# Investigation on the Influx Transport Mechanism of Pentazocine at the Blood-Brain Barrier in Rats Using the Carotid Injection Technique

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**The influx transport mechanism of pentazocine (PTZ) at the blood-brain barrier (BBB) was investigated in rats using the carotid injection technique. The uptake kinetics of PTZ into the rat brain exhibited saturability, which occurred by both nonsaturable and carrier-mediated transport processes. The *in vivo* kinetic parameters were estimated as follows: the maximal uptake rate ($J_{max}$), 3.6±1.2 $\mu$mol/min/g brain and the apparent Michaelis constant ($K_t$), 3.7±1.7 mM for the saturable component of PTZ into the brain, and the nonsaturable uptake rate constant ($K_d$), 0.06±0.04 ml/min/g brain. The uptake of PTZ by the brain was strongly inhibited by lidocaine, imipramine and propranolol, and also by $H_1$-antagonists such as mepyramine, diphenhydramine. In addition, narcotic-antagonist analgesic (buprenorphine, butorphanol or eptazocine) and an opioid antagonist (naloxone) significantly inhibited PTZ transport. These results suggest that PTZ permeates into the brain *via* a carrier-mediated transport system, which may widely recognize the cationic drugs.**

**Key words** pentazocine; blood-brain barrier; carrier-mediated transport; brain uptake index; rat

Pentazocine (PTZ), a narcotic-antagonist analgesic, is widely used in the management of patients with postoperative pain or initial carcinogenic pain.[1)] PTZ is a cationic drug having physicochemical properties of high lipophilicity,[2)] and immediately reaches the brain in rats when the drug is administered parenterally.[3,4)] In rats, brain–plasma concentration ratio is relatively constant, and PTZ concentration in the brain is much higher than that in the corresponding plasma.[5,6)] The blood-brain barrier (BBB) appears to have little restricting effect on the uptake of this drug by the brain after parenteral administration in rats.

We recently demonstrated that the major factor governing the uptake of PTZ into the brain was not only nonsaturable process but also carrier-mediated transport with a low-affinity saturable process, using the *in situ* rat brain perfusion technique.[7)] The advantage of this *in situ* technique is the high sensitive ability to estimate the kinetic parameters representing the individual rate process.[8,9)] Moreover, this technique has greater advantages to the use of perfusate because the composition and flow rate can be adjusted according to the needs of the individual experiments.[8,9)] However, this technique is too complex technically, because at least 3 arteries and veins must be ligated before perfusion.[8,9)] On the other hand, the carotid injection technique can maintain the cerebral endothelial cells and vasculature of a brain in their normal physiological states and anatomical positions in the animal. Furthermore, the carotid injection technique is technically simpler than the brain perfusion technique.[8,9)] Therefore, we investigated the influx transport mechanism of PTZ at the BBB in rats using the carotid injection technique, and compared the results with those from the *in situ* perfusion technique.

## MATERIALS AND METHODS

**Radioisotopes and Chemicals** [*Ring*-1,3-$^3$H]-(+)-pentazocine ([$^3$H]-PTZ, specific activity 1036.0 GBq (28.0 Ci)/mmol) and 3-*O*-[*methyl*-$^3$H]-methyl-D-glucose ([$^3$H]-3OMG, specific activity 2782.4 GBq (75.2 Ci)/mmol) were purchased from NEN™ Life Science Products, Inc. (Boston, MA, U.S.A.). *N*-[1-$^{14}$C] butanol ([$^{14}$C]-butanol, specific activity 74 MBq (2 mCi)/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). The radiochemical purity of the [$^3$H]-PTZ used for the experiment was greater than 99%. PTZ (Sosegon® injection) was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). The composition of Sosegon® injection was PTZ (30 mg), lactic acid (12 $\mu$l) and sodium chloride (2.8 mg) in 1 ml of distilled water for injection. The PTZ powder used as a free base was from Kobayashi Kako Co., Ltd. (Fukui, Japan), which was used to adjust the drug concentration of the injection solution after dissolving in 0.1 M hydrochloric acid. Xylazine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) and ketamine hydrochloride (Ketaral® 50; Sankyo Co., Ltd., Tokyo, Japan) were used as anesthetics. Amantadine hydrochloride, choline chloride, cimetidine, desipramine hydrochloride, ketotifen fumarate salt, hemicholinium-3, imipramine hydrochloride, lidocaine hydrochloride, mepyramine maleate, naloxone hydrochloride, propranolol hydrochloride and tetraethylammonium chloride (TEA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diphenhydramine hydrochloride, phenylalanine and HEPES were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Procainamide hydrochloride was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). Buprenorphine hydrochloride (Lepetan injection) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), butorphanol tartrate (Stadol® injection) was purchased from Bristol-Myers Squibb K.K. (Tokyo, Japan), eptazocine hydrobromide (Sedapain® injection) was purchased from Nihon Iyakuhin Kogyo Co., Ltd. (Toyama, Japan), and levallorphan tartrate (Lorfan® injection) was purchased from Takeda Chemical Industries, Ltd. (Osaka, Japan). Tramadol hydrochloride was provided by Kowa Co., Ltd. (Nagoya, Japan). Soluene®-350, Pico-Fluor™ 40 and Hionic-Fluor™ were purchased from Packard Instruments

