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Detection and Determination of Pharmacologically Active Benzodiazepines in Rat Brain after the Administration of 2-*o*-Chlorobenzoyl-4-chloro-N-methyl-N′-glycylglycinanilide, using a Combination of High-Pressure Liquid Chromatography and Radioreceptorassay

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In the present work we attempted to detect and quantitate central nervous system (CNS)–active benzodiazepines in rat brain after administration of a peptido-aminobenzophenone. The results can be summarized as follows.

1. Radioreceptorassay (RRA) using ^{3}H-diazepam as the labeled ligand and rat-brain crude synaptosomes was a simple, reliable and sensitive assay method for benzodiazepines.

2. A benzene extract of a rat brain homogenate prepared 15 min after *i.p.* administration of 2-*o*-chlorobenzoyl-4-chloro-N-methyl-N′-glycylglycinanilide (**1**) was subjected to high pressure liquid chromatography (HPLC). By RRA analysis of the eluted fractions, four benzodiazepines having high affinity for benzodiazepine receptors were detected.

3. Changes in the levels of each benzodiazepine in rat brains after *i.p.* administration of **1** were determined by a combination of HPLC and RRA. The levels can account for the CNS activities of **1**. **1** itself could not be detected throughout the experiments.

These results strongly indicate that **1** exerts its CNS activities through conversion into CNS–active benzodiazepines *in vivo*.

Keywords——2-*o*-chlorobenzoyl-4-chloro-N-methyl-N′-glycylglycinanilide; benzodiazepine; high pressure liquid chromatography (HPLC); radioreceptorassay (RRA); HPLC-RRA; the benzodiazepine levels in rat brains

In an accompanying paper,[2)] we report that peptido-aminobenzophenones, which are ring-opened derivatives of benzodiazepines and exhibit pronounced central nervous system (CNS) activities in experimental animals,[3)] have low affinity for benzodiazepine receptors themselves but are converted into compounds having higher affinity by incubation with crude synaptosomes from rat brains or rat liver homogenates. We inferred that peptido-aminobenzophenones exert their CNS activities through *in vivo* conversion into compounds having the benzodiazepine ring. In the present work, we report the detection and quantitation of CNS-active benzodiazepines in rat brain after *i.p.* injection of a peptido-aminobenzophenone, 2-*o*-chlorobenzoyl-4-chloro-N-methyl-N′-glycylglycinanilide (**1**). We also report that the combination of high pressure liquid chromatography (HPLC) and radioreceptor assay (RRA) is useful for the separation and determination of nanogram or smaller quantities of pharmacologically active benzodiazepines in the brain.

Experimental

Materials——All materials were obtained from the sources described in the preceding paper.[2)]

Preparation of the Crude Synaptosomal Fraction——Crude synaptosomes from rat brains were prepared as described in the preceding paper, except that the 30000×***g*** pellet was suspended in five times the original

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2) M. Fujimoto, Y. Tsukinoki, H. Hirose, K. Hirai, and T. Okabayashi, *Chem. Pharm. Bull.*, **28**, 1374 (1980).
3) K. Hirai, T. Ishiba, H. Sugimoto, K. Sasakura, T. Fujishita, Y. Tsukinoki, and K. Hirose, *Chem. Pharm. Bull.*, **26**, 1947 (1978).

tissue weight of 50 mM Tris HCl buffer (pH 7.4), and portions were stored at −70°. The frozen suspension was thawed and diluted 1: 10 in the same buffer prior to use for RRA. Binding activity to ^{3}H-diazepam was stable for at least 3 months.

Preparation of Brain Extract——**1** was injected into male Wistar rats (body weight 180—200 g) at a dose of 10 mg/kg, and whole forebrains were removed at various times after the administration.

Two methods of extracting benzodiazepines were used.

1. Benzene Extraction: Unless otherwise indicated, this extraction method was used. A brain was homogenized in 20 ml of benzene plus 20 ml of H_2O. The homogenate was shaken for 20 min and then centrifuged at $1500 \times g$ for 20 min. A 10 ml portion of the benzene layer was evaporated to dryness under a vacuum, dissolved in 2 ml of methanol plus 0.4 ml of H_2O, and centrifuged again for 20 min at $10000 \times g$ at 0°. The supernatant was evaporated to dryness and dissolved in 100 μl of methanol.

