Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.10, No.8, 315-323, 2023

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Flunitrazepam alters the toxicokinetics of chlorpromazine enhancing its toxicity

Kota Nakajima, Asuka Kaizaki-Mitsumoto and Satoshi Numazawa

Department of ToxicologyShowa University Graduate School of Pharmacy,
1-5-8, Hatanodai, Shinagawa-ku Tokyo 142-8555, Japan

(Received November 29, 2023; Accepted December 15, 2023)

ABSTRACT — Flunitrazepam, a highly potent benzodiazepines, has a wide safety margin and widely used for insomnia treatment. However, a number of fatal poisoning cases involving a combination of flunitrazepam and other drugs have been reported. In instances of drug overdose deaths involving flunitrazepam in Japan, antipsychotic drugs like chlorpromazine are frequently used concomitantly. This study seeks to elucidate the pharmacokinetic interactions during the overdose of concurrent drugs, with a specific focus on the toxic effects of elevated doses of flunitrazepam and chlorpromazine in mice. Male ICR mice were intraperitoneally administered chlorpromazine (90 mg/kg) and flunitrazepam (200 mg/kg) either alone or concurrently. Body temperature was measured up to 24 hr after administration, and the number of deaths within 24 hr was quantified for each group. Additionally, flunitrazepam, chlorpromazine, and its active metabolite 7-hydroxychlorpromazine concentrations in serum and brain extracellular fluid were measured up to 24 hr after administration. Flunitrazepam enhanced the hypothermic effect of chlorpromazine, and acute intoxication deaths occurred only in the combination group. Cmax and $AUC_{0\text{-}24h}$ of flunitrazepam in serum and brain were not affected by concomitant administration with chlorpromazine. While flunitrazepam significantly increased the $AUC_{0\text{-}24h}$ of chlorpromazine, and the Cmax and $AUC_{0\text{-}24h}$ of 7- hydroxychlorpromazine in serum. Flunitrazepam was shown to alter the toxicokinetics of chlorpromazine when administered in combination, thereby augmenting the toxicity of chlorpromazine and leading to lethal drug intoxication. This study underscores the significance of comprehending toxicokinetics not only for individual agents but also when utilized in combination.

Key words: Flunitrazepam, Chlorpromazine, Toxicokinetics, Drug Intoxication

INTRODUCTION

Drug overdoses pose a significant and ongoing concern for global medical and public health. In the United States, the overdose-related mortality rate per 100,000 individuals surged by more than fivefold between 2000 and 2021 (Spencer *et al.*, 2022). Furthermore, drug overdoses surpassed traffic accidents in 2013, emerging as the primary cause of injury-related deaths (McCarthy, 2015). In Japan, drug abuse has witnessed a rise since 1996, with sleep medications and anxiolytics becoming the predominant contributors to drug-related psychiatric disorders (Matsumoto *et al.*, 2022). Some central nervous system (CNS)-acting drugs, characterized by narrow safety margins, pose a heightened risk of fatality due to overdose (Hikiji *et al.*, 2016). Benzodiazepines (BZDs), notably, have been frequently implicated in CNS-acting drug overdoses (Okumura *et al.*, 2017). They are generally perceived as safe in terms of lethal toxicity and side effects compared to other sleep medications like barbiturates and bromovalerylurea. Flunitrazepam (FNZ), a highly potent BZD widely used for insomnia treatment

Correspondence: Asuka Kaizaki-Mitsumoto (E-mail: asuka.0110@pharm.showa-u.ac.jp)

especially in Japan, remains subject to abuse. FNZ boasts a wide safety margin based on its LD50/ED50 ratio and, in fact, has low lethal toxicity in single-drug overdoses. Our previous mouse model study demonstrated relatively mild increases of FNZ in blood and brain concentrations with escalating doses and low central toxicity in overdose situations (Inoue *et al.*, 2022). However, FNZ abuse has prompted regulatory measures in many countries, including the United States and Europe (Saum and Inciardi, 1997; Victorri-Vigneau *et al.*, 2003). In Japan, fatal poisonings associated with FNZ have been reported, (Kinoshita *et al.*, 2008; Namera *et al.*, 2012), highlighting its potential for high-risk death due to pharmaceutical overdose (Hikiji *et al.*, 2016). However, given that many of these poisoning cases involve a combination of FNZ and other drugs, it is conceivable that an overdose of FNZ may potentiate the effects of the concurrent drug, leading to poisoning symptoms. Potential factors contributing to this phenomenon include pharmacodynamic interactions with multiple psychotropic drugs, as well as pharmacokinetic interactions such as competition at protein binding sites and saturation of metabolism and excretion. Indeed, the simultaneous use of FNZ and buprenorphine has been identified as predisposing patients to respiratory depression, with a suggested interaction in the metabolic process (Pirnay *et al.*, 2008).