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Co., Inc. (Downers Grove, IL, U.S.A.). All other solvents and reagents were commercial products of analytical grade and were used without further purification.

**Experimental Animals** Female Wistar/ST rats (11—12 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed in stainless steel cages with a 12 h light/dark cycle (light on 8 : 00 a.m.—8 : 00 p.m.) under conditions of controlled temperature maintained at 23±1 °C with a humidity of 55±10% for at least 1 week before use. The rats were fed and given water *ad libitum* prior to experiments. Rats weighing 250—320 g were used throughout all experiments. The experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (College of Pharmacy, Nihon University, Chiba, Japan).

***In Vivo* Brain Uptake Study: Carotid Injection Technique** The first pass brain extraction of [$^{3}$H]-PTZ relative to [$^{14}$C]-butanol was measured using the carotid injection technique in rats.[10] Rats were anesthetized with an intramuscular dose (4.7 ml/kg) of ketamine (235 mg/kg) and xylazine (2.3 mg/kg). Body temperature was maintained at 36.5±0.5 °C using heat lamps during the experiment. The injection solution consisted of Ringer-HEPES buffer (141 mM; NaCl, 4.0 mM; KCl, 2.8 mM; $CaCl_2 \cdot 2H_2O$, 10 mM; HEPES–NaOH, pH 7.4) containing trace concentrations (0.35 $\mu$M) of [$^{3}$H]-PTZ (10 $\mu$Ci/ml) and [$^{14}$C]-butanol (0.5 $\mu$Ci/ml) as references. An approximately 200 $\mu$l solution was rapidly injected (0.5 s) into the right common carotid artery *via* a 27-gauge×1/2 needle. Rapid arterial injection is minimal mixing (7—9%) of the injection solution bolus with circulating rat plasma.[11] For the experiment of varying pH, hydrochloric acid was used to adjust the solution to pH 5.5 and 6.5. To measure the saturation of PTZ uptake at pH 7.4 and 5.5, various concentrations of unlabeled PTZ were dissolved in the injection solution containing [$^{3}$H]-PTZ (0.35 $\mu$M) and [$^{14}$C]-butanol. The pH was adjusted by buffering the injection mixture before the injection. For the inhibition experiment, various compounds were added to the injection solution to yield the final concentrations. At 15 s after the injection, the rats were scarified by decapitation. The brain was quickly freed from the cranium, and the right hemisphere was placed on ice-chilled filter paper moistened with 0.9% NaCl. The arachnoid membrane and meningeal vessels were carefully removed. A sample of the hemisphere was solubilized in a scintillation vial containing 1 ml of Soluene®-350 at 50 °C for 2—4 h. Then, 10 ml of Hionic-fluor™, the liquid scintillation cocktail, was added to the brain sample. A 50-$\mu$l aliquot of the injection solution was transferred to a scintillation vial to determine the radioactivity of the injection solution and was dissolved in 3 ml of Pico-fluor™ 40. The disintegrations per minute of [$^{3}$H] and [$^{14}$C] radioactivity in the brain and solution samples were determined by dual-channel scintillation counting using a Tricarb 2050CA liquid scintillation counter (Packard Instruments Co., Downvers Grove, IL, U.S.A.). The counting efficiency and crossover correction were determined using the external standard channel ratio technique.