For the recovery experiment, benzene–H_2O homogenates of brains were prepared from rats which had not received the drug. Known amounts of 7-chloro-1,3-dihydro-1-methyl-5-(2′-chlorophenyl)-2H-1,4-benzodiazepin-2-one (**2**), 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2H-1,4-benzodiazepin-2-one (**3**), 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-(2′-chlorophenyl)-2H-1,4-benzodiazepin-2-one (**4**), and lorazepam (**5**) (Fig. 1) were added, and after the extraction, the amounts were determined by a combination of HPLC and RRA (see below). The recoveries (percent) of **2**, **3**, **4**, and **5** were 106.2±8.6, 90.4±4.9, 80.0±6.0, and 58.2±5.2, respectively ($n=15$). These values were used to correct the values determined in the experiment presented in Fig. 6.

2. Ethanol Extraction: A brain was homogenized in nine volumes of ethanol, centrifuged at $30000 \times g$ for 20 min, and the supernatant was evaporated to dryness under a vacuum. The residue was suspended in an appropriate volume of 10% ethanol and used in experiments to detect **1** in brain extracts. By this procedure **1**, **2**, **3**, **4**, and **5** were recovered quantitatively in the ethanol extracts.

HPLC——HPLC of benzodiazepines was performed by the method of Konaka and Kuruma.[4] 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 and Co.). The solvent used for the mobile phase was a mixture of methanol–water (60: 40 by volume) 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 30 sec. Each fraction was evaporated to dryness under a vacuum and redissolved in 100 μl of a 10% methanol aqueous solution, then 10 μl portions were subjected to RRA.

For rechromatography of peak II in Fig. 2, fractions of the peak were combined, evaporated to dryness, and redissolved in 100 μl of methanol, then a 20 μl portion was charged on the column preequilibrated with methanol–water (45: 55 by volume). The column was flushed with the same solvent at a flow rate of 1.5 ml/min, and the eluate fractions were processed as described above.

RRA——RRA was performed in a manner similar to that used for determining the affinity of drugs for benzodiazepine receptors[2] with slight modifications. Briefly, 10 μl of standard solution or unknown sample

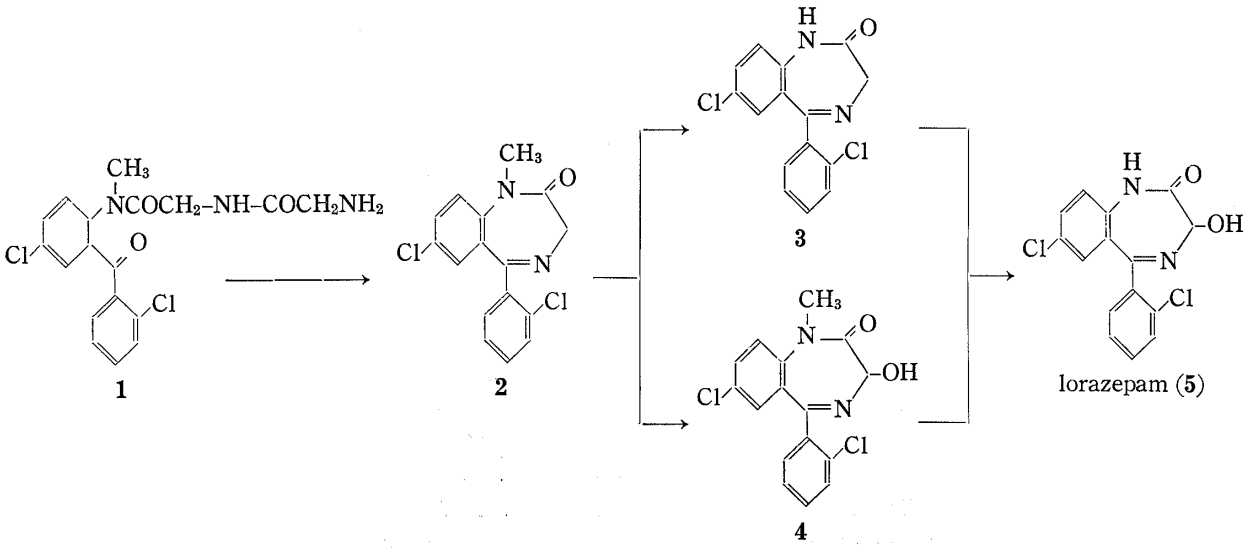

Fig. 1. Postulated Metabolic Pathways of **1** in the Rat

5) R. Konaka and K. Kuruma, manuscript, in preparation.

was incubated in an ice bath for 30 min with 525 μl of P_2-crude synaptosomal suspension in 50 mM Tris-HCl (pH 7.4) containing 2 nM ^{3}H-diazepam. After filtration through a Whatman GF/C glass fiber filter and subsequent washing, the filter was counted for tritium. The amounts of **2**, **3**, **4**, and **5** in the HPLC peaks (Figs. 2 and 3) were calculated from the standard curve prepared for each benzodiazepine.

