------------------------------------|-------------------|----------------|--------------------|
| M1 | C ₁₄ H ₁₁ BrN ₃ O ₂ | 332.0029 | 332.0023 | -1.8 |
| M2 | C ₁₄ H ₈ BrN ₂ O ₂ | 314.9764 | 314.9758 | -1.9 |
| M3 | C ₁₄ H ₁₂ BrN ₂ O | 303.0128 | 302.9996 | -43.6 |
| M4 | C ₁₃ H ₈ BrN ₂ O | 286.9815 | 286.9810 | -1.7 |

**Fig. 3.** MS/MS spectrum of unknown compound detected in the positive ion mode. Unknown peak UK-1 detected in Fig. 2 was subjected to LC-Q/TOF-MS analysis. The assignment of precursor and product ions of UK-1 is summarized in Table 1.**Fig. 4.** Effects of FeCl₂, FeCl₃, Hb, DMTS-induced metHb, catalase (CAT), glutathione (GSH), Hb/CAT, and Hb/GSH on bromazepam concentration (a, c, e, and g) and 3-hydroxybromazepam (3-OH BZ) peak area (b, d, f, and h). Ten µg/mL of bromazepam was incubated with each of the above factors for 24 hr. Data are expressed as means and standard deviations (SD). Significant difference is indicated by ** at $p < 0.01$.

Unlike the FeCl₃ treatment, the FeCl₂ treatment produced 3-hydroxybromazepam (Fig. 4b).

Hb treatment significantly reduced the concentration of bromazepam compared with control and the DMTS-induced metHb treatment at 24 hr (Fig. 4c), the reduction rate being 24.6%. Although DMTS-induced metHb produced 3-hydroxybromazepam at 24 hr, the amount produced was significantly smaller than that produced by the Hb treatment (Fig. 4d).

The decrease of bromazepam concentration by the Hb

treatment was attenuated by CAT treatment, and the Hb plus CAT treatment significantly diminished the oxidation of bromazepam, i.e., the production of 3-hydroxybromazepam, at 24 hr (Figs. 4e and 4f).

Although the Hb and Hb plus GSH treatments did not result in significant changes in the concentration of bromazepam, the Hb plus GSH treatment significantly suppressed the oxidation of bromazepam at 24 hr (Figs. 4g and 4h).

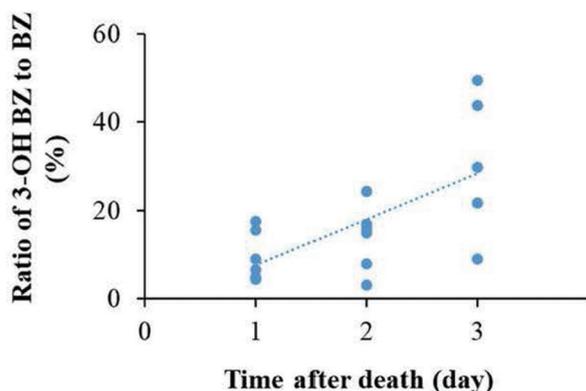


Fig. 5. Effect of time after death on ratio of 3-hydroxybromazepam (3-OH BZ) to bromazepam (BZ) peak area in blood. Blood samples obtained from seventeen cases were used, in which bromazepam was detected by drug screening performed in our laboratory.

Post-mortem changes in 3-hydroxybromazepam concentration in blood samples from bromazepam poisoning subjects

Bromazepam and 3-hydroxybromazepam were detected in blood collected from autopsied subjects who intentionally ingested bromazepam. The ratio of 3-hydroxybromazepam peak area to bromazepam peak area increased with time after death (Fig. 5). The ratio of 3-hydroxybromazepam to bromazepam was calculated on the basis of their peak areas in the chromatograms, and was plotted versus the estimated time after death. The average ratios on days 1, 2, and 3 after death were 9.6%, 13.8%, and 30.7%, respectively. A positive linear relationship was noted between the time after death and the ratio of 3-hydroxybromazepam to bromazepam (Fig. 5), and the correlation coefficient (r^2) was 0.413.