**Calculations** The percentage of brain uptake index (BUI) was calculated as follows:

$$\mathrm{BUI}=\frac{\{[^{3}\mathrm{H}](\mathrm{dpm})/[^{14}\mathrm{C}](\mathrm{dpm})\}\ \text{in brain}}{\{[^{3}\mathrm{H}](\mathrm{dpm})/[^{14}\mathrm{C}](\mathrm{dpm})\}\ \text{in injection solution}}\times 100 \quad (1)$$

$$\mathrm{BUI}=E_{test}/E_{ref}\times 100 \quad (2)$$

where $E_{test}$ and $E_{ref}$ are functional extractions of PTZ and butanol, respectively, at 15 s after the injections. Since the $E_{ref}$ value of [$^{14}$C]-butanol was reported as 64% for the brain,[12] the value of $E_{test}$ was estimated using the following Equation 3:

$$E_{test}=\mathrm{BUI}\times 0.64 \quad (3)$$

**Estimation of Kinetic Parameters** To estimate the kinetic parameters of PTZ, the brain uptake rate ($J$, $\mu$mol/min/g brain) and mean capillary concentration ($C_{cap}$, mM) were calculated from the following Equations 4 and 5, respectively,

$$J=(E_{test}/100)\times F\times C_{in} \quad (4)$$

$$C_{cap}=C_{in}\times(-E_{test}/100)/\ln(1-E_{test}/100) \quad (5)$$

where $F$ and $C_{in}$ are the cerebral blood flow rate (0.93 ml/min/g brain)[13] and the concentration of PTZ (mM) in the carotid injection solution, respectively. $J$ values with various $C_{cap}$ were fitted to the following Equation 6, consisting of a saturable component and a non-saturable component, using a non-linear least-squares regression program (WinNONLIN)

$$J=\frac{J_{max}\times C_{cap}}{K_t+C_{cap}}+K_d\times C_{cap} \quad (6)$$

where $J_{max}$ ($\mu$mol/min/g brain), $K_t$ (mM) and $K_d$ (ml/min/g brain) represent the maximal transport rate of the saturable uptake, the half-saturation concentration (Michaelis constant) and the nonsaturable uptake rate constant, respectively.

**Statistical Analysis** Statistical analysis of the results was performed using Student's *t*-test. A value of $p<0.05$ was considered significant.

## RESULTS

**Concentration Dependence of PTZ Uptake by the Brain** Figure 1A shows the concentration dependence of PTZ uptake by the brain. With an increase in the PTZ concentration from 0.35 $\mu$M (without addition of unlabeled PTZ) to 1 mM, the BUI value increased significantly. However, the BUI of PTZ decreased as the PTZ concentration in a range of 1 to 40 mM in the injection solution increased, suggesting the participation of a saturable uptake of PTZ by the brain. Since no inhibitory effect of the high concentration of PTZ (40 mM) was observed on the BUI of [$^{3}$H]-3OMG (data not shown), the concentration dependent uptake of PTZ by the brain was not be attributed to a toxic effect of PTZ on the BBB, but rather to saturation of the carrier-mediated transport system. As shown in Fig. 1B, the BUI values of PTZ in the range of 1 to 40 mM were analyzed by plotting the brain uptake rate ($J$) against the mean capillary concentration of PTZ ($C_{cap}$). Brain uptake of PTZ was saturable and Eadie–Hofstee plot showed a single straight line. Nonlinear least-squares analysis indicated a $J_{max}$ of 3.6±1.2 $\mu$mol/min/g brain, a $K_t$ of 3.7±1.7 mM, and a $K_d$ of 0.06±0.04 ml/min/g brain.

**Effects of pH of the Carotid Injection Solution on PTZ

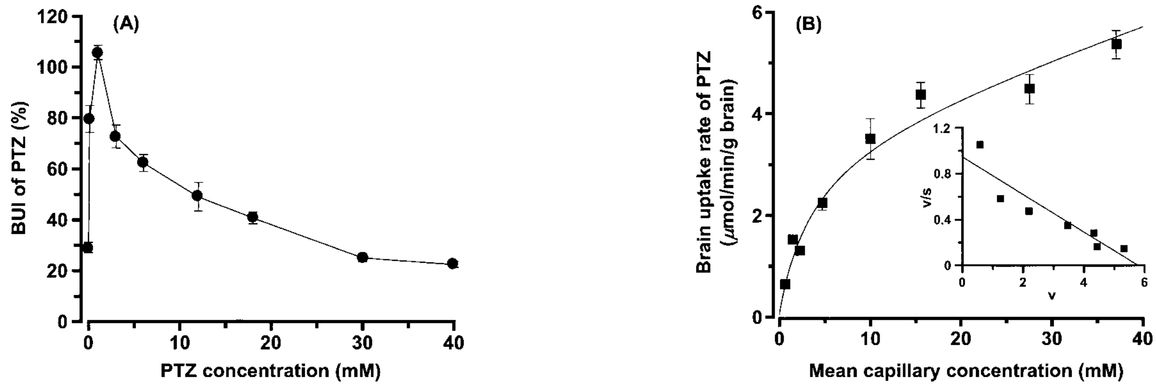


Fig. 1. Concentration Dependence for BUI (Panel A) and Brain Uptake Rate of PTZ (Panel B) at pH 7.4