Thin-Layer Chromatography (TLC)——An appropriate volume of an ethanol extract or an HPLC eluate was spotted on a silica gel plate and developed with ethyl acetate–*n*-hexane–ethanol (4: 2: 0.5). The chromatogram was cut into 0.5 cm pieces, and each piece was extracted with methanol. Portions of the extract were determined for inhibition potency in ^{3}H-diazepam binding.

Combined Gas Chromatography-Mass Spectrometry (GC-MS)——Mass spectra were taken on a Varian MAT 44S GC/MS system equipped with split-splitless injector at an ionizing voltage of 75 eV. The column was a glass capillary (Varian), 20 m × 0.25 mm I.D., coated with SE-54. The column oven temperature was programmed from 125° (1 min) to 250° at 25°/min. The inlet pressure was 10.0 psi. The temperatures of the injector, separator, and ion source were kept at 251°, 247°, and 175°, respectively.

Results

Detection of Pharmacologically Active Benzodiazepines in Rat Brain after Administration of 1

1 was administrated to a rat. After 15 min, the rat was killed and the whole forebrain

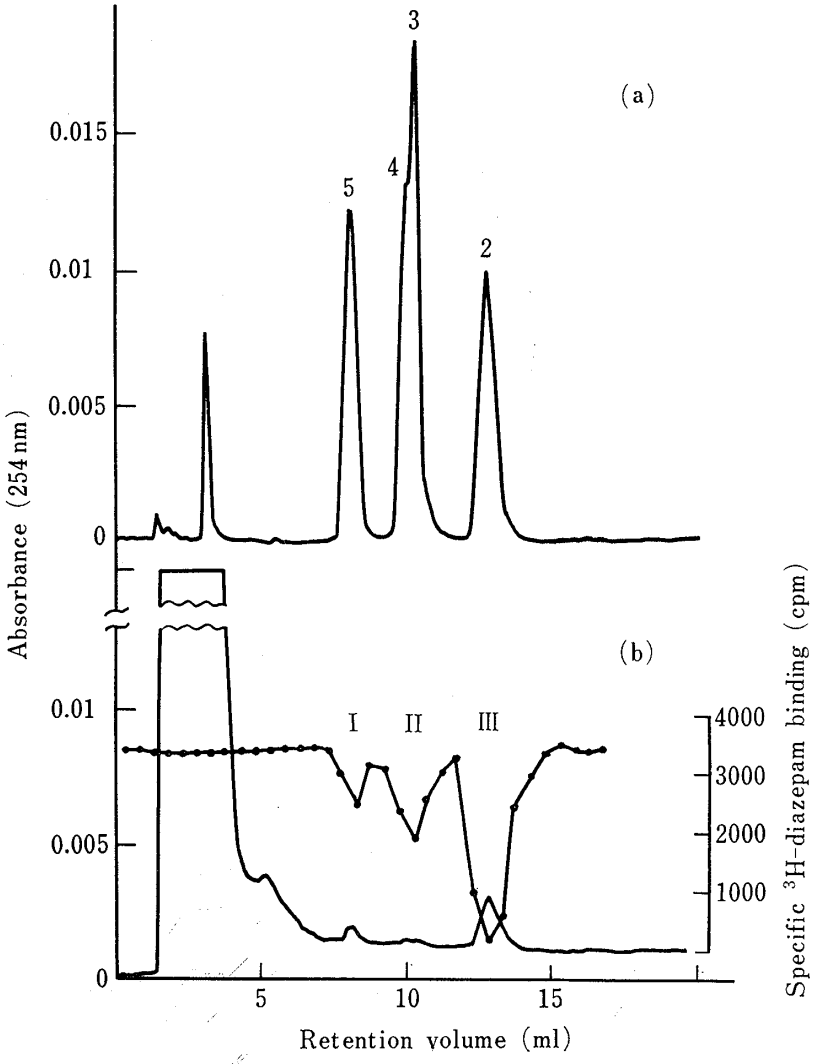

Fig. 2. HPLC Elution Profile of Rat Brain Extract

1 was administered to a rat and the brain extract was subjected to HPLC. Chromatographic conditions are described in the text [eluant, methanol–water (60: 40)].