In instances of drug overdose deaths involving FNZ in Japan, antipsychotic drugs like chlorpromazine (CPZ) are frequently used concomitantly (National Research Institute of Police Science, 2021; Kinoshita *et al.*, 2008). However, the interactions of these drugs in overdose situations remain unknown. CPZ, a classical antipsychotic, shares a wide safety margin with FNZ, and overdose deaths solely attributable to CPZ are rare. There have been isolated cases of fatal intoxication involving CPZ in combination with other drugs, particularly "Begetamine," a fixed-dose combination with phenobarbital and promethazine (Maebashi *et al.*, 2005). Begetamine, identified as a high-risk factor for overdose death (Hikiji *et al.*, 2016), was ultimately discontinued in 2016. Consequently, CPZ may exhibit increased toxicity through drug-drug interactions when overdosed in combination with other drugs.

Pharmacokinetic parameters, such as alterations in blood concentrations, play a crucial role in the management of acute drug intoxication. Two well-established methods for pharmacokinetic analysis are compartmental model analysis and physiologically based pharmacokinetic (PBPK) model analysis. In recent times, PBPK models have gained prominence for predicting the concentrations of drugs in both blood and tissues (Ministry of Health, Labour and Welfare, 2020). However, the application of PBPK models have primarily focused on pharmacokinetic modeling at therapeutic doses, neglecting toxicokinetics associated with intoxication. During drug development, toxicological studies, encompassing toxicokinetics, are imperative, and blood drug concentrations have been routinely measured in these studies (Ministry of Health, Labour and Welfare, 2014). However, the tissue distribution and concentration of the drugs have not been investigated.

Given the pivotal role of brain drug levels in the manifestation of acute intoxication symptoms induced by CNS-acting drugs, elucidating the relationship between brain and blood drug levels is imperative. Our prior research involved characterizing the pharmacokinetic parameters of CNS drugs in mice, focusing on changes in brain concentrations following an overdose of a single drug (Inoue *et al.*, 2022). It is crucial to recognize that even drugs that are safe when overdosed as single agents the toxicity of certain drugs may escalate when combined with others. Consequently, understanding pharmacokinetic alterations during drug combination overdoses is essential for optimizing the treatment of acute drug intoxication. This study seeks to elucidate the pharmacokinetic interactions during the overdose of concurrent drugs, with a specific focus on the toxic effects of elevated doses of FNZ and CPZ in mice. The objective is to establish a correlation between blood and brain concentrations and the resultant toxicity.

MATERIALS AND METHODS

Materials

Flunitrazepam and chlorpromazine hydrochloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 7-hydroxychlorpromazine (7-OH-CPZ) hydrochloride was purchased from Cayman Chemical Company (Michigan, USA). Diazepam-D_5 in methanol was purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other reagents used in this study were of the highest grade and commercially available.

Animals

ICR male mice (8 weeks old) were obtained from Sankyo Lab Service Corporation (Tokyo, Japan). Mice were housed in plastic cages in a temperature-controlled room (22 ± 1°C) and maintained on a 12 hr light-dark cycle with free access to food and water. All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the

Institutional Animal Care and Use Committee at Showa University. Every effort was made to minimize the number of animals used and their suffering.

Microdialysis surgery

The mice, anesthetized with a combination of medetomidine (0.75 mg/kg, i.p.), midazolam (4 mg/kg, i.p.), and butorphanol (5 mg/kg, i.p.), were placed in a stereotaxic apparatus. A microdialysis probe (D-I-6-02; 0.22 mm outer diameter, 2 mm membrane length; Eicom Co. Ltd., Kyoto, Japan) was implanted into the striatum at the following coordinates: AP: +0.5 mm, Ml: +1.7 mm relative to bregma and DV: −4.4 mm the skull (Kaizaki *et al.*, 2014). The probes were secured onto the skull using dental acrylic. The mice were allowed to recover for at least 24 hr before the experiment was begun. After the experiments, the mice were decapitated and brain tissue was removed to confirm that the probe had been embedded in the striatum.

Drug treatment

The drugs used in the present study are taken orally in humans; however, most of their toxicological information comes from intraperitoneal administration to animals. In addition, in order to avoid the factor of intestinal absorption of the drug, intraperitoneal administration to mice was employed for all drugs in this study. The toxic doses of each drug were determined as follows.

The LD50 of CPZ in Random-Swiss mice was reportedly 136 mg/kg (Fujimori and Cobb, 1965). In our preliminary study, acute death was observed at a dose of 100 mg/kg in ICR mice. Consequently, for the present study, a severe toxic dose of 90 mg/kg of CPZ was selected. The LD50 value of FNZ is reported to be 1050 mg/kg (Miyagawa *et al.*, 1985), but the sub-intoxication dose was set at 200 mg/kg because of its solubility (Inoue *et al.*, 2022).

In vivo microdialysis

The probes were perfused at 2 μL/min with artificial cerebrospinal fluid (Kaizaki *et al.*, 2014). One hour after the reflux, the dialysate sample was collected in 10-min fractions. Samples were collected up to 24 hr after drug administration. Samples at 0.5, 1, 2, 4, 8, and 24 hr after drug administration were subjected to liquid chromatography-mass spectrometry (LC-MS/MS) analysis directly.