Each point with a vertical bar represents the mean±S.E. ($n$=3—19). Data points without vertical bars include the S.E. within the points. (A): The BUI of PTZ relative to [$^{14}$C]-butanol was plotted against the concentration of PTZ in a range of 0.35 $\mu$M (without addition of unlabeled PTZ) to 40 mM in the injection solution. (B): The BUI values of PTZ in a range of 1 to 40 mM were analyzed by plotting the brain uptake rate ($J$) against the mean capillary concentration of PTZ ($C_{cap}$) as described in Materials and Methods. Line for total uptake was drawn using the parameters obtained from a nonlinear least-squares regression analysis (WinNONLIN). Inset: Eadie-Hofstee plot of PTZ uptake in a range of 1 to 40 mM. v, brain uptake rate of PTZ in $\mu$mol/min/g brain; s, substrate concentration in mM.

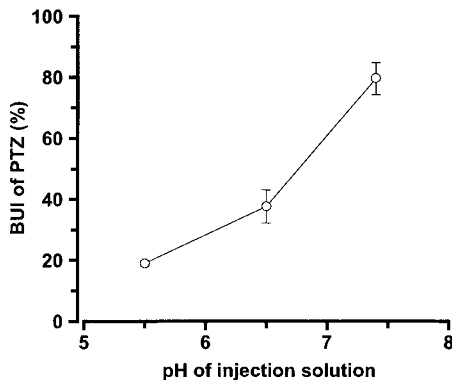


Fig. 2. Effects of pH in the Carotid Injection Solution on PTZ Uptake by the Brain

Each point with a vertical bar represents the mean±S.E. ($n$=3—4). Data points without vertical bars include the S.E. within the points. The BUI values were determined [$^{3}$H]-PTZ (0.35 $\mu$M) with 0.1 mM unlabeled PTZ added to the injection solution.

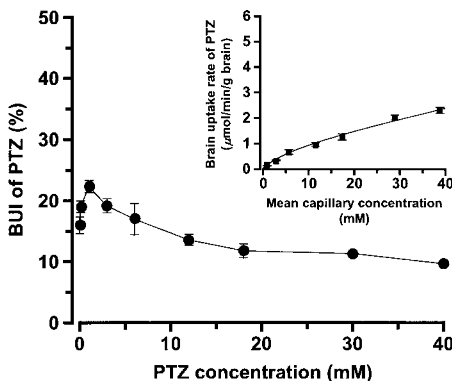


Fig. 3. Concentration Dependence for BUI of PTZ at pH 5.5

Each point with a vertical bar represents the mean±S.E. ($n$=3—7). Data points without vertical bars include the S.E. within the points. The BUI of PTZ relative to [$^{14}$C]-butanol was plotted against the concentration of PTZ in a range of 0.35 $\mu$M (without addition of unlabeled PTZ) to 40 mM in the injection solution adjusted to pH 5.5. Inset: the BUI values of PTZ in a range of 1 to 40 mM were analyzed by plotting the brain uptake rate ($J$) against the mean capillary concentration of PTZ ($C_{cap}$) as described in "Materials and Methods". Line for total uptake was drawn using the parameters obtained from a nonlinear least-squares regression analysis (WinNONLIN).

**Uptake by the Brain** The effect of the pH in the carotid injection solution on PTZ uptake by the brain is illustrated in Fig. 2. The BUI value of PTZ at the concentration of 0.1 mM was decreased when the pH of the injection solution was changed from 7.4 to 6.5 or 5.5. As shown in Fig. 3, the concentration dependent uptake of PTZ by the brain from the injection solution at pH 5.5 was similar to that at pH 7.4 (Figs. 1A, 1B). Nonlinear least-squares analysis of the results at pH 5.5 yielded the following kinetic parameters: The $J_{max}$, $K_t$ and $K_d$ were 0.9±0.4 $\mu$mol/min/g brain, 7.6±3.5 mM, and 0.04±0.01 ml/min/g brain, respectively.