(a) Separation of known amounts of authentic **2**, **3**, **4**, and **5**. (b) Separation of an extract of rat brain.

————, optical density at 254 nm; ——●——, ^{3}H-diazepam binding.

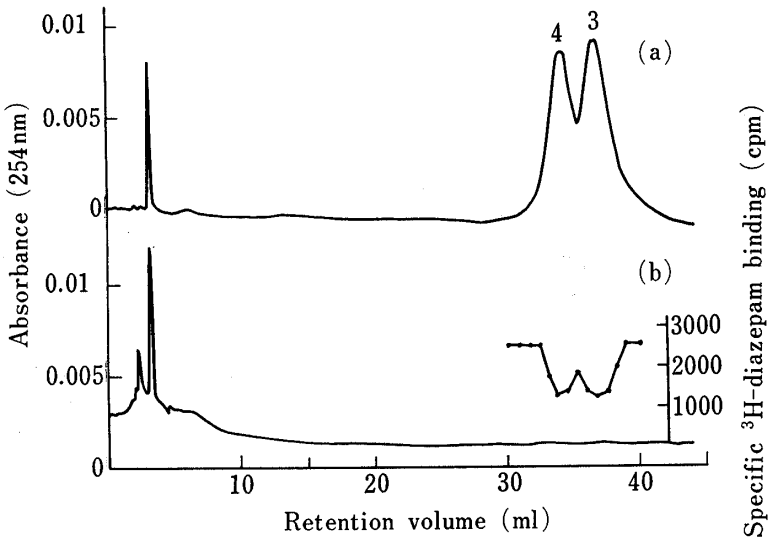

Fig. 3. Rechromatography of Peak II in Fig. 2

Chromatographic conditions are described in the text [(eluant, methanol–water (45: 55)].
(a) Reference elution profile of **3** and **4**. (b) Elution profile of peak II in Fig. 2.
———, optical density at 254 nm; —●—, ³H-diazepam binding.

was extracted with benzene–H_2O then subjected to HPLC using 60% methanol as an eluent (see "Experimental"). As indicated in Fig. 2b, one small peak and two barely visible peaks were found, judging from the optical density of the eluate at 254 μm. However, we could detect three distinct peaks in terms of the reduction of ³H-diazepam binding determined by RRA. Comparison of the retention times with those of reference compounds (Fig. 2a) indicated that peaks I and III were **5** and **2**, respectively. Rechromatography of peak II with methanol–H_2O (45: 55) resolved two more peaks, whose retention times were the same as those of **4** and **3** (Fig. 3).

