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DETECTION AND DETERMINATION OF ACTIVE METABOLITES OF 1-(2-*o*-CHLOROBENZOYL-4-CHLOROPHENYL)-5-GLYCYL-AMINOMETHYL-3-DIMETHYLCARBAMOYL-1*H*-1,2,4-TRIAZOLE HYDROCHLORIDE DIHYDRATE, (450191-S), IN RAT TISSUES, USING A RADIORECEPTOR ASSAY FOR BENZODIAZEPINES

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Abstract—1-(2-*o*-Chlorobenzoyl-4-chlorophenyl)-5-glycyl-aminomethyl-3-dimethylcarbamoyl-1*H*-1,2,4-triazole hydrochloride dihydrate, (450191-S), exhibits pronounced central nervous system (CNS) activities similar to those of benzodiazepines, but it has only low affinity for benzodiazepine receptors. However, when 450191-S was administered to rats at a dose of 10 mg/kg, brain extracts markedly inhibited [^{3}H]diazepam binding to the receptors. Thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and radioreceptor assay (RRA) were used to isolate three metabolites that could inhibit [^{3}H]diazepam binding prominently. These were identified by gas chromatography-mass spectrometry (GC/MS) as compounds having the triazolo-benzodiazepine skeleton. They showed high affinities for benzodiazepine receptors (K_i = 0.9 to 2.1 nM) and exerted potent pharmacological effects similar to those of 450191-S. In addition, their levels in the brain were sufficient to explain the pharmacological activity of 450191-S, which could not be detected in tissue extracts 15 min after administration. These results indicate that the pharmacological activity of 450191-S is largely due to the action of active metabolites, although some points remain to be elucidated to fully account for the large attenuation of the side effect (ataxia) compared with the major effects (anti-convulsant and hypnotic). We also determined the brain levels of metabolites following the administration of 450191-S and evaluated the extent to which each active metabolite contributes to the pharmacological activities of this drug.

High-affinity specific binding of benzodiazepines to their receptors (benzodiazepine receptor) in mammalian brains [1, 2] has not only become an invaluable probe for studying the mechanism(s) of action of benzodiazepines but also provides an effective method of searching for or developing new anxiolytic or sedative drugs. Also, radioreceptor assay (RRA) is a useful means for determining the blood level of benzodiazepines [3] and, when combined with high performance liquid chromatography (HPLC), can be used to detect and measure not only the administered benzodiazepines but also their active metabolites in biological materials [4]. In the present work, we demonstrate that the method is also effective in searching for and identifying the active metabolites.

1-(2-*o*-Chlorobenzoyl-4-chlorophenyl)-5-glycyl-aminomethyl-3-dimethylcarbamoyl-1*H*-1,2,4-triazole hydrochloride dihydrate, (450191-S), is one of the 1*H*-1,2,4-triazoyl benzophenone derivatives developed as ring-opened derivatives of triazolobenzodiazepines [5]. In experimental animals, this compound has strong sleep-inducing, anxiolytic, and anticonvulsant effects, with an effect on muscle relaxation that is surprisingly weaker than other known benzodiazepines.† In humans, 450191-S has a strong sleep-inducing effect, with a very small unfavorable residual effect after awakening.‡ As reported below, 450191-S has very low affinity for benzodiazepine receptors, but RRA, HPLC, and gas chromatography-mass spectrometry (GC/MS) results showed that it is converted *in vivo* into at least three active metabolites that display high affinity for benzodiazepine receptors and mimic the central nervous system (CNS) activity of 450191-S.

MATERIALS AND METHODS

Compounds

[^{3}H]Diazepam, sp. act. 90–97 Ci/mmole, radiochemical purity >99%, was purchased from Amersham International, U.K. Leucine aminopeptidase Type VI from porcine kidney microsomes, lyophilized powder, was obtained from the Sigma

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† K. Yamamoto and K. Hirai, in *Abstracts of the Twenty-second Annual Meeting of the Association for the Psychophysiological Study of Sleep*, p. 63. San Antonio, TX (1982).