Measurement of body temperature

The body temperature was employed to measure the grade of hypothermia. Rectal temperature as an index of total body heat was measured before and 0.5, 1, 2, 4, 8, and 24 hr after treatment using a thermometer BWT-100A (Bio Research Center Co., Nagoya, Japan).

Blood Sample collection and extraction

At 0.5, 1, 2, 4, 8, and 24 hr after drug administration, 30 μL of blood was collected from the tail vein of mice using microhematocrit tubes (Fisher Scientific, Tokyo, Japan). After standing for 30 min, the blood was centrifuged for 10 min at 1,500 × g at 20°C and the serum was collected. The drug in the serum was extracted according to the QuEChERS method (Mathiaux *et al.*, 2014). Ten microliters of serum were added to 290 μL of water, 100 mg of Agilent Bond Elut QuEChERS (Agilent, Tokyo, Japan), and 300 μL of acetonitrile containing 20 ng of diazepam-D_5 as internal standard (IS) and mixed well using a vortex mixer. The mixture was centrifuged for 10 min at 15,000 × g at room temperature. The supernatant was collected and subjected to LC-MS/MS analysis.

LC-MS/MS analysis

LC-MS/MS was performed using an LC-40ADXR and LCMS-8045 (Shimadzu, Tokyo, Japan). Chromatographic separation was achieved on a Phenomenex Kinetex XB-C18 column (2.1 mm I.D. × 100 mm., 1.6 μm; Shimadzu) with an equivalent Phenomenex Security Ultra C18 guard column (2.1 mm ID; Shimadzu). The column temperature was set at 40°C. The mobile phases were (A) 10 mmol/L ammonium formate and 0.1% formic acid, (B) methanol containing 10 mmol/L ammonium formate and 0.1% formic acid. The gradient started at 5% B, increased to 95% over 7.5 min, was held for 2.5 min, then immediately returned to 5%, and then held for 5 min. The flow rate was set at 0.3 mL/min and injection volume was 5 μL. After ionization with electrospray ionization (ESI) in positive mode, the samples were analyzed in multiple reaction monitoring mode (MRM). Flow rate of the nebulizer gas, the drying gas, and the heating gas were set at 3 L/min, 10 L/min, and 10 L/min, respectively. Temperatures of the interface, the desolvation line, and the heat block were set to 300°C, 250°C, and 400°C, respectively. The instrumental conditions for each drug are indicated in Table 1.

Pharmacokinetic parameters

Pharmacokinetic parameters such as Cmax, Tmax, T1/2, and area under the drug concentration-time curve (AUC) were calculated by non-compartmental analyses.

Statistical analysis

The disparities in body temperature data before and

Table 1. Liquid chromatography-mass spectrometry (LC-MS/MS) conditions.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Retention time (min)
CPZ	319.1	86.1	22	6.64
7-OH-CPZ	335.1	264.1	13	5.8
FNZ	314.0	268.0	26	5.98
Diazepam-D_5	290.2	198.2	34	6.89

after drug administration were assessed utilizing analysis of variance (ANOVA) followed by Dunnett's test. Additionally, a comparison between the CPZ single drug administration group and the FNZ combination group was conducted employing Student's t-tests. The number of deaths resulting from acute drug intoxication was subjected to analysis using the chi-square test. To discern variations in Cmax, Tmax, T1/2, and $AUC_{0\text{-}24}$ between the CPZ single drug administration groups (20 or 90 mg/kg) and the FNZ (200 mg/kg), the Mann–Whitney U test was employed. Moreover, distinctions in serum and brain drug concentration data between the CPZ single drug administration and the FNZ combination group were analyzed using Student's *t*-tests. All statistical analyses were performed on JMP Pro 16.0 (SAS, Cary, NC, USA).

RESULTS AND DISCUSSION

Changes in toxicity of CPZ when treated in combination with FNZ in mice

We investigated whether the hypothermic effects of CPZ and FNZ were potentiated by their combined administration. CPZ (90 mg/kg, a severely toxic dose) and FNZ (200 mg/kg, a mildly toxic dose) were intraperitoneally administered to mice either alone or concurrently. In the CPZ group, a significant reduction in body temperature occurred 30 min post-administration compared to pre-administration levels, reaching its nadir at -8.8 ± 0.2°C after 4 hr. Although a gradual recovery trend was observed thereafter, a significant decrease persisted 24 hr post-administration (Fig. 1). Conversely, the FNZ group exhibited a transient, yet significant, decrease in body temperature, with the maximum decline of -0.97 ± 0.3°C observed at 30 min post-administration. Notably, this temperature-lowering effect was markedly weaker than that observed in the CPZ group. In the CPZ and FNZ combination group, temperature changes up to 4 hr post-administration mirrored those in the CPZ group. However, a sustained temperature decrease manifested after 8 hr, with no recovery observed at 24 hr post-administration. The body temperature at 24 hr was -10.9 ± 1.2°C in the combination group, significantly lower than the -5.0 ± 0.6°C observed in the CPZ group. These results suggest that FNZ enhanced the temperature-lowering effect of CPZ.

