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Semi-micro column HPLC of triazolam in rat plasma and brain microdialysate and its application to drug interaction study with itraconazole

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

Semi-micro column high-performance liquid chromatographic method with ultraviolet detection for the determination of triazolam (TZ) in rat plasma and brain microdialysate is described. The separation was achieved on a 250 × 1.5 mm, i.d. C_{18} column and the column effluent was monitored at 222 nm. The detection limits at a signal-to-noise ratio of 3 obtained using spiked plasma and artificial cerebrospinal fluid were 2.1 and 0.7 ng/ml, respectively. The method was applied to drug–drug interaction study of TZ with itraconazole (ITZ). The peak concentration (C_{max}) and the area under the curve (AUC) of TZ in brain microdialysate after simultaneous administration of TZ (2.5 mg/kg, intravenously (i.v.)) and ITZ (25 mg/kg, p.o.) to rats increased 3.4-folds ($P < 0.001$) and 2.9-folds ($P < 0.001$), respectively, compared to those of TZ alone. Also, the AUC of TZ in plasma increased 2.6-folds and remarkable delay in its elimination half-life ($t_{1/2}$) was observed. The concentrations of TZ in brain microdialysate and plasma were also measured after single administration of TZ (2.5 mg/kg, i.v.) to rats pretreated with daily administration of ITZ (25 mg/kg, p.o.) once a day for a week. There was no significant difference in TZ's C_{max} in both ITZ treatments ($P > 0.2$) however its $t_{1/2}$ after the daily pretreatment with ITZ was significantly increased ($P < 0.05$). In plasma, the AUC of TZ after daily pretreatment of ITZ was lower than the single combined treatment, but significantly different from TZ's AUC in the absence of ITZ ($P < 0.05$). As a result, single simultaneous administration of TZ with ITZ and single administration of TZ after daily pretreatment with ITZ to rats, ITZ seriously interfered with the pharmacokinetic parameters of TZ in plasma and brain micodialysate.

Keywords: Triazolam; Semi-micro column HPLC; Rat plasma; Rat brain microdialysis; Itraconazole; Drug–drug interaction

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1. Introduction

Triazolam (TZ), 8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-*s*-triazolo-(4,3-*a*) (1,4)-benzodiazepine, is a well known short-acting hypnotic with sedative, anxiolytic and anticonvulsant properties being prescribed for short-term treatment of insomnia [1–3]. The clearance of TZ is dependent on hepatic oxidative metabolism by CYP3A isoforms to α-hydroxytriazolam and 4-hydroxytriazolam [4]. Therefore, the inhibition of CYP3A isoforms by other co-administered drugs can result in clinically significant interactions. One of the well-studied interactions of benzodazepines including TZ is their combination with the azole antifungal agents, which are potent inhibitors of CYP3A isoforms [5–7]. Itraconazole (ITZ), (±)-1-*sec*-butyl-4-[*p*-[4-[*p*[[(2*R*,4*S*)-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl-Δ^2-1,2,4-triazolin-5-one, an azole antifungal, inhibits CYP3A4 in human and other isoforms involved in xenobiotic metabolism in the liver [8]. Recently, it has been reported that ITZ cause serious side effects due to drug–drug interactions [9–11]. In addition, the effect of ITZ on the pharmacokinetic parameters of TZ is well established in plasma [5,6]. However, there are no studies reported the interference of ITZ with the pharmacokinetic parameters of TZ in brain.

Many methods have been developed for the determination of TZ in biological fluids including plasma and brain tissue. Most of these methods used HPLC accompanied with UV [2,12,13], photodiode array [14,15] and mass spectrometric [16–18] detections. Previously, we reported a simple and highly sensitive semi-micro column HPLC method for the determination of TZ in rat plasma and brain microdialysate [19]. The method was applied for the monitoring of TZ levels in plasma and brain microdialysates obtained from rats administered with a single dose of 2.5 mg/kg of TZ, intravenously (i.v.).