‡ I. Namura, Y. Saito, Y. Shimaoka, and H. Uesaka, in *Abstracts of the Twenty-second Annual Meeting of the Association for the Psychophysiological Study of Sleep*, p. 64. San Antonio, TX (1982).

Chemical Co. 450191-S was donated by Dr. Kentaro Hirai of these laboratories.

RRA for benzodiazepines

RRA was performed as described previously [4]. For receptor preparation, a P_2-crude synaptosomal fraction obtained from whole forebrains of rats was suspended in five times the original tissue weight of 50 mM Tris–HCl buffer (pH 7.4), and portions were stored at −70°. The frozen tissue was thawed and diluted 1:10 in the same buffer prior to use for RRA. In the binding assay, 500 μl of this suspension was incubated for 60 min in an ice bath, with 25 μl of [^{3}H] diazepam, at a final concentration of about 1.0 nM, and 10 μl of a standard solution of corresponding benzodiazepines or a sample. After filtration through a Whatman GF/C glass fiber filter and subsequent washing, the filter was counted for tritium. Specific binding was calculated by subtracting the nonspecific binding obtained in the presence of 3 μM diazepam. The amounts of three active metabolites were calculated from the standard curves prepared for the corresponding compounds. The level of receptor-reactive substances, which is hereafter designated as diazepam activity, in tissue extracts was obtained from the standard curve for diazepam, as described previously [6]. The binding inhibition equal to that caused by 1 μg of diazepam was defined as 1 unit. K_i values for benzodiazepine receptors were calculated using the equation $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of the radioactive ligand, K_d is its dissociation constant, and IC_{50} is the concentration causing 50% inhibition of specific [^{3}H]diazepam binding.

Preparation of tissue extracts

Animals used in the metabolism experiments were male Wistar rats (200–220 g). 450191-S, suspended in 2% arabic gum at a concentration of 2.5 mg/ml, was injected i.p. at a dose of 10 mg/kg. Rats were decapitated at different times after the administration, and fresh blood from the carotid artery was collected in a tube and frozen with dry ice. Forebrains, lungs, livers, kidneys, and jejunums were quickly excised and placed on dry ice. Total blood was used instead of plasma, to avoid further metabolism of the drug during preparation of plasma samples. Frozen tissue was weighed, homogenized in 10 vol. of ice-cold ethanol using a Polytron PT 10/35 (Kinematica, setting 6.5 for 20–30 sec), and centrifuged at 10,000 *g* for 20 min.

Thin-layer chromatography (TLC)

The first step in purification of active metabolites and product identification after incubation of 450191-S with an aminopeptidase was performed by TLC, employing Eastman silica gel plates (Eastman code 13181) of 0.1-mm layer thickness on inactive poly(ethylenetelephthalate) film. The solvent was chloroform–methanol–benzene–water (7:6:5:1, by vol.). Tissue extract was spotted on a plate and developed to 10 cm from the origin. The chromatogram was cut into 0.5-cm pieces, and each piece was extracted with methanol. Portions of the extracts were subjected to RRA. Fractions containing receptor reactive substances were collected.

HPLC

A liquid chromatograph was equipped with a Waters Assoc. model 6000A solvent delivery system, a Waters Assoc. model U6K injector, and a stainless steel tube (30 cm × 4 mm i.d.) packed with Nucleosil 10C_8 (Macherey, Nagel & Co.). The solvent used for the mobile phase was a mixture of methanol–water (55:45, v/v) which had been passed through a 0.5 μm membrane filter. The eluent was passed through the column at a flow rate of 1.0 ml/min at room temperature. The methanol solution of the sample was injected into the column. The eluate was monitored with an ultraviolet detector (Waters Assoc. model 440) at 254 nm and fractionated every 1 min. A portion of each fraction was evaporated to dryness under vacuum and subjected to RRA.