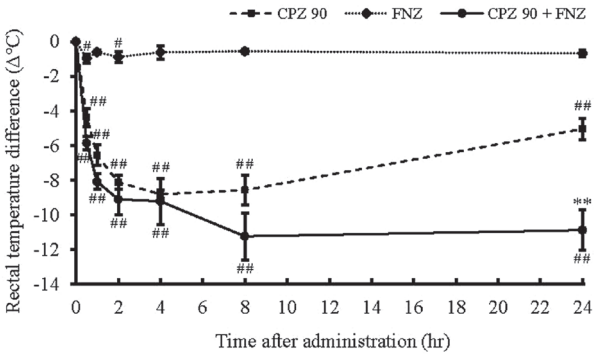

Fig. 1. Effects of FNZ and CPZ on body temperature in mice. Values represent the mean ± SE. Pre- and post-administration statistical analyses were performed using ANOVA followed by Dunnett's test. Student's *t*-test was used for analysis between the CPZ single-drug group and the FNZ combination group. CPZ (90 mg/kg), n=5; FNZ (200 mg/kg), n=6; CPZ + FNZ combination, n=6. ** $p<0.01$ vs the CPZ group, # $p<0.05$, ## $p<0.01$ vs pre-administration.

Notably, mortality was observed in the combination group 12–24 hr post-administration. Deaths within 24 hr were termed "acute drug intoxication deaths," and their incidence was quantified for each group. While all mice in the CPZ and FNZ single-drug groups survived until 24 hr post-administration, six mice in the combination group succumbed, a significantly higher rate compared to that in the CPZ and FNZ single-drug groups (Table 2). These findings imply that the acute drug intoxication-related deaths in the combination group resulted from increased CPZ toxicity facilitated by FNZ co-administration.

The hypothermic effect of CPZ has been reported to be due to 5-HT_2 receptor inhibition in the CNS and adrenergic α1 receptor inhibition in the peripheral nervous system (Boschi *et al.*, 1987; Yamada *et al.*, 1995). Since FNZ binds to GABA-A receptors and the hypo-

Table 2. Number of deaths in acute drug intoxication.

Treatment	Survival	Death
CPZ	11	0
FNZ	12	0
CPZ + FNZ	12	6*§

Deaths from acute drug intoxication in mice treated with CPZ (90 mg/kg) and FNZ (200 mg/kg) as single agents or in combination. Values were analyzed using the chi-square test. * $p<0.05$ vs the CPZ (90 mg/kg) group, § $p<0.05$ vs the FNZ (200 mg/kg) group.

thermic effect exhibited by GABA-A receptor agonists (Zaretsky *et al.*, 2003), FNZ-induced body temperature reduction is likely mediated by GABA-A receptors. While the distinct mechanisms of CPZ and FNZ-induced hypothermia suggest a potential for synergistic effects, the considerably weak hypothermic effect of FNZ in this study indicates that factors beyond pharmacodynamic interactions may contribute to the severe and prolonged hypothermia observed in the CPZ and FNZ combination group. The potentiation of FNZ on the lethal effects of CPZ overdose may be considered within the same context.

Toxicokinetics (TK) of CPZ and FNZ

To investigate the factors contributing to the heightened toxicity of CPZ in the presence of FNZ, we assessed the trends in blood and brain drug concentrations of CPZ when administered alone or in conjunction with FNZ. Additionally, we conducted a comparative analysis of the pharmacokinetic parameters of these drugs. For this study, two doses of CPZ were utilized: a mildly toxic dose of 20 mg/kg and a severely toxic dose of 90 mg/kg. These doses were administered both independently and in combination with FNZ at a fixed dosage of 200 mg/kg. Toxicokinetics during FNZ single-drug administration were previously investigated in a study conducted by Inoue *et al.*, 2022. In adherence to the 3R principle, FNZ single-drug administration was omitted in the present study.

TK parameters for FNZ

The Cmax and Tmax values of FNZ in both serum and brain did not exhibit significant differences between the 20 and 90 mg/kg CPZ combination groups (Table 3). While the serum T1/2 of FNZ was notably prolonged in the CPZ 90 mg/kg combination group in comparison to the CPZ 20 mg/kg combination group, there was no significant variance in serum $AUC_{0\text{-}24h}$ (Table 3). The equivalence of brain T1/2 and $AUC_{0\text{-}24h}$ between the CPZ 20 mg/kg and 90 mg/kg combination groups was suggested, despite the occurrence of fatalities in the latter group (Table 3, S1). Due to an insufficient number of mice at 24 hr post-administration, statistical analysis could not be conducted. Based on the above results and those of a previous study (Inoue *et al.*, 2022) on FNZ single-drug administration, it is postulated that concurrent CPZ administration or an escalation in CPZ dosage leads to a prolongation of FNZ serum T1/2. Nevertheless, the Cmax and $AUC_{0\text{-}24h}$ in both serum and brain remained unaffected, implying that the combination with CPZ is unlikely to enhance the effects of FNZ.

