Involvement of P-glycoprotein in Blood–Brain Barrier Transport of Pentazocine in Rats Using Brain Uptake Index Method

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The involvement of P-glycoprotein (P-gp) in pentazocine (PTZ) transport at the blood–brain barrier (BBB) in rats was evaluated by means of an *in vivo* study using the brain uptake index (BUI) method. The amount of radioactivity in the brain was estimated at different intervals (up to 240 s) after carotid injection in rats. The apparent elimination rate constant (k_{test}) due to efflux of PTZ from the brain was calculated as 0.22 min^{-1} . The observed BUI values of [^3H]-PTZ ($0.35 \mu\text{M}$) were not significantly different between 5 and 15 s after the carotid injection. The concentration-dependent uptake of PTZ by the brain was increased gradually by increasing the concentration (0.01 – 1 mM) of PTZ in the injection solution. The apparent uptake of PTZ by the brain increased in the presence of P-gp inhibitors such as cyclosporin A, quinidine, verapamil and vinblastine after the carotid injection. These results suggest that the increment of PTZ uptake by the brain could be explained by the saturable efflux transport system involving a P-gp-mediated efflux mechanism of PTZ transport at the BBB.

Key words pentazocine; blood–brain barrier; P-glycoprotein; brain uptake index; efflux transport

Pentazocine (PTZ), a narcotic-antagonist analgesic, is widely used in the management of patients with postoperative pain or initial carcinogenic pain.¹⁾ The analgesic effects of PTZ are caused by interactions with specific opioid receptors in the brain. We recently demonstrated that PTZ is transported predominantly *via* a carrier-mediated influx system at the blood–brain barrier (BBB) in rats, using both the brain uptake index (BUI) method²⁾ and an *in situ* brain perfusion technique.³⁾ Furthermore, we previously found an increase in the BBB permeability of PTZ in the presence of non-radiolabeled PTZ within the lower concentration range examined in these studies.^{2,3)} These results may be explained by the saturation of the efflux transport from the brain capillary endothelial cells.

P-glycoprotein (P-gp) is expressed at high levels on the luminal (blood) side of endothelial cells, which comprise the BBB.^{4–6)} Substrates for the P-gp or multidrug-resistance efflux transporters tend to be basic, aromatic and lipophilic compounds.^{7,8)} PTZ is a small, highly lipophilic-basic compound that is positively charged at physiological pH. Previous *in vitro* experiments have shown that PTZ can be a substrate for P-gp-mediated efflux at the BBB using multidrug-resistant cells.^{9,10)} We have found *in vivo* that the apparent influx transport of PTZ is increased markedly in the presence of a P-gp inhibitor (verapamil), in a brain perfusion study.³⁾

Although the brain efflux index (BEI) method is useful for investigating an efflux transport system for substrates from the cerebrum to the circulating blood across the BBB,^{11–14)} this method involves a microinjection of the drug and a reference compound into the cerebral Par2 region. In contrast to the BEI method, the modified BUI method by Pardridge *et al.*¹⁵⁾ enables the efflux of substrates to be measured at various times after injection. This method involves decapitating the animals at different time intervals after the initial phase and determining the extraction of the drug and reference compound. The advantage of this technique is that it is simpler technically than the BEI method and may be most convenient for quick and initial *in vivo* experiments with substrates for the P-gp or multidrug-resistance efflux trans-

porters.

The primary objective of the present study was to demonstrate *in vivo* the involvement of P-gp in PTZ transport at the BBB using the BUI method.