Synthesis of authentic samples of active metabolites

N,*N*-Dimethyl-6-(*o*-chlorophenyl)-8-chloro-4*H*-*S* - triazolo[1,5 - *a*][1,4]benzodiazepine - 2 - carboxamine(M-1),*N*-methyl-6-(*o*-chlorophenyl)-8-chloro-4*H*-*S*-triazolo[1,5-*a*][1,4]benzodiazepine-2-carboxamide (M-2), and 6 - (*o* - chlorophenyl) - 8 - chloro - 4*H* - *S* - triazolo[1,5 - *a*][1,4]benzodiazepine - 2-carboxamide (M-3) were synthesized by the procedure described by Gagneux *et al.* [7].

GC/MS

The gas chromatograph-mass spectrometer was a Varian-MAT 44S coupled to a Varian model 3700 gas chromatograph with a split-splitless injector (Varian-MAT, Bremen, FRG). Column: SCOT column coated with OV-17 (3 m × 0.23 mm i.d.) (Gasukuro Kogyo Co., Ltd., Tokyo, Japan). Operating temperatures: injector, 300°; transfer line, 270°; ion source, 200°; column, M-1: 175° isothermal for 1 min followed by temperature programming at 40° min^{-1} to 280°. M-2 and M-3: 100° isothermal for 1 min followed by temperature programming at 40° min^{-1} to 310°. Carrier gas: He; inlet pressure, 4 psi. Injection was done in the splitless mode; at 60 sec after the injection, the split exit vent was opened to give a splitting ratio of 1:20. Typical ion source operating conditions: for M-1 (E1 mode), ion box potential 8.4 V, pusher potential 1.6 V, draw-out plate potential 1.0 V, lens potential 25 V, electron energy 75 eV, emission current 0.5 mA, and voltage of secondary electron multiplier 1750 V; for M-2 and M-3 (negative ion chemical ionization mode), ion box potential 9.9 V, pusher potential 0 V, draw-out plate potential 0 V, lens potential 150 V, electron energy 160 eV, emission current 0.25 mA, and voltage of secondary electron multiplier 2450 V. Reagent gas: ammonia (*ca.* 0.4 Torr).

Aminopeptidase treatment

450191-S at a concentraton of 55 μg/ml was incubated with an aminopeptidase (0.5 unit) in 0.5 ml Tris–HCl (50 mM, pH 7.4) containing 5 mM $CaCl_2$ for 20 min at 37°. The incubation was terminated by adding 0.1 ml of 0.1 N HCl. The reaction mixture was evaporated, and then the reaction product was extracted with 2 ml of ethanol.

Pharmacological activity

The experiments were conducted on albino male

mice (DS-strain, Aburahi Farm, Shionogi, 20–23 g, and ddY-strain, Shizuoka Laboratory Center, Japan, 20–23 g). All drugs were suspended in an aqueous solution of arabic gum and administered orally in a volume of 0.01 ml/g weight. The test was done with a group of ten mice.

Rotating rod test. The procedure was based on the method of Dunham and Miya [8]. At 60 min after dosing, each mouse (ddY) was put on the wooden rod (3 cm in diameter) turning at 5 rpm, and the number which fell off the rod within 2 min was counted. The ED_{50} was calculated by the probit method.

Potentiation of the effect of chlorprothixene (CPT). Mice (DS) were treated with a combination of test drugs and CPT (2 mg/kg, i.p.) [9]. After 60 min, each animal was placed on its back, and the duration of the loss of the righting reflex was measured. The number of animals remaining in the supine position for more than 3 min was counted, and the ED_{50} was calculated by the probit method.

Anti-pentetrazole (PTZ)-induced convulsion. Mice (ddY) were challenged with 125 mg/kg, s.c., of PTZ at 60 min after dosing. The dose required to prevent convulsions and death in 50% of the animals during observation for 2 hr was calculated by the probit method.