Table 1. Effects of Various Compounds on PTZ Uptake by the Brain

| Compound | Concentration (mM) | Relative BUI (%) |
|---|---|---|
| Lidocaine | 20 | 12.6±1.7* |
| Imipramine | 20 | 24.0±3.4* |
| Propranolol | 20 | 32.9±4.6* |
| Mepyramine | 20 | 17.8±3.3* |
| Ketotifen | 20 | 21.0±5.2* |
| Diphenhydramine | 20 | 28.5±4.7* |
| Amantadine | 20 | 71.9±10.4* |
| Cimetidine | 20 | 62.3±9.5* |
| Desipramine | 20 | 36.8±5.9* |
| Procainamide | 20 | 43.4±2.8* |
| TEA | 20 | 44.7±9.2* |
| Buprenorphine | 0.2 | 46.1±6.5* |
| Butorphanol | 2 | 33.7±4.8* |
| Eptazocine | 20 | 18.2±2.6* |
| Naloxone | 20 | 29.3±4.3* |
| Levallorphan | 2 | 64.2±8.8* |
| Tramadol | 20 | 48.0±6.4* |
| Choline | 20 | 60.1±9.9* |
| Hemicholinium-3 | 20 | 64.6±9.4* |
| Phenylalanine | 20 | 96.9±14.1 |

Each value represents the mean±S.E. ($n$=3—5). The control value was experimentally determined BUI of [$^{3}$H]-PTZ (0.35 $\mu$M) in the injection solution at pH 7.4 with addition of unlabeled PTZ (0.1 mM). *Significantly different from the control value by Student's $t$-test ($p<0.05$).

**Effects of Various Compounds on PTZ Uptake by the Brain** Table 1 summarizes the effect of various compounds on PTZ uptake by the brain. The uptake of PTZ (0.1 mM) was strongly inhibited by lidocaine, imipramine and propranolol, and also by the $H_1$-antagonists such as mepyramine, ketotifen and diphenhydramine. Similarly, the narcotic-antagonist such as buprenorphine, butorphanol, or eptazocine, opioid antagonist such as naloxone and, the centrally acting analgesic tra-

madol, significantly inhibited the transport of PTZ at the BBB. A relatively weak inhibitory effect was observed by amantadine, cimetidine, levallorphan, choline or hemicholinium-3 among the compounds tests. However, phenylalanine of the endogenous amino acid showed no inhibitory effect against PTZ uptake.

## DISCUSSION

Our previous *in situ* brain perfusion study demonstrated that PTZ is transported *via* a carrier-mediated system, and shares a common carrier system specific for cationic drugs such as lidocaine, propranolol and diphenhydramine.[7] To clarify the characterization of PTZ transport at the BBB under physiological conditions, which maintain intact cerebral endothelial cells and vasculature of the brain, the present study was performed using the *in vivo* carotid injection technique.

Concentration dependence of PTZ transport was examined to re-evaluate whether the saturable uptake system participates in BBB permeation of PTZ in this technique. Increasing the PTZ concentration from 0.35 $\mu$M to 1 mM increased the BUI value of PTZ (Fig. 1), suggesting the existence of a saturable efflux mechanism at the BBB (as discussed later). In contrast, the decrease in the BUI value with the increasing PTZ concentration range between 1 and 40 mM suggests the participation of a saturable influx process at that concentration range (Fig. 1). Assuming the efflux transport of PTZ was saturated, the kinetic parameter on the apparent influx transport of PTZ was analyzed in the concentration range between 1 and 40 mM. The analysis of uptake kinetics provided apparently one saturable component with a $K_t$ value of 3.7 mM, a $J_{max}$ value of 3.6 $\mu$mol/min/g brain, and a non-saturable component with a $K_d$ value of 0.06 ml/min/g brain. According to the kinetic parameters, the ratio of $J_{max}/K_t$ at pH 7.4 was estimated to be 0.97 ml/min/g brain, which was 16-fold greater than the value of $K_d$. Thus, PTZ is transported into the brain predominantly by a carrier-mediated mechanism. It was reported that the saturable BBB influx transport of cationic drugs showed an apparent low-affinity system for propranolol ($K_t$=9.8 mM)[14] or mepyramine ($K_t$=4.4 mM),[15] using the *in vivo* carotid injection technique. When the uptake of PTZ was measured by the *in situ* brain perfusion technique, the apparent influx transport of PTZ was saturable, with a $K_m$ value of 2.9 mM.[7] In the present study, the apparent value of $K_t$ (3.7 mM) was in good agreement with that reported previously. A high similarity between these techniques was shown in the influx transport system of PTZ with low-affinity at the BBB.