MATERIALS AND METHODS

Radioisotopes and Chemicals [*Ring*-1,3- ^3H]-(+)-pentazocine ([^3H]-PTZ, specific activity 1036.0 GBq (28.0 Ci)/mmol; 99% purity) and 3-*O*-[methyl- ^3H]-methyl- D -glucose ([^3H]-3OMG, specific activity 2782.4 GBq (75.2 Ci)/mmol; 99% purity) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA, U.S.A.). *N*-[1- ^{14}C] butanol ([^{14}C]-butanol, specific activity 74 MBq (2 mCi)/mmol; 99% purity) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). PTZ (Sosegon® injection) was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Cyclosporin A, quinidine sulfate salt dihydrate, verapamil hydrochloride and vinblastine sulfate salt were purchased from Sigma Chemical Co. (St. Louis, MO, 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 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 *in vivo* BBB transport was evaluated by the carotid injection technique reported previously.^{2,16)} Rats (250–320 g) were anesthetized with an intramuscular injection of ketamine-xylazine. An aliquot of $200 \mu\text{l}$ of injection solution was injected rapidly into the right common carotid artery. The injection solution consisted of Ringer's/HEPES buffer (pH 7.4) containing both a test compound, [^3H]-PTZ ($0.35 \mu\text{M}$; $10 \mu\text{Ci/ml}$), and a reference compound, [^{14}C]-butanol ($0.5 \mu\text{Ci/ml}$), with or without unlabeled PTZ. Rats were sacrificed by decapitation at different times (5, 15, 30, 60,

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Table 1. Concentration Dependence of the Uptake of PTZ by the Rat Brain after Carotid Injection

Condition	Concentration (mM)	5 s		15 s ^{a)}	
		BUI (%)	Relative uptake (% of control)	BUI (%)	Relative uptake (% of control)
Control		30.1±1.2	100	29.1±2.0	100
+PTZ	0.01	32.2±3.3	107	49.1±3.7	169*
	0.1	35.4±2.5	118	79.6±5.3	274*
	1	39.4±2.1	131	105.6±2.8	363*

The control value represents the BUI of [³H]-PTZ (0.35 μM) without addition of unlabeled PTZ to the injection solution. Each value represents the mean±S.E. (n=3–19).
 * Significantly different from the control value (p<0.05). a) Taken from Suzuki *et al.*²⁾

120, 240 s) after the injection. Various concentrations of unlabeled PTZ were dissolved in the injection solution containing [³H]-PTZ (0.35 μM) and [¹⁴C]-butanol in order to measure the self-inhibition. The BUI values of PTZ at 15 s were quoted from our previous work.²⁾ For the cross-inhibition experiment, unlabeled typical P-gp inhibitors such as cyclosporin A, quinidine, verapamil and vinblastine were added to the injection solution to yield the final concentrations. The radioactivity in the injection solution and the hemisphere ipsilateral to the injection were determined according to the method described previously.²⁾

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

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

The value of BUI(*t*) at different times after the carotid artery injection was expressed as follows:

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

where $E_{\text{test}}(t)$ and $E_{\text{ref}}(t)$ are the extraction by the brain of PTZ and butanol, respectively, at the respective time (*t*) points after the injection. $E_{\text{test}}(t)$ and $E_{\text{ref}}(t)$ were determined by the following equations:

$$E_{\text{test}}(t) = E_{\text{test}}(0) \times e^{-k_{\text{test}}(t)} \quad (3)$$

$$E_{\text{ref}}(t) = E_{\text{ref}}(0) \times e^{-k_{\text{ref}}(t)} \quad (4)$$

where $E_{\text{test}}(0)$ and $E_{\text{ref}}(0)$ are the maximal functional extraction of PTZ and butanol at time zero (0), respectively, *t* is time after the injection, k_{test} (min⁻¹) is the apparent elimination rate constant due to efflux of PTZ from the brain, and k_{ref} (min⁻¹) is the rate constant of removal of radioactive butanol from the brain. k_{test} was calculated from the following equation (5) by the combination of equations (2)–(4):

$$\text{BUI}(t) = \text{BUI}(0) \times e^{-k_n(t)} \quad (5)$$

where $\text{BUI}(0) = E_{\text{test}}(0)/E_{\text{ref}}(0) \times 100$ and $k_n = k_{\text{test}} - k_{\text{ref}}$. Therefore, a log plot of BUI(*t*) versus time gives a *y*-intercept (BUI(0)) and slope (k_n).¹⁷⁾ The apparent half-life ($t_{1/2}$) of PTZ retention by the brain was given by $0.693/k_{\text{test}}$. The efflux rate constant (k_{ref}) for butanol has been reported as 0.92 min⁻¹ in rat brains under ketamine/xylazine anesthesia.¹⁸⁾

Statistical Analysis Statistical analysis of the results was performed using analysis of variance with the Bonferroni correction for multiple comparisons. A value of *p*<0.05 was considered significant.