Recently, microdialysis technique is playing an important role in the field of pharmacokinetic studies of drugs [20,21]. Thus the aim of this work was to apply our previously developed HPLC method to elucidate the interaction of ITZ with the pharmacokinetic parameters of TZ simultaneously in plasma and brain microdialysate samples of rats administered with TZ and ITZ. By the resulted data, the interaction of ITZ with TZ pharmacokinetic parameters in plasma and brain microdialysate was discussed.

2. Experimental

2.1. Materials

TZ was obtained from Yoshitomi Pharmaceuticals (Osaka, Japan). ITZ (Itorizole capsule 50) was purchased from Kyowa Hakkou (Tokyo, Japan). Nitrazepam (NZ) as an internal standard (I.S.), HPLC grade acetonitrile and methanol were purchased from Wako (Osaka). Water was deionized and passed through a pure line WL21P (Yamato Kagaku, Tokyo, Japan). All other chemicals were of analytical reagent grade and used as received.

Standard stock solutions of TZ and NZ of 1.0 mM were prepared in methanol. Working solutions were prepared by dilution with the mobile phase or artificial cerebrospinal fluid (CSF).

The composition of artificial CSF used was: 7.3 g NaCl, 78 mg Na_2PO_4, 895 mg Na_2HPO_4, 186 mg KCl, 203 mg $MgCl_2$ and 133 mg $CaCl_2$ in 1 l water, pH 7.4.

Artificial CSF and rat plasma were spiked with TZ to give concentrations in the range of 3.4–1716.1 and 8.6–3432.1 ng/ml, respectively. Calibration curves were constructed by plotting the ratio of UV absorbance of TZ as peak height to that of I.S. against the concentration in ng/ml.

For administration to rats, TZ was dissolved in 35% polyethylene glycol 300. ITZ capsule was dissolved in 1.0 M HCl and neutralized with 1.0 M NaOH.

2.2. Microdialysis system and animal experiments

The microdialysis system and operation conditions used were the same as those in the previous work [19]. Male Wistar rats (260–370 g) were used. After animals were anesthetized with urethane (1.5 g/kg, 0.5 ml/100 g, i.p.), a heparinized

polyethylene tube was inserted into the left femoral vein for TZ administration and right femoral vein for blood sampling. Rats were then mounted on a stereotaxic instrument (Narishige Scientific Instruments, Tokyo) and a small hole was drilled into the skull to place the microdialysis probe (CMA/PC10, membrane length of 3.0 mm and outer diameter of 0.5 mm, molecular cut-off 20 000) into the medial prefrontal cortex (coordinates: rostral +2.7 mm, lateral −0.8 mm, ventral −5.5 mm, relative to bregma) according to Paxinos and Watson Atlas [22]. Artificial CSF was perfused at flow rate of 1 μl/min.

Rats were divided into three groups. The first group was administered with TZ (2.5 mg/kg, i.v., $n = 5$), the second group was simultaneously administered with ITZ aqueous solution (25 mg/kg) orally followed by TZ (2.5 mg/kg, i.v.) ($n = 3$). The third group was daily pretreated with ITZ (25 mg/kg, p.o.) once a day for 7 days, after 24 h of the last dose of ITZ a single dose of TZ (2.5 mg/kg, i.v.) was administered ($n = 4$). For blood sampling, blood (200 μl) was collected after 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 300 min of drugs administration in tubes containing EDTA, centrifuged and then plasma was separated and stored at −20 °C until analysis. Brain microdialyates were collected at the same intervals as blood samples.

2.3. Sample pretreatment

TZ was extracted from plasma by liquid–liquid extraction method as previously described [19]. In brief, after the addition of the I.S. (10 μl, 7.5 μM) to 90 μl of plasma, isopropanol (100 μl) was added and the resultant was centrifuged. To the obtained supernatant, borate buffer and chloroform were added. Sample was centrifuged and the organic layer was pipetted into a vial and evaporated. The residue was reconstituted with 40 μl of a 1:1 mixture of acetonitrile in water and 10 μl of the resultant were injected onto the column. For microdialysate samples, the I.S. (20 μl, 1.0 μM) was added and the volume was adjusted to 200 μl with artificial CSF. Ten microliters were injected onto the column.