Inclined screen method. A modification of the procedure described by Ueki *et al.* [10] was used. Muscle relaxation was measured 60 min after dosing. The mouse (DS) was placed with its head downwards on a canvas screen, and the angle of the slope was elevated at the rate of 1.2/sec until the animal slipped down the slope. The measurement was done twice per test. The ED_{50} was calculated by regression analysis as the dose needed to decrease the degree of inclination by 10° compared to the saline control.

RESULTS

Diazepam activity in tissue extracts of 450191-*S-treated rats*

As shown in Fig. 1, 450191-S had low affinity for benzodiazepine receptors (K_i = 2500 nM), being about 1/1000 of that of diazepam. However, when 450191-S was administered to rats at a dose of 10 mg/kg, brain extracts of the rats markedly inhibited [^{3}H]diazepam binding to the brain receptors. The

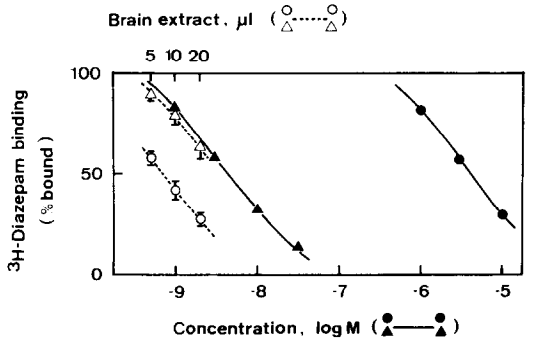

Fig. 1. Inhibition of [^{3}H]diazepam binding by 450191-S (●), diazepam (▲), and brain extracts prepared 15 min after a single dose 10 mg/kg) of 450191-S (○) or diazepam (△). Brain extracts were prepared as described in Materials and Methods.

Table 1. Diazepam activity in tissue extracts of rats after a single i.p. dose (10 mg/kg) of 450191-S*

Tissue	Diazepam activity (units/g tissue)					
	15 min	30 min	60 min	2 hr	6 hr	18 hr
Jejunum	49.38 ± 3.96	36.15 ± 1.78	18.35 ± 0.75	7.83 ± 1.17	1.65 ± 0.56	
Liver	120.34 ± 5.53	107.52 ± 3.63	59.71 ± 1.83	20.57 ± 1.13	3.17 ± 0.79	
Blood	5.20 ± 0.03	6.74 ± 0.24	5.53 ± 0.15	1.65 ± 0.23	0.27 ± 0.09	0.0069 ± 0.0003
Lung	10.94 ± 0.24	13.18 ± 1.81	10.09 ± 1.36	2.09 ± 0.41		
Kidney	33.88 ± 2.56	34.24 ± 2.24	24.89 ± 0.75	18.78 ± 2.62	1.95 ± 0.64	
Brain	2.32 ± 0.11	3.96 ± 0.12	2.91 ± 0.08	0.90 ± 0.14	0.13 ± 0.04	0.0093 ± 0.0005

* Each value represents the mean ± S.E.M. of five rats. One unit of diazepam activity results in the inhibition of [^{3}H]diazepam binding equal to that caused by 1 µg of diazepam.

potency was five times as high as that of rats to which diazepam had been injected at the same dose.

Table 1 gives the diazepam activity in various tissues of rats each of which had received an i.p. injection of 450191-S at a dose of 10 mg/kg. The activity in the liver was the highest throughout the experimental period. The activities in the liver and the jejunum reached the maximum levels 15 min after the administration, while the other tissues showed the maximum activity at 30 min. The activity in the brain was the lowest, being 1/30 to 1/20 of the liver activity, but it was sufficient to explain the CNS activity of 450191-S when compared with the level obtained with diazepam (Fig. 1). After 18 hr, the levels in the brain and the blood decreased to about 0.1% of the maximum, but they were still detectable.

These results strongly suggested that 450191-S was transformed into pharmacologically active benzodiazepines *in vivo* as has been observed with a peptide-aminobenzophenone [5].