TK parameters for CPZ

The serum concentrations of CPZ in the CPZ (20 mg/kg) and FNZ combination group were significantly higher than those in the CPZ (20 mg/kg) group 4 and 8 hr after administration (Fig. 2 A). The serum $AUC_{0\text{-}24h}$ was also significantly higher in the FNZ combination group than in the CPZ (20 mg/kg) group (Table 3). However, no significant differences were observed in the serum Cmax, Tmax, and T1/2 (Table 3). There were also no significant differences in the CPZ concentration trends or pharmacokinetic parameters in the brain (Fig. 2 B, Table 3).

In the CPZ (90 mg/kg) and FNZ combination group, the serum CPZ concentration 24 hr after administration was significantly higher than that in the CPZ (90 mg/kg) group (Fig. 2C). Although there were no significant differences in the serum Cmax and Tmax, a significant prolongation of T1/2 and a significant increase in $AUC_{0\text{-}24h}$ were observed (Table 3). In contrast, in the brain, no significant differences were observed in CPZ concentration changes, Cmax, and Tmax (Fig. 2D, Table 3). Although statistical analysis could not be performed because of insufficient mouse numbers 24 hr after administration, brain T1/2 and $AUC_{0\text{-}24h}$ did not seem to differ between the two groups (Fig. 2D, Table 3). The CPZ (90 mg/kg) and FNZ combination group showed a decrease in brain CPZ concentration over time, whereas the blood CPZ concentration remained high 24 hr after administration. These results suggest that the prolonged hypothermia and acute drug intoxication-related deaths observed in the CPZ (90 mg/kg) and FNZ combination group may be related to higher serum CPZ concentrations. Serum CPZ concentration trends and T1/2 suggest that FNZ may have a significant effect on CPZ pharmacokinetics (Figs. 2A and C, Table 3).

In humans, CPZ is mainly metabolized by CYP2D6, but CYP1A2, CYP3A4, and CYP2C19 are also involved (Muralidharan *et al.*, 1996; Wójcikowski *et al.*, 2010),