DISCUSSION

Hb is present in abundance in red blood cells in the human body, and is released from red blood cells via hemolysis after death (Hirabayashi, 1953). We recently reported that Hb plays a unique and specific role in the post-mortem decomposition of methomyl (Yamagishi *et al.*, 2021). Hence, we initially speculated that benzodiazepines are decomposed by Hb. Indeed, the concentrations of almost all types of benzodiazepines examined were decreased by the Hb treatment, with bromazepam concentration showing the greatest decrease. Therefore, we focused on bromazepam to uncover the mechanisms underlying the decrease in benzodiazepine

concentrations by the Hb treatment.

The results obtained by LC-Q/TOF-MS clearly indicated that bromazepam was transformed into 3-hydroxybromazepam by the Hb treatment, suggesting that bromazepam was oxidized by Hb. As Hb has four irons in its molecule, we speculated that these irons are involved in the oxidation. The most typical oxidation reaction involving iron is the Fenton reaction. Therefore, we evaluated the Fenton reaction caused by iron in Hb.

In the Fenton reaction, divalent iron catalyzes the dismutation of hydrogen peroxide, and hydroxyl radical and hydroxide ion are produced with trivalent iron (Barb *et al.*, 1949). It is known that hydroxyl radical reacts with drugs such as ibuprofen to form oxidized metabolites (Illés *et al.*, 2013). In this study, the oxidized form of bromazepam, 3-hydroxybromazepam, was produced by Hb treatment. DMTS induced the oxidation of divalent iron to trivalent iron in Hb, namely, Hb was converted into metHb. As expected, trivalent iron and DMTS-induced metHb had no or little effects on the oxidation of bromazepam. These results also support that the divalent iron in Hb plays a role in bromazepam oxidation. CAT is the most typical enzyme for hydrogen peroxide decomposition (Northrop, 1925). Hydroxyl radicals are scavenged by GSH (Fiser *et al.*, 2013). Antioxidants CAT and GSH inhibited the Fenton reaction, thereby suppressing bromazepam oxidation. The results also suggest that the Fenton reaction involving the divalent iron in Hb occurs in post-mortem blood. This is the first observation that bromazepam was oxidized by Hb via the Fenton reaction after death.

In forensic toxicology, accurate determination of blood bromazepam concentration is mandatory to prove death by bromazepam poisoning. However, it was reported that benzodiazepine concentration was changed in post-mortem blood (Dylan *et al.*, 2020). In addition to our *in vitro* study, we detected 3-hydroxybromazepam in blood sampled from autopsied subjects who intentionally ingested bromazepam, and 3-hydroxybromazepam concentration in post-mortem blood showed a time-dependent increase after death. This indicates that 3-hydroxybromazepam is useful as a biomarker of bromazepam poisoning in clinical practice. On the other hand, 3-hydroxybromazepam is the major metabolite of bromazepam in human, and is detected in blood, feces, and urine (Schwartz *et al.*, 1973; Allen *et al.*, 1984). Bromazepam is converted into 3-hydroxybromazepam by cytochrome P450 (CYP) in human (Oda *et al.*, 2003). However, the activities of CYP enzymes, such as CYP1A2 and CYP3A4, are lost or severely reduced after death (Hansen *et al.*, 2019). Because the amount of 3-hydroxybromazepam

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increased *in vivo* with time after death, we thought that the increase was driven by the Fenton reaction rather than CYP in post-mortem blood. The issue of how to distinguish 3-hydroxybromazepam produced while alive from that produced after death in post-mortem blood remains unresolved. We also need to evaluate the metabolic fate of 3-hydroxybromazepam, particularly its disappearance in post-mortem blood in future studies.

In conclusion, almost all types of benzodiazepines were decomposed by Hb, and bromazepam was the most markedly reduced. Bromazepam was oxidized by the Fenton reaction with Hb to form 3-hydroxybromazepam. The oxidized metabolite was also detected in blood sampled from an autopsied subject who intentionally ingested bromazepam, and thus could be used as a biomarker of bromazepam poisoning. Because the ratio of 3-hydroxybromazepam in blood increased with time after death, we expect that blood bromazepam concentration would be estimated on the basis of the amount of 3-hydroxybromazepam in post-mortem blood.

Conflict of interest---- The authors declare that there is no conflict of interest.

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