2.4. HPLC system and operating conditions

The HPLC system and operation conditions used were the same as those in our previous report [19]. The system was consisted of an LC-10AS pump (Shimadzu, Kyoto, Japan), a 7125 Rheodyne injector with a 10 μl loop (Cotati, CA), a Develosil ODS-5 column (250 × 1.5 mm i.d., 5 μm, Nomura Chemicals, Seto, Japan), an SPD-10AV UV-Vis spectrophotometer equipped with a 5 μl flow cell (Shimadzu) and set at 222 nm. Separations were carried out with water–acetonitrile (62:38 v/v) at a flow rate of 100 μl/min for microdialysate samples and water–acetonitrile–methanol (60:38:2 v/v/v) at a flow rate of 80 μl/min for plasma samples.

2.5. Pharmacokinetics and statistical analysis

The concentrations of TZ in rat plasma and brain microdialysate were calculated from the corresponding calibration curves. The brain microdialysate concentrations were corrected to the in vitro recovery (14.1%), and the pharmacokinetic parameters were performed using the corrected data.

Pharmacokinetic calculations were processed by the nonlinear least-squares method [23] for plasma levels of TZ and analyzed by the program MULTI, which is based on the moment theory [23] for brain microdialysate samples. All data are presented as means ± standard error of mean (SEM). Statistical analysis was assessed by student t-test with $P < 0.05$ being considered significant.

3. Results and discussion

3.1. Chromatographic separation

The chromatographic conditions used in our previous work [19] were applied here. For microdialysates, water–acetonitrile (62:38 v/v) at a flow rate of 100 μl/min was used as the mobile phase. The retention times of TZ and I.S. were 17 and 14 min, respectively. For plasma samples, the mobile phase was water–acetonitrile–methanol (60:38:2 v/v/v) at a flow rate of 80 μl/min and the retention

times were 19 and 17 min for TZ and I.S., respectively. The obtained detection limit of TZ in brain microdialysate was 0.7 ng/ml and in plasma was 2.1 ng/ml.

In this study, only TZ was monitored in rat brain microdialysate and plasma. ITZ levels in samples obtained from rats co-treated with TZ did not interfere with the determination of TZ. Fig. 1 illustrates chromatograms obtained from rat plasma before (A) and after administration with a single i.v. dose of 2.5 mg/kg of TZ (B) and after 24 h of daily pretreatment with ITZ (25 mg/kg, p.o.) (C). B and C chromatograms were obtained after 90 min of the administration and TZ peaks represent 29.8 and 102.4 ng/ml, respectively.

3.2. Pharmacokinetics studies

3.2.1. Pharmacokinetic parameters of TZ in rat plasma

Fig. 2 shows the plasma concentration-time profiles of TZ in the presence or absence of ITZ. The effect of the co-administration of ITZ with TZ was studied with single oral dose of 25 mg/kg ITZ and after 24 h of daily pretreatment of 25 mg/kg ITZ, once a day for a week. The pharmacokinetic parameters of TZ in plasma are shown in Table 1.

In human, ITZ impairs the clearance of TZ, which is consistent with the inhibition of CYP3A4, the enzyme that is responsible for the metabolism