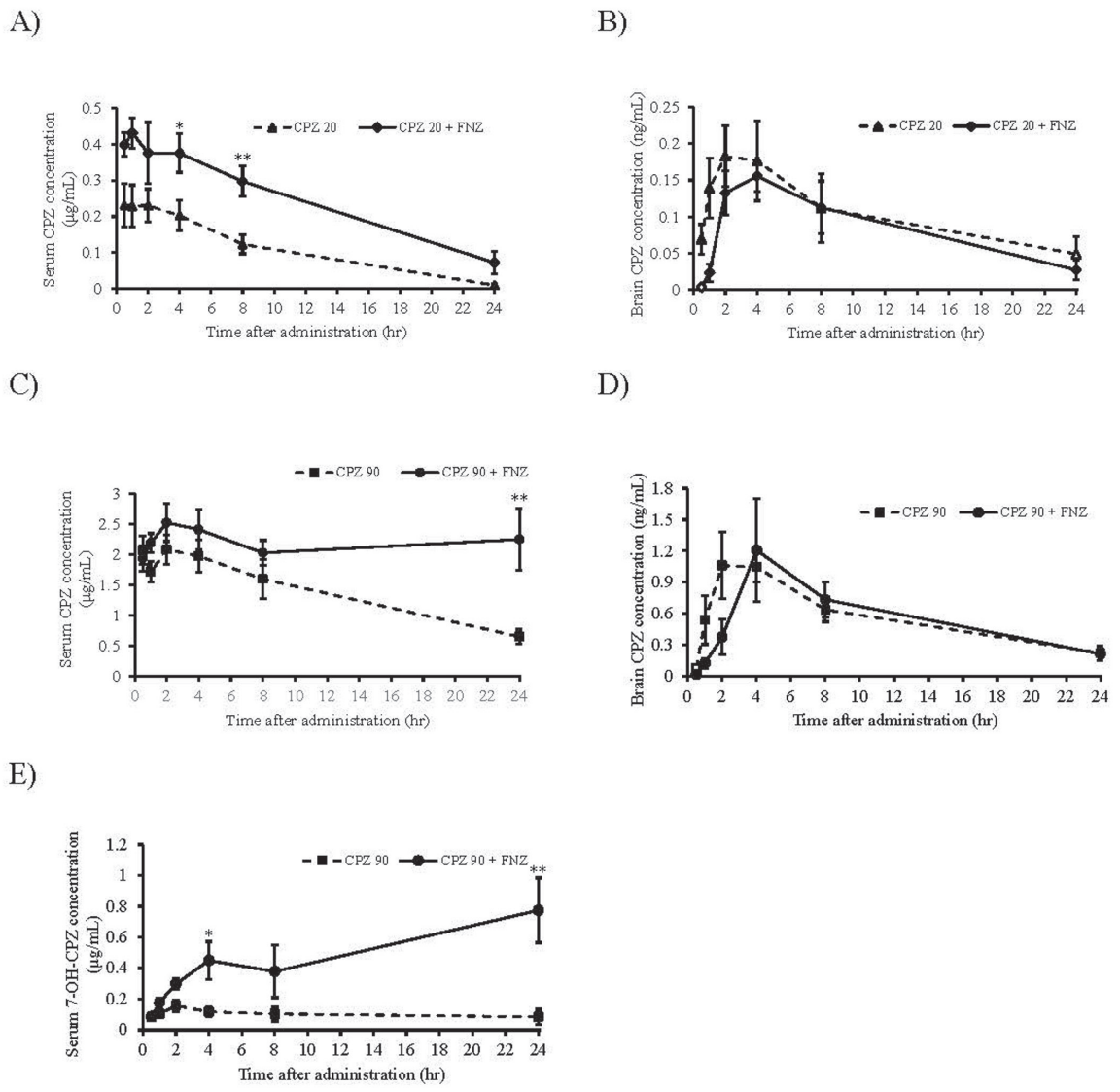

Fig. 2. Concentrations of CPZ and 7-OH-CPZ in serum and brain extracellular fluid. Changes in CPZ concentrations in serum (A, C) and brain (B, D) after administration of CPZ (20, 90 mg/kg) or CPZ (20, 90 mg/kg) and FNZ (200 mg/kg). Changes in 7-OH-CPZ concentrations in serum (E) after administration of CPZ (90 mg/kg) or CPZ (90 mg/kg) and FNZ (200 mg/kg). Values represent the mean ± SE and analyzed using Student's *t*-test. * $p<0.05$, ** $p<0.01$ vs the CPZ group. [A, B: CPZ (20 mg/kg), n=5; CPZ (20 mg/kg) and FNZ combination (n=4). C: CPZ (90 mg/kg), n=10; CPZ (90 mg/kg) and FNZ combination (n = 8), except at 24 hr (n=6). D, E: CPZ (90 mg/kg), n=5. D: CPZ (90 mg/kg) and FNZ combination (n = 3), except at 24 hr (n=2). E: CPZ (90 mg/kg) and FNZ combination, n=5, except at 24 hr (n=4)]. Intervals containing samples with brain CPZ concentrations below the limit of quantitation are indicated by white marks. When the brain concentration of CPZ of the sample was above the limit of detection and below the limit of quantitation, 0.001 ng/mL were applied, and when below the limit of detection, 0 ng/mL was applied.

and FNZ is metabolized by CYP3A4 and CYP2C19 (Kilicarslan *et al.*, 2001). Metabolism in mice is unknown; however, because the major metabolizing enzymes are different from those in humans, no significant interactions are expected to occur at therapeutic doses or in combination up to a certain dose. However, when CPZ and FNZ are combined at high doses, metabolic saturation of CPZ by CYP2D and competition with

Table 3. Pharmacokinetic parameters for mice treated with chlorpromazine, flunitrazepam, or those combined.

Compound	Dose		Cmax				Tmax				T1/2				$AUC_{0\text{-}24}$			
	CPZ	FNZ	Serum		Brain		Serum		Brain		Serum		Brain		Serum		Brain	
	(mg/kg)	(mg/kg)	(μg/mL)	(n)	(ng/mL)	(n)	(hr)	(n)	(hr)	(n)	(hr)	(n)	(hr)	(n)	(μg hr/mL)	(n)	(ng hr/mL)	(n)
FNZ	20	200	7.6 ± 0.6	(4)	97.7 ± 2.7	(4)	4.5 ± 1.3	(4)	4.5 ± 1.3	(4)	6.7 ± 1.5	(4)	7.1 ± 1.5	(4)	101.7 ± 17.5	(4)	1262.9 ± 222.6	(4)
	90	200	7.8 ± 2.5	(8)	78.5 ± 15.8	(3)	6.1 ± 1	(8)	5.3 ± 1.3	(3)	34.6 ± 16.1†	(4)	11.4	(2)	160.4 ± 54.3	(6)	1129	(2)
CPZ	20	-	0.23 ± 0.06	(5)	0.18 ± 0.04	(5)	1.8 ± 0.6	(5)	2.8 ± 0.5	(5)	10.7 ± 0.9	(5)	13.6 ± 5.4	(5)	2.6 ± 0.5	(5)	2.5 ± 0.9	(5)
	20	200	0.43 ± 0.04	(4)	0.16 ± 0.02	(4)	2 ± 0.7	(4)	4.5 ± 1.3	(4)	18 ± 3.8	(4)	15.5 ± 3.2	(4)	5.8 ± 0.7*	(4)	2 ± 0.4	(4)
	90	-	2.1 ± 0.2	(10)	1.1 ± 0.3	(5)	3.2 ± 1.1	(10)	3.6 ± 0.4	(5)	31.3 ± 5.5	(10)	20.7 ± 3.9	(5)	32.5 ± 4.5	(10)	13.4 ± 2.2	(5)
	90	200	2.5 ± 0.3	(8)	1.2 ± 0.5	(3)	6.1 ± 2.8	(8)	5.3 ± 1.3	(3)	184.1 ± 101.6*	(4)	17.5	(2)	52.5 ± 7.3*	(6)	13.5	(2)
7-OH-CPZ	90	-	0.16 ± 0.04	(5)	N.A.		7.4 ± 4.3	(5)	N.A.		24.3 ± 7.3	(5)	N.A.		2.4 ± 1	(5)	N.A.	
	90	200	0.77 ± 0.2*	(5)	N.A.		16.4 ± 4.7	(5)	N.A.		N.C.		N.A.		10 ± 1.7*	(5)	N.A.	