Isolation and identification of active metabolites from rat tissues

To identify active metabolites in rat tissues, we first attempted to isolate them, by TLC and HPLC, from the 15-min liver extract, in which we found the highest diazepam activity. As TLC resulted in a broad band in terms of the inhibition of [^{3}H] diazepam binding, the entire activity band was collected, extracted with methanol, concentrated, and applied to HPLC. As shown in Fig. 2, we detected two large peaks (peaks I and II) and two small peaks (peaks III and IV) and expressed as diazepam activity. The fractions of peaks I and II were collected individually, purified again by TLC, and identified by GC/MS as M-3 and M-2 respectively. The retention times of M-2 and M-3 on HPLC were the same as those of peaks II and I respectively. On TLC, R_f values of M-2 and M-3 coincided with those of peaks II (0.71) and I (0.65) respectively. We could not obtain enough of the metabolites forming peaks III and IV for GC/MS analysis.

Next, we examined the 10-min extract of the brain. Peak III was the largest, peak I was the smallest, and peak IV was not detected. The fraction of peak III was collected, purified by TLC, analyzed by GC/MS, and identified as M-1. The chromatographic behaviour of M-1 on HPLC and TLC also was the same as that of peak III. Again peaks I and II were identified as M-3 and M-2 by GC/MS respectively (results not shown).

RRA showed no major peaks other than peaks I, II, and III in all the tissue extracts tested. Although peak IV was detected as a minor peak in some tissues, it may not be behaviorally significant because it was not detected in the brain.

Enzymatic formation of M-1 from 450191-S

Incubation of peptido-aminobenzophenones with rat brain or liver homogenate results in cleavage of the intramolecular peptide bond and subsequent formation of a benzodiazepine [11]. The first step in *in vivo* conversion of 450191-S may similarly be the cleavage of the peptide bond in the molecule. To check this, we incubated 450191-S with a partially purified amino peptidase M and then extracted the reaction mixture with ethanol. The extract strongly inhibited [^{3}H]diazepam binding, and examination of its TLC under an ultraviolet lamp showed a single spot which comigrated with M-1 and had an R_f (0.78) different from that of 450191-S (0.49). The latter was quantitatively converted into the former under the

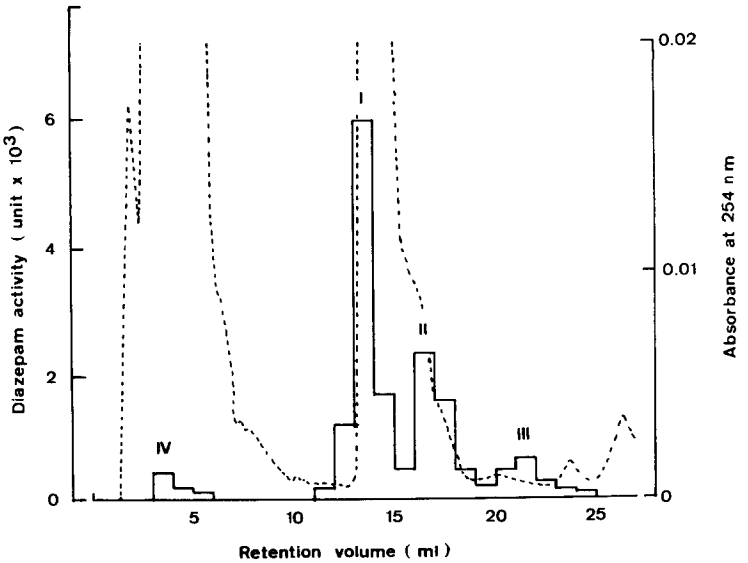

Fig. 2. HPLC-RRA chromatogram of a liver extract prepared 15 min after a single dose (10 mg/kg) of 450191-S. This experiment was performed with 300 μl of a liver extract, prepared as described in Materials and Methods. After TLC, fractions containing receptor-reactive substances were collected, evaporated to dryness under vacuum, and resolved in 50 μl of methanol. A 20-μl portion was subjected to HPLC. A 100-μl sample of each fraction was evaporated in an assay tube for RRA and subjected to RRA Key: (——) diazepam activity, and (----) absorbance at 254 nm.