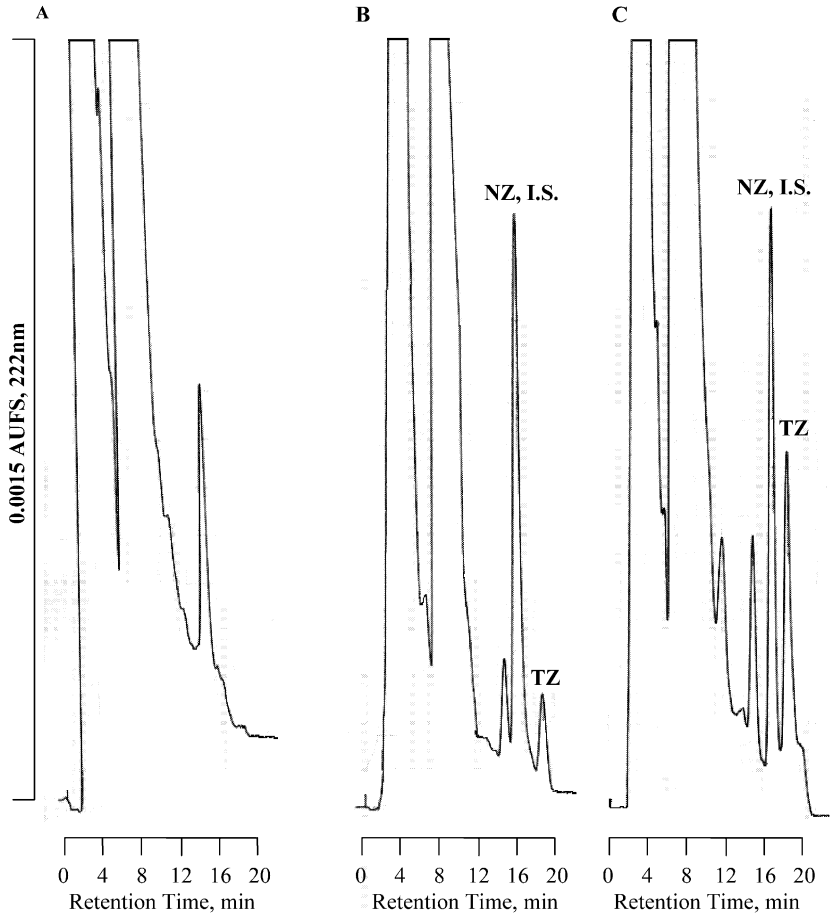

Fig. 1. Chromatograms obtained from rat plasma (A) before drugs administration, (B) administered with TZ (2.5 mg/kg, i.v.) and (C) administered with TZ after 24 h of daily pretreatment with ITZ (25 mg/kg, p.o.). TZ peaks represent 29.8 ng/ml and 102.4 ng/ml in B and C, respectively. TZ, triazolam; NZ, nitrazepam, I.S., internal standard.

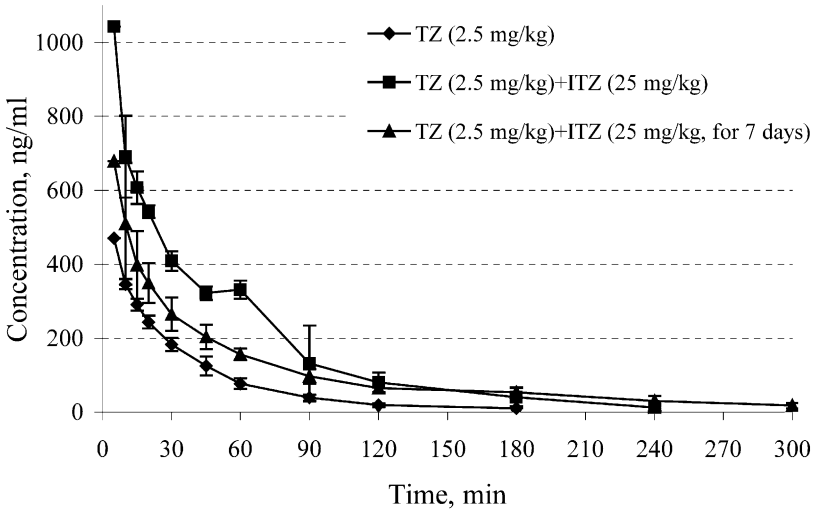

Fig. 2. Mean concentrations of TZ in rat plasma after administration of TZ (25 mg/kg, i.v.) without and with ITZ (25 mg/kg, p.o.). TZ, triazolam; ITZ, itraconazole.

of TZ [5–7]. The results obtained in this study are in agreement with these findings.