Values represent the mean ± SE and were analyzed using the Mann–Whitney U test. * $p<0.05$ vs the CPZ single dose group (20 or 90 mg/kg), † $p<0.05$ vs the CPZ (20 mg/kg) and FNZ combination group. N.A.; data not available because the brain concentration was below the detection limit. N.C.; data not calculated because the serum concentration was non-linear.

FNZ in the metabolic pathway by CYP3A and CYP2C may occur, resulting in a significant prolongation of T1/2 and an increase in the $AUC_{0\text{-}24h}$ for CPZ. Therefore, we measured the concentration of 7-OH-CPZ, an active CPZ metabolite.

TK parameters for 7-hydroxy chlorpromazine

Serum concentrations of 7-OH-CPZ at 4 and 24 hr post-administration in the combination group were significantly elevated compared to those in the CPZ group (Fig. 2 E). In the combination group, the serum concentration at 24 hr exceeded that at 8 hr post-administration. Consequently, the T1/2 could not be precisely calculated, but it was definitely longer than that in the CPZ group (Fig. 2 E). Furthermore, the serum Cmax and $AUC_{0\text{-}24h}$ in the combination group exhibited a significant increase compared to those in the CPZ group (Table 3). However, the determination of 7-OH-CPZ concentration in the brain was unattainable due to it being below the detection limit. Given that 7-OH-CPZ, produced through hydroxylation, is more polar than CPZ and exhibits reduced brain permeability, a substantial elevation in 7-OH-CPZ levels within the brain is deemed unlikely. These results suggest that FNZ may significantly affect the pharmacokinetics of both CPZ and 7-OH-CPZ. Although the metabolic pathway of 7-OH-CPZ remains unclear, existing literature suggests its excretion in feces as a glucuronide conjugate, in addition to urinary excretion (Muralidharan, 1996; Dingell and Sossi, 1977). Therefore, the observed high serum concentration of 7-OH-CPZ in the combination group could be attributed to urinary excretion and potential saturation during glucuronidation.

In conclusion, FNZ was shown to alter the toxicokinetics of CPZ when administered in combination, thereby augmenting the toxicity of CPZ and leading to acute drug toxicity. This study underscores the significance of comprehending toxicokinetics not only for individual agents but also when utilized in combination. We anticipate that the outcomes of this study will lay the groundwork for future toxicokinetic investigations and contribute to the advancement of treatments for drug poisoning.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant No. 23K08429.

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

REFERENCES

Boschi, G., Launay, N. and Rips, R. (1987): Neuroleptic-induced hypothermia in mice: lack of evidence for a central mechanism. Br. J. Pharmacol., **90**, 745-751.

Dingell, J.V. and Sossi, N. (1977): Studies on the glucuronidation of 7-hydroxychlorpromazine *in vitro*. Drug Metab. Dispos., **5**, 397-404.

Evaluation and licensing division, pharmaceutical and food safety bureau, ministry of health, labour and welfare (2014): Iyakuhinnkaihatsu ni okeru seitaisiryoutyuuyakubutunoudobunnsekihou (riganndoketugouhou) no baride-syonn ni kannsuru gaidorainn. (in Japanese), https://www.pmda.go.jp/files/000206206.pdf

Fujimori, H. and Cobb, D.P. (1965): Central nervous system depressant activity of MA1337, 3-(3-(4-m-Chlorophenyl-1-piperazyl) propyl)-2,4(1H,3H)quinazolinedione hydrochloride. J. Pharmacol. Exp. Ther., **148**, 151-157.

Guidelines of analysis reports for physiologically based pharmacokinetic models, ministry of health, labour and welfare (2020): Seirigakutekiyakubutsusokudoronn moderu no kaisekihoukokusho ni kannsuru gaidorainn. (in Japanese), https://www.pmda.go.jp/files/000238191.pdf

Hikiji, W., Okumura, Y., Matsumoto, T., Tanifuji, T., Suzuki, H., Takeshima, T. and Fukunaga, T. (2016): Identification of psychotropic drugs attributed to fatal overdose -a case-control study by data from the Tokyo medical examiner's office and prescriptions. Seishin Shinkeigaku Zasshi, **118**, 3-13.

Inoue, Y., Kaizaki-Mitsumoto, A. and Numazawa, S. (2022): Toxicokinetic evaluation during intoxication of psychotropic drugs using brain microdialysis in mice. J. Toxicol. Sci., **47**, 99-108.

Kaizaki, A., Tanaka, S. and Numazawa, S. (2014): New recreational drug 1-phenyl-2-(1-pyrrolidinyl)-1-pentanone (alpha-PVP) activates central nervous system via dopaminergic neuron. J. Toxicol. Sci., **39**, 1-6.