RESULTS

Time Dependence of PTZ Uptake by the Brain The brain uptake of [³H]-PTZ (0.35 μM) was 30±1, 29±2, 43±3, 60±5, 181±18 and 499±91%, respectively, at 5, 15, 30, 60, 120 and 240 s after the carotid injection. The observed BUI value of [³H]-PTZ at 5 s was not significantly different from that at 15 s after the injection. However, circulation times beyond 30 s after the injection resulted in a decay of brain PTZ radioactivity owing to washout of PTZ from the brain. A plot of the natural logarithm of BUI(*t*) against times after the injection up to 240 s was linear (*r*=0.991) with a *y*-intercept BUI(0) of 31%, and a slope (k_n) of 0.70 min⁻¹. Since k_{ref} is 0.92 min⁻¹ for butanol efflux in rat brains,¹⁸⁾ the apparent elimination rate constant (k_{test}) due to efflux of PTZ from the brain was calculated as 0.22 min⁻¹, which is equivalent to an apparent elimination half-life ($t_{1/2}$) from the brain of 3.2 min.

Concentration Dependence of PTZ Uptake by the Brain Table 1 shows the concentration dependence of PTZ uptake by the brain at the two different time points of 5 and 15 s after the carotid injection. The brain uptake of PTZ at 5 s after the injection was increased gradually by increasing the concentration (0.01–1 mM) of PTZ in the injection solution. Similarly, the BUI value of PTZ at 15 s increased significantly with an increase in the concentration. When the concentration of PTZ was increased to 3 mM, the brain uptake of PTZ at 15 s was decreased to 69%, suggesting the participation of a saturable influx transport at the BBB, as shown in our previous report.²⁾

In Vivo Evaluation of Involvement of P-gp at the BBB Transport of PTZ The effects of the P-gp inhibitors on the BBB transport of PTZ after carotid injection were examined. Table 2 shows that P-gp inhibitors, such as cyclosporin A, quinidine, verapamil and vinblastine, increased the apparent uptake of PTZ by the brain at 15 s after the injection. Cyclosporin A, among the P-gp inhibitors examined, exhibited the most potent inhibitory effects on P-gp. A similar tendency was also observed at the time point of 5 s after the injection.

To test whether the concentrations of these P-gp inhibitors in the injection solution have a non-specific effect on BBB permeability, the BUI of [³H]-3OMG, relative to [¹⁴C]-butanol, was measured at 15 s after the carotid injection. The BUI values of [³H]-3OMG in the presence of the P-gp inhibitors were unchanged compared with the control BUI in the absence of P-gp inhibitors (data not shown).

Table 2. Effects of P-gp Inhibitors on the Uptake of PTZ by the Rat Brain after Carotid Injection

Inhibitor	Concentration (mM)	5 s		15 s	
		BUI (%)	Relative uptake (% of control)	BUI (%)	Relative uptake (% of control)
Control		30.1±1.2	100	29.1±2.0 ^{a)}	100 ^{a)}
Cyclosporin A	0.1	90.1±10.7	299*	137.5±14.1	473*
Quinidine	1	87.1±9.3	289*	71.6±7.6	246*
Verapamil	0.5	51.8±4.5	172	65.8±4.5	226*
Vinblastine	0.1	34.1±5.0	113	49.4±4.7	170

The control value represents the BUI of [³H]-PTZ (0.35 μ M) without addition of unlabeled PTZ to the injection solution. Each value represents the mean±S.E. ($n=3-19$).
* Significantly different from the control value ($p<0.05$). a) Taken from Suzuki *et al.*³⁾

DISCUSSION

The involvement of P-gp in PTZ transport at the BBB in rats was evaluated by means of an *in vivo* study using the BUI method. The present study provided evidence that the BUI method is convenient for quick and initial *in vivo* experiments for confirming substrates for the P-gp efflux transporter.