As listed in Table 1, after single administration of TZ to rats, TZ clearance significantly decreased from 151.4±13.8 ml/min/kg for rats administered with TZ alone to 56.8±3.5 for the single dose of ITZ with TZ and 83.7±12.9 ml/min/kg for the daily administration of ITZ with TZ ($P < 0.01$). The area under the curve (AUC) of TZ increased by 2.6 ($P < 0.001$) and 1.9-folds ($P < 0.05$) for single and daily administration of ITZ, respectively, compared to single individual administration of TZ. As well, $t_{1/2}$ and MRT were significantly prolonged in the presence of ITZ. The obtained $t_{1/2}$ for TZ in the absence of ITZ was 28.5±1.4 min and prolonged to 40.0±2.7 min ($P < 0.01$) and 45.0±7.0 min ($P < 0.05$) after single simultaneous administration and daily pretreatment with ITZ, respectively, while TZ's MRT was prolonged from 34.1±1.6 min for individual treatment to 43.7±2.0 min ($P < 0.01$) with single dose of ITZ and 66.8±13.3 min ($P < 0.05$) with daily pretreated rats with ITZ. On the other hand there was no difference in these two parameters between the ITZ groups. Although TZ clearance was higher when administered after daily pretreatment of ITZ (83.7±12.9 ml/min/kg) but was not significantly different ($P > 0.1$) when compared with single dose of ITZ (56.8±3.5 ml/min/kg). The AUC of TZ was 1.4-fold higher ($P > 0.08$) when it

Table 1
Pharmacokinetic parameters for 2.5 mg/kg intravenous TZ administration either without ITZ or with ITZ (25 mg/kg, p.o.) or after 24 h of daily pretreatment of ITZ for 7 days in rats plasma

	Elimination $t_{1/2}$ (min)	CL (ml/min/kg)	AUC (ng min/ml)	MRT (min)
TZ ($n = 5$)	28.5±1.4	151.4±13.8	17 064±1539	34.1±1.6
TZ+ITZ ($n = 3$)	40.0±2.7†	56.8±3.5†	44 339±2768‡	43.7±2.0†
TZ+ITZ (daily for 7 days) ($n = 4$)	45.0±7.0*	83.7±12.9†	31 942±4565*	66.8±13.3*

Date are expressed as mean±SEM.
† Significantly different ($P < 0.01$) from the TZ group.
‡ Significantly different ($P < 0.001$) from the TZ group.
* Significantly different ($P < 0.05$) from the TZ group.

was simultaneously administered with ITZ to that of its administration after 24 h of daily pretreatment with ITZ.

The elimination half-life of ITZ has been reported in the range 3–6 h [24,25] in rats. Although TZ was administered after 24 h of the last ITZ dose, the pharmacokinetic parameters of TZ were still markedly affected by ITZ. This result suggests that enough intervals should be spaced between stopping ITZ regimen and starting the TZ regimen assuring the complete clearance of ITZ from the body.

3.2.2. Pharmacokinetic parameters of TZ in rat brain microdialysate

Many studies evaluated the disposition of TZ in rat brain tissue [2,26,27]. To our knowledge, no papers reported the effect of ITZ on the pharmacokinetic parameters of TZ in rat brain microdialysates. In this study, the pharmacokinetic parameters of TZ in rat brain microdialysates in the absence and presence of ITZ were also examined. The brain microdialysate concentration-time profiles are shown in Fig. 3. The Pharmacokinetic parameters are listed in Table 2.

The mean C_{max} of TZ was significantly increased 3.4-fold ($P < 0.001$) when administered simultaneously with ITZ and 2.9-fold ($P < 0.001$) when administered after 24 h of daily pretreatment for 7 days with ITZ.

ITZ increased the AUC of TZ by 2.9-fold ($P < 0.001$) when administered in combination and by 3.8-fold ($P < 0.001$) after 24 h of daily pretreatment for 7 days with ITZ. On the other hand, there was no significant difference in TZ's C_{max} and AUC between the two treatments with ITZ.

There was no significant difference in the T_{max} and MRT of TZ with or without ITZ. T_{max} and MRT values of TZ were 19.0 ± 2.0 and 63.1 ± 12.8 min, respectively, in the absence of ITZ, 15.8 ± 1.7 and 65.2 ± 7.4 with single ITZ dose and 20.0 ± 3.1 min and 93.6 ± 5.9 min after 24 h of daily pretreatment with ITZ. But there was a significant difference in TZ's MRT between the two ITZ treatments ($P < 0.05$). This result could be due to the considerable variation in MRT values obtained from rats administered with TZ in the absence of ITZ as indicated by the SEM (Table 2). Also, there was no significant difference in the $t_{1/2}$ of TZ when administered in combination with ITZ (57.2 ± 8.9 min, $P > 0.1$) compared to its single administration (37.0 ± 8.4). On the other hand, $t_{1/2}$ was significantly prolonged when TZ was administered after 24 h of ITZ pretreatment (78.6 ± 8.7 min, $P < 0.05$).