Kilicarslan, T., Haining, R.L., Rettie, A.E., Busto, U., Tyndale, R.F. and Sellers, E.M. (2001): Flunitrazepam metabolism by cytochrome P450S 2C19 and 3A4. Drug Metab. Dispos., **29**, 460-465.

Kinoshita, H., Nishiguchi, M., Kasuda, S., Takahashi, M., Ouchi, H., Minami, T., Matsui, K., Yamamura, T., Motomura, H., Ohtsu, N., Yoshida, S., Adachi, N., Ohta, T., Komeda, M., Ameno, K. and Hishida, S. (2008): Forensic toxicological implication of an autopsy case of mixed drug overdose involving clomipramine, chlorpromazine and flunitrazepam. Soud. Lek., **53**, 28-30.

Maebashi, K., Shigeta, A., Fukui, K., Abe, S., Murata, S. and Takatsu, A. (2005): Iyakuhin ni yoru chuudoku: begetaminn hukuyou ga kannyo sita boukennrei. Leg. Med. (Tokyo), **59**, 83. (in Japanese)

Mathiaux, F., Dulaurent, S., Julia, F. and Gaulier, J.M. (2014): Case report of ivabradine intoxication. J. Anal. Toxicol., **38**, 231-232.

Matsumoto, T., Usami, T., Funada, D., Murakami, M., Okita, K., Tanibuchi, Y., Yamamoto, T. and Yamaguchi, S. (2022): Zennkoku no seisinnkairyousisetu ni okeru yakubutukannrennseisinnsikkann no jittaityousa. (in Japanese.), National Institute of Mental Health, National Center of Neurology and Psychiatry (Matsumoto, T. ed.): https://www.ncnp.go.jp/nimh/yakubutsu/report/pdf/J_NMHS_2022.pdf

McCarthy, M. (2015): Drug overdose has become leading cause of

death from injury in US. BMJ, **350**, h3328.

Miyagawa, H., Watanabe, T., Ogura, K., Sumigama, S., Nakanowatari, J. and Ohgou, T. (1985): Flunitrazepam no kyuusei oyobi akyuusei dokuseisikenn. Clin. Rep., **19**, 1277-1295. (in Japanese.)

Muralidharan, G., Cooper, J.K., Hawes, E.M., Korchinski, E.D. and Midha, K.K. (1996): Quinidine inhibits the 7-hydroxylation of chlorpromazine in extensive metabolisers of debrisoquine. Eur. J. Clin. Pharmacol., **50**, 121-128.

Namera, A., Makita, R., Saruwatari, T., Hatano, A., Shiraishi, H. and Nagao, M. (2012): Acute intoxication caused by overdose of flunitrazepam and triazolam: high concentration of metabolites detected at autopsy examination. Am. J. Forensic Med. Pathol., **33**, 293-296.

National Research Institute of Police Science. (2021): Annual case reports of drugs and toxic poisoning in Japan, nos. 63 (in Japanese). National Police Agency, Kashiwa

Okumura, Y., Sakata, N., Takahashi, K., Nishi, D. and Tachimori, H. (2017): Epidemiology of overdose episodes from the period prior to hospitalization for drug poisoning until discharge in Japan: an exploratory descriptive study using a nationwide claims database. J. Epidemiol., **27**, 373-380.

Pirnay, S., Megarbane, B., Declèves, X., Risède, P., Borron, S.W., Bouchonnet, S., Perrin, B., Debray, M., Milan, N., Duarte, T., Ricordel, I. and Baud, F.J. (2008): Buprenorphine alters desmethylflunitrazepam disposition and flunitrazepam toxicity in rats. Toxicol. Sci., **106**, 64-73.

Saum, C.A. and Inciardi, J.A. (1997): Rohypnol misuse in the United States. Subst. Use Misuse, **32**, 723-731.

Spencer, M.R., Miniño, A.M. and Warner, M. (2022): Drug Overdose Deaths in the United States, 2001-2021. NCHS Data Brief, **457**, 1-8.

Victorri-Vigneau, C., Basset, G., Bourin, M. and Jolliet, P. (2003): Impacts de la nouvelle réglementation du flunitrazépam sur la consommation d'hypnotiques (in French). Therapie, **58**, 425-430.

Wójcikowski, J., Boksa, J. and Daniel, W.A. (2010): Main contribution of the cytochrome P450 isoenzyme 1A2 (CYP1A2) to N-demethylation and 5-sulfoxidation of the phenothiazine neuroleptic chlorpromazine in human liver--A comparison with other phenothiazines. Biochem. Pharmacol., **80**, 1252-1259.

Yamada, J., Sugimoto, Y. and Horisaka, K. (1995): Serotonin2 (5-HT2) receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) inhibits chlorpromazine- and haloperidol-induced hypothermia in mice. Biol. Pharm. Bull., **18**, 1580-1583.

Zaretsky, D.V., Zaretskaia, M.V. and DiMicco, J.A. (2003): Stimulation and blockade of GABA(A) receptors in the raphe pallidus: effects on body temperature, heart rate, and blood pressure in conscious rats. Am. J. Physiol. Regul. Integr. Comp. Physiol., **285**, R110-R116.