We measured the efflux of PTZ (0.35 μ M without addition of unlabeled PTZ) from the brain at different time intervals from 5 to 240 s after carotid artery injection. Following the injection, PTZ disappeared from the brains exhibiting k_{test} of 0.22 min⁻¹. Pardridge *et al.* similarly characterized the kinetics of the drug transport and sequestration of propranolol and lidocaine in rat brains *in vivo* using the present BUI method.¹⁷⁾ The apparent $t_{1/2}$ of PTZ from the brain was 3.2 min, which was shorter than that of 6–7 min for both propranolol and lidocaine.

Our previous study demonstrated the increase in the BBB permeability of PTZ with an increasing PTZ concentration, from 0.35 μ M to 1 mM, in the injection solution, by using the BUI method.²⁾ The following two possibilities are proposed to explain the increase in the BUI value with an increasing PTZ concentration range: 1) the saturation of the efflux transport from the brain capillary endothelial cells, or, 2) a toxic effect on the BBB at the increase of PTZ concentration. We re-examined the concentration dependence of PTZ transport to determine whether the saturable efflux transport system participates in the BBB permeation of PTZ. To avoid simple back-diffusion and metabolism,¹⁹⁾ the present study was performed by decapitating the animals at 5 s and making evaluations the same as in previous study.²⁾ The concentration-dependent uptake of PTZ by the brain at 5 s from the injection solution was similar to that at 15 s (Table 1). Similar increases in BBB permeability have been reported with the H₁-antagonist ebastine-metabolite carebastine,²⁰⁾ and the quinolone antibacterial agent HSR-903,²¹⁾ which are both P-gp substrates. Therefore, the increment of PTZ uptake by the brain implies the involvement of a P-gp efflux mechanism of PTZ transport at the BBB.

We have found that the apparent influx transport of PTZ increased markedly in the presence of verapamil, a P-gp inhibitor, in a brain perfusion study.³⁾ In the present study, we examined the effect of various P-gp inhibitors on the BBB transport of PTZ at 5 or 15 s after carotid injection. P-gp inhibitors such as cyclosporin A, quinidine, verapamil and vinblastine increased the BBB permeability of PTZ (Table 2). In

contrast, the uptake of 3-OMG by the brain did not change by the addition of various P-gp inhibitors to the injection solution (data not shown). These results suggest that the dramatic increase of PTZ uptake by the brain can not be attributed to a toxic effect of various P-gp inhibitors on the BBB. Recently, Kawamura *et al.* reported that the brain uptake of [³H]-PTZ was greatly enhanced by pre-treatment with cyclosporin A in mice.²²⁾ The P-gp-mediated efflux of opioids at the BBB has been demonstrated with a variety of *in vitro* and *in vivo* approaches.^{23,24)} P-gp probably participates in the brain distribution of PTZ and limits accumulation of PTZ in the brain *via* active efflux, which may influence the onset, magnitude, and duration of analgesic action.

In conclusion, our present study suggested that the increase in the BBB permeability of PTZ in the presence of non-radiolabeled PTZ could be explained by the saturable efflux transport system involving a P-gp-mediated efflux mechanism of PTZ transport at the BBB. Further studies may be needed to characterize quantitatively *in vivo* the P-gp-mediated efflux transport system using the BEI method.

Acknowledgments This work was supported in part by a Grant from the Ministry of Education, Culture, Sports, Science and Technology to promote multi-disciplinary research projects in Japan. The authors express their sincere gratitude to Mr. Naoki Yasuda and Ms. Mitsuko Iiyama, who kindly provided technical assistance in this study.

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