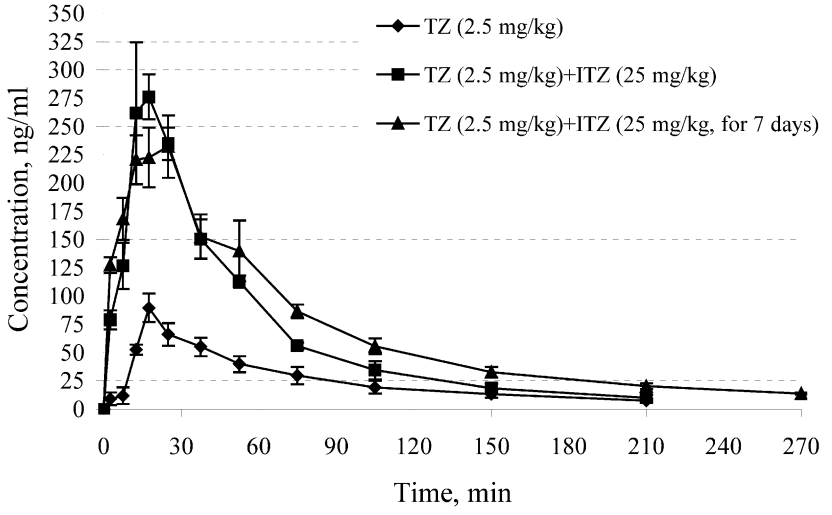

Fig. 3. Mean concentrations of TZ in rat brain microdialysate after administration of TZ (25 mg/kg, i.v.) without and with ITZ (25 mg/kg, p.o.). TZ, triazolam; ITZ, itraconazole.

Table 2
Pharmacokinetic parameters for 2.5 mg/kg intravenous TZ administration either without ITZ or with ITZ (25 mg/kg, p.o.), or after 24 h of daily pretreatment of ITZ for 7 days in rats brain microdialysates

	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	CL (ml/min/kg)	AUC (ng min/ml)	MRT (min)
TZ ($n=5$)	91±12	19.0±2.0	37.0±8.4	565.3±138.1	5292±937	63.1±12.8
TZ+ITZ ($n=3$)	312±40‡	15.8±1.7	57.2±8.9	163.6±10.1	15404±1012‡	65.2±7.4
TZ+ITZ (daily for 7 days) ($n=4$)	268±12‡	20.0±3.1	78.6±8.7*	129.0±13.3*	19939±1821‡	93.6±5.9**

Date are expressed as mean±SEM.
‡ Significantly different ($P<0.001$) from the TZ group.
* Significantly different ($P<0.05$) from the TZ group.
** Significantly different ($P<0.05$) from TZ+ITZ group.

Although the clearance of TZ in the absence of ITZ from the brain microdialysate was faster, the difference was not significant (565.3±138.1 ml/min/kg) when compared to its simultaneous administration with ITZ (163.6±10.1 ml/min/kg, $P=0.09$). Conversely, the daily pretreatment of ITZ significantly decreased TZ clearance (129.0±13.3 ml/min/kg, $P<0.05$).

The pharmacokinetics of TZ in the brain is obviously affected by ITZ. Fig. 4 shows the difference in AUC and CL of TZ in plasma and brain microdialysate. The ratio of TZ's AUC in brain microdialysate and plasma were 0.31, 0.35 and 0.62 for TZ alone, simultaneous administration with single oral dose of ITZ and after 24 h of daily oral pretreatment with ITZ for 7 days, respectively. This result indicates that the increase in the AUC of TZ in brain microdialyste when simultaneously administered with ITZ is parallel to that in plasma, which suggests the elevation in the AUC of TZ in plasma and brain could be related to the inhibition of TZ metabolism by ITZ. On the other hand, the ratio increased to 0.62 when ITZ was pretreated for 7 days before TZ administration. Although there was no difference in the C_{max} of TZ in the two treatments of ITZ but the increase in TZ's AUC in the brain was higher than that in the plasma in the continuous treatment indicating the accumulation of TZ in the brain, which is supported by the decrease in its clearance.