Table 2. Affinities for benzodiazepine receptors and pharmacological potencies of 450191-S, its active metabolites, and reference drugs

Compound	K_i values for [^{3}H]diazepam binding (nM) (relative affinities)	ED_{50} (mg/kg) (ddY mouse)		Ratio B/A	ED_{50} (mg/kg) (DS mouse)		Ratio D/C
		Anti-PTZ-induced convulsion (A)	Rotating rod test (B)		Potentiation of CPT hypnosis (C)	Inclined screen test (D)	
450191-S	2500 (0.0008)	0.11 (0.06–0.18)	236.9 (166.7–410.3)	2154	0.52 (0.25–0.96)	142.6 (109.4–178.5)	274.2
M-1	2.1 (1.0)	0.05 (0.04–0.07)	67.7 (46.4–104.8)	1354	0.22 (0.13–0.56)	8.7 (7.5–10.1)	39.5
M-2	0.9 (2.3)	0.05 (0.03–0.07)	81.5 (50.7–170.8)	1630	0.33 (0.24–0.46)	9.2 (6.7–12.8)	27.9
M-3	0.9 (2.3)	0.07 (0.05–0.10)	128.9 (82.9–292.4)	1842	0.53 (0.34–0.79)	16.9 (13.0–21.9)	26.8
Diazepam	3.2 (0.7)	0.76 (0.58–0.99)	17.7 (13.4–23.4)	23	3.06 (2.17–4.28)	6.9 (4.9–9.1)	2.3
Nitrazepam	4.4 (0.5)	0.13 (0.10–0.18)	5.3 (3.7–7.3)	41	0.30 (0.19–0.52)	0.8 (0.7–1.0)	2.7

incubation conditions. GC/MS analysis of the methanol extract of the spot revealed that the spot was M-1.

Absence of 450191-*S from tissue extracts* 15 *min after its administration*

When all tissue extracts, obtained 15 min after the administration of 450191-S, were incubated with aminopeptidase M, the ability of the extracts to inhibit [^{3}H]diazepam binding did not increase. Thus, there was practically no 450191-S in the extracts.

Affinities for benzodiazepine receptors and pharmacological potencies of active metabolites

Table 2 lists the K_i values in [^{3}H]diazepam binding and the ED_{50} values in pharmacological tests determined with 450191-S, M-1, M-2, and M-3. Diazepam and nitrazepam were also tested as reference drugs. 450191-S showed very low affinity for the receptors, while all metabolites had 2- or 4-fold higher affinities than that of diazepam or nitrazepam. In experiments using ddY mice, metabolite ED_{50} values for anti-PTZ-induced convulsions were slightly lower than that of the parent compound, which is comparable to that of nitrazepam and seven times lower than that of diazepam. The ED_{50} values of metabolites for the rotating rod test were slightly lower than that of the parent compound but were distinctly higher than those of the reference drugs. Thus, as is evident from the table, the ratios of the ED_{50} values in the rotating rod and the anti-PTZ tests for the metabolites were comparable to that of the parent compound but were 30- to 80-fold larger than those of the reference drugs. In DS mice in which we compared potentiation of CPT hypnosis and the inclined screen test, the activities of metabolites in the former test were comparable to that of the parent compound but, in the latter test, the metabolites were about ten times as active as 450191-S. The ratios of the metabolite ED_{50} values from the CPT hypnosis and the inclined screen test were one order of magnitude larger than that of diazepam or nitrazepam but were about ten times smaller than that of 450191-S.