To characterize the saturable influx transport system for PTZ, the pH dependence of PTZ uptake was investigated. The BUI value of PTZ was greatest at pH 7.4 and fell significantly at pH 6.5—5.5 (Fig. 2), indicating that PTZ is taken up into the brain in a pH-dependent manner as in a previous study.[7] This observation is in agreement with the effect of pH for cationic drugs such as lidocaine, mepyramine or propranolol using *in vivo* carotid injection technique.[14,15] The concentration-dependent uptake of PTZ at pH 5.5 showed the participation of a saturable uptake process (Fig. 3). At pH 5.5 of the injection solution, $J_{max}/K_t$ and $K_d$ were 0.12 and 0.04 ml/min/g brain, respectively. $J_{max}/K_t$ is 3-fold greater than the value of $K_d$. Reducing the pH from 7.4 to 5.5, $J_{max}/K_t$ was decreased to 88%, but $K_d$ was not changed. These results suggest that the decrease in the transport efficiency ($J_{max}/K_t$) in a pH-dependent manner is caused by a pH-dependent conformational change in the transport carrier. The apparent nonsaturable uptake process ($K_d$) appears to reflect the passive diffusion of PTZ at the BBB. However, the process has another possibility that the other low affinity carrier-mediated transport system remains as suggested in our previous perfusion study.[7]

PTZ transport at the BBB was inhibited by several cationic drugs such as lidocaine,[14] propranolol,[14] mepyramine,[15—17] diphenhydramine[18] and amantadine,[19] which are transported into the brain *via* the specific carrier system for cationic drugs (Table 1). The inhibitory effect on the influx transport was especially shown in lidocaine, propranolol or diphenhydramine by both the *in vivo* carotid injection and *in situ* brain perfusion techniques. These observations strongly suggest that PTZ is transported *via* a common carrier-mediated system for cationic drugs. In addition, narcotic-antagonist analgesics such as buprenorphine (0.2 mM), butorphanol (2 mM) and eptazocine (20 mM) effectively inhibited the brain uptake of PTZ, because of the specific structural similarity for PTZ. Since various cationic drugs inhibited the apparent influx transport of PTZ (Table 1), it is suggested that the carrier system for PTZ widely recognizes the broad structural characteristics of cationic drugs. Our previous brain perfusion study showed no inhibitory effect by choline on the influx transport of PTZ.[7] In the present study, however, choline or hemicholimium-3 inhibited slightly the PTZ uptake. Although the choline transport system[20—22] may be involved in the influx transport of PTZ at the BBB, this apparently different result for choline between *in vivo* and *in situ* techniques may be due to the differences between the experimental techniques.

The BUI value of PTZ was significantly increased within the lower concentration range (0.35 $\mu$M—1 mM) examined (Fig. 1). A similar finding was observed in our previous *in situ* study,[7] although it was not significant. It is anticipated that the saturation of the efflux transport from the brain capillary endothelial cells occurred at the low concentration range of PTZ. Since we previously found that the apparent influx transport of PTZ increased markedly in the presence of verapamil, a P-glycoprotein (P-gp) inhibitor, in the brain perfusion study,[7] the observed increment of BUI may be attributed to the involvement of P-gp in the active efflux transport of PTZ at the BBB. *In vitro* experiments using multidrug-resistant cells have shown that PTZ can be a substrate for P-gp-mediated efflux from the brain.[23,24)]

In conclusion, the findings from the present study suggest that PTZ is transported predominantly *via* a carrier-mediated influx system at the BBB as previously suggested in our brain perfusion study. The apparent low-affinity in the influx transport system of PTZ at the BBB showed high similarities between the *in vivo* carotid injection and *in situ* brain perfusion techniques. Furthermore, it is also suggested that this carrier system for PTZ is responsible for the transport of various cationic drugs such as propranolol and mepyramine. The increase in BBB permeability of PTZ at the low concentration range may be explained by the saturation of P-gp-mediated efflux transport from the brain capillary endothelial

cells. Further studies are needed to elucidate both the influx and P-gp-mediated efflux transport system at the BBB transport mechanism of PTZ.

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