The results obtained from this study are in agreement with other studies that reported the role of ITZ as an inhibitor of the plasma membrane transporter *P*-glycoprotein, which is an important molecular determinant of brain penetration through the blood–brain barrier [28–31]. Although the obtained results require further

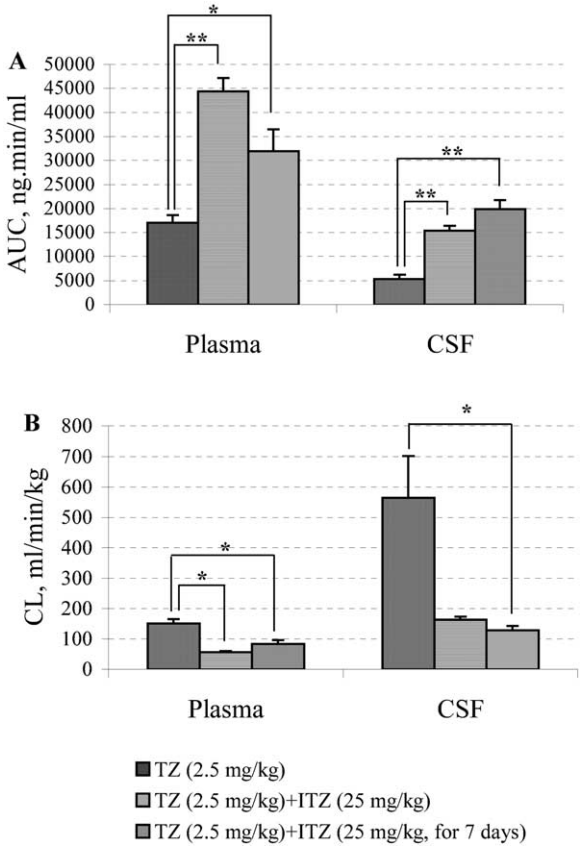

Fig. 4. Effect of ITZ on the pharmacokinetic parameters of TZ. (A) AUC, (B) CL. Data are depicted as the mean ±SEM; $n=5$ (TZ), 3 (TZ+ITZ), 4 (TZ+ITZ, daily for 7 days). *$P<0.05$, **$P<0.01$. TZ, triazolam. AUC, area under the curve; CL, clearance.

investigations, it is clear that ITZ inhibited the efflux of TZ from brain to the blood. The conflict in the AUC results in plasma and brain microdialyste for TZ administered after 24 h of 7 days pretreatment of ITZ (i.e. in plasma, TZ's AUC with single ITZ dose is higher that after 24 h of ITZ daily pretreatment, while in brain microdialysate, TZ's AUC in the latter was higher than the former) could be related to the longer half-life of ITZ in brain compared to that in plasma. More experiments are required to clarify this point.

4. Conclusions

The present paper describes a drug–drug interaction study using a simple and sensitive semi-micro column HPLC method for the determination of TZ in rat plasma and brain microdialysate. ITZ remarkably increased TZ levels in plasma and brain after single simultaneous administration of TZ (2.5 mg/kg, i.v.) with ITZ (25 mg/kg, p.o.) and after 24 h of 7 days pretreatment with ITZ (25 mg/kg, p.o. daily). ITZ is not recommended to be prescribed with TZ; as well enough period should be elapsed between the discontinuation of ITZ regimen and the beginning of TZ regimen. Also, it is necessary to consider not only the inhibition of CYP3A4 mediated metabolism of TZ by ITZ but also the central nervous system side effects that could be caused by the accumulation of TZ in the brain, which might be induced by ITZ mediated inhibition of *P*-glycoprotein activity. To clarify this phenomenon, further investigations are in progress.

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