Time course study

By combining TLC, HPLC, and RRA and using

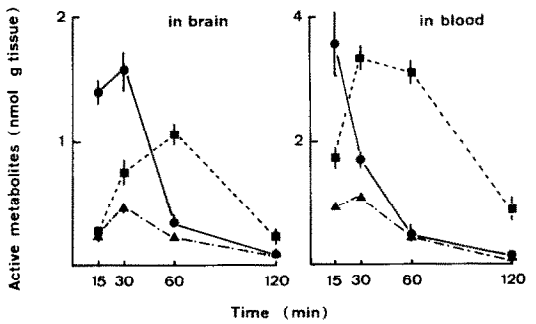

Fig. 3. Levels of M-1 (●), M-2 (▲), and M-3 (■) in brain and blood extracts presented in Table 1. Metabolites were purified by TLC and HPLC and then determined by RRA, as described in Materials and Methods. Values represent the mean ± S.E.M. of five rats.

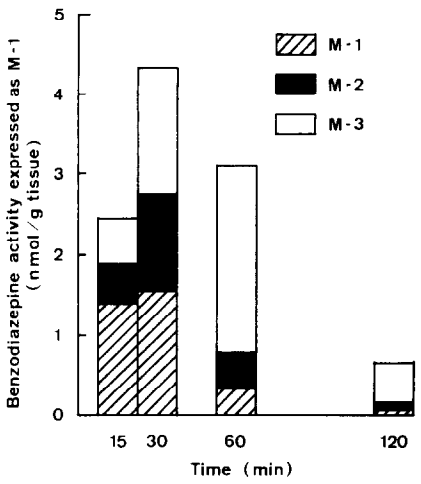

Fig. 4. Contribution of M-1, M-2, and M-3 to pharmacological activity of 450191-S. Benzodiazepine activity of each metabolite was calculated by multiplying the content by the relative affinity to M-1.

the standard curves obtained for M-1, M-2, and M-3, we determined the levels of these metabolites in the brain and blood listed in Table 1 (Fig. 3). In blood, the maximum level of M-1 occurred within 15 min after drug administration. This level decreased rapidly to 46% at 30 min, 13% at 60 min, and 4% at 120 min as compared with that obtained at 15 min. M-2 reached maximum between 15 min and 30 min and then gradually decreased. M-3 reached maximum between 30 min and 60 min, and one fourth of this level was maintained at 120 min. The time courses of metabolite levels in the brain closely resembled those in the blood, but the appearances of maximum levels of the metabolites were delayed by about 15 min.

Figure 3 also shows that the concentration ratio of active metabolites in the brain markedly changed with time. Therefore, it seem pertinent to evaluate which metabolite contributed to the CNS activity of 450191-S at a given moment. Thus, the brain level of each metabolite indicated in the figure was multiplied by its relative affinity (M-1 = 1.0), as shown in Fig. 4. The values obtained express the amounts of M-1 equivalent to the receptor reactivity of each metabolite and probably reflect its CNS activity at the given time. At 15 min after drug administration, more than half of the effect of 450191-S was ascribed to M-1. On the other hand, three metabolites almost equally contributed to the activity at 30 min, and, after 60 min, most of the activity depended upon M-3. The sum of the activities of the three metabolites was roughly equivalent to the level of diazepam activity in the brain (see Table 1).

DISCUSSION

Our previous paper reported that a combination of HPLC and RRA for benzodiazepines is effective in the determination of active metabolites of a peptido-aminobenzophenone [4]. The present study shows that this method is also effective for searching for unknown active metabolites. Thus, our results show that, although 450191-S by itself has a very low affinity for the brain benzodiazepine receptor, it is transformed *in vivo* into at least three active metabolites having even higher affinitiy for the receptor than that of diazepam or nitrazepam.

The experiment with the aminopeptidase suggests that, as was observed with peptido-aminobenzophenone, the first step in transformation *in vivo* of 450191-S is the enzymatic cleavage of an intramolecular peptide bond, with subsequent nonenzymatic ring closure. This gives rise to M-1, and stepwise demethylation of M-1 leads to the formation of M-2 and M-3 (Fig. 5). We confirmed these assumptions by the time course study summarized in Fig. 3.

The above facts, together with the lack of 450191-S in the brain, strongly suggest that the CNS activity of this compound is mainly due to the active metabolites. We therefore compared the potencies in some pharmacological tests of M-1, M-2, and M-3 with that of the parent compound and found that all metabolites mimicked the effect of 450191-S on PTZ-induced convulsions and CPT hypnosis (Table 2).

Figure 3 shows that the increase and decrease of the active metabolites in the brain follows different time courses. We thus decided to evaluate to what extent each metabolite contributes to the CNS activity of 450191-S at a given time after its administration. This was calculated by taking account of the content of each metabolite and its affinity. The result

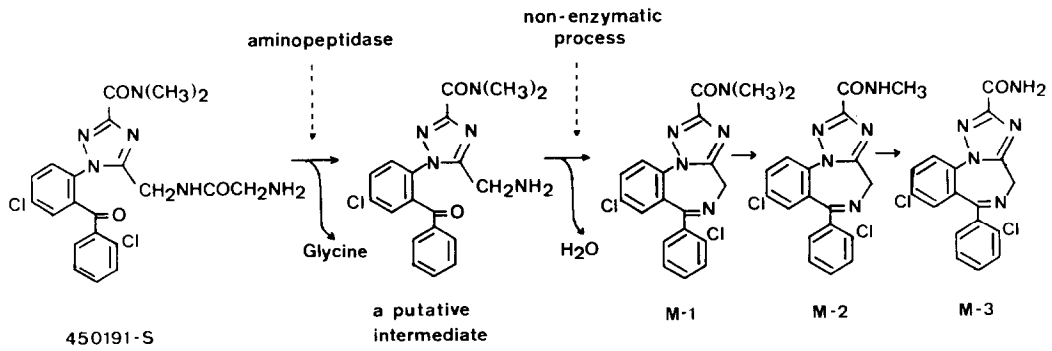

Fig. 5. Postulated metabolic pathway of 450191-S.

shows that, within 30 min after administration, all three metabolites had roles in the CNS activity but, after this, the role of M-3 was the most important. Figure 3 also shows that, although the changes in the levels of each metabolite in the brain and blood paralleled each other, a longer period of time was required for the maximum levels to be reached in the brain. This means that the blood levels of the active metabolites do not necessarily reflect the pharmacological significance in the brain.

With preexisting benzodiazepines or triazolobenzodiazepines, it has been impossible to dissociate the anti-PTZ or hypnotic properties from the ataxia. Thus, it is interesting that the ataxic effect of 450191-S is extremely weak compared with the other two effects.*† The ratios of the ED_{50} values from the anti-PTZ convulsion and rotating rod tests using ddY mice, listed in Table 2, suggested that the extremely weak ataxic effect of 450191-S was due to the extremely weak effect of the active metabolites identified in this experiment. However, the attenuation cannot be fully accounted for by the ratios of the ED_{50} values in the CTP hypnosis and inclined screen tests with DS mice, although the ratios for the metabolites were at least 10-fold higher than those for diazepam and nitrazepam. In addition, we were informed recently (Ken'ichi Yamamoto, personal communication) that, in rhesus monkeys, the ratio of the ED_{50} values for gait disturbance and sleep-inducing effect is several-fold higher with 450191-S than with active metabolites. Finally using cerebellar and hippocampal benzodiazepine receptors [12], we compared the binding profiles of M-1, M-2, and M-3 with those of CL218872 and diazepam. Our results did not support the assumption that the attenuation in the ataxic effect observed with the metabolites is due to their ability to discern so-called subtypes of benzodiazepine receptors [13]. Further work is needed to elucidate the mechanism by which the large attenuation of the ataxic effect takes place with the metabolites and the even greater attenuation that occurs with 450191-S.

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