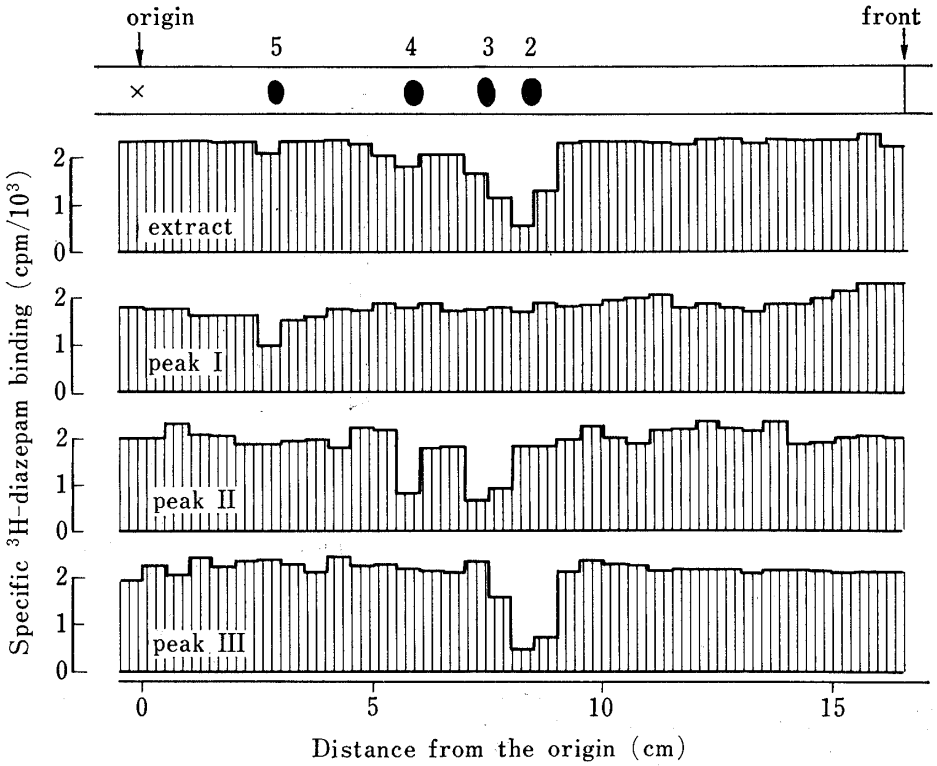

Fig. 4. TLC of the Ethanol Extract and HPLC Eluates

Identification of 2, 3, 4, and 5

On TLC, substances which inhibit ^{3}H-diazepam bindingin peaks I and III in Fig. 2 moved the same distances as **5** and **2**, respectively (*Rf*'s are 0.19 and 0.53, Fig. 4). The chromatography of peak II gave two substances, the mobilities of which (*Rf*'s 0.37 and 0.48) coincided with those of **4** and **3**, respectively. The figure also shows that the results obtained with an unfractionated ethanol extract are consistent with those obtained with peaks I, II, and III.

We also analyzed the HPLC eluates by GC–MS. **5**, **2**, and **3** and **4** were detected in peaks I, III, and II, respectively (results not shown).

RRA for Benzodiazepines

Fig. 5a shows standard curves obtained for **2**, **3**, **4**, and **5** (lorazepam). Each curve could be converted into a straight line over a fairly wide range of benzodiazepine concentrations after log–logit transformation (Fig. 5b). Statistical analyses were done with **2**. The maximum sensitivity was 0.04 ng. An extract of the whole forebrain of a rat which had received **1** was prepared and portions were subjected to HPLC for **2** in ten consecutive assays. The interassay coefficient of variation was 4.1%. The intraassay coefficient of variation for 10 aliquots from the same sample determined in a single assay was 3.7%.

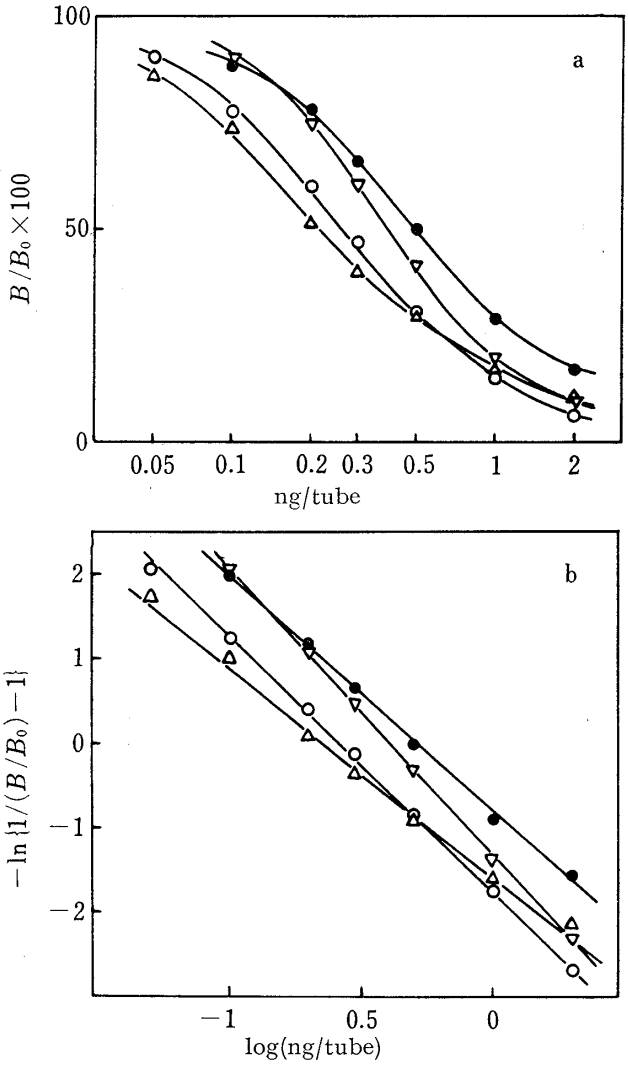

Fig. 5. Standard RRA Curves for **2**, **3**, **4**, and **5**

(a) B/B_0 is plotted versus log concentration of each benzodiazepine, where B_0 and B denote specific ^{3}H-diazepam binding determined in the absence or presence of designated amounts of each benzodiazepine, respectively. (b) Log-logit plot.
—○—, **2**; —△—, **3**; —▽—, **4**; —●—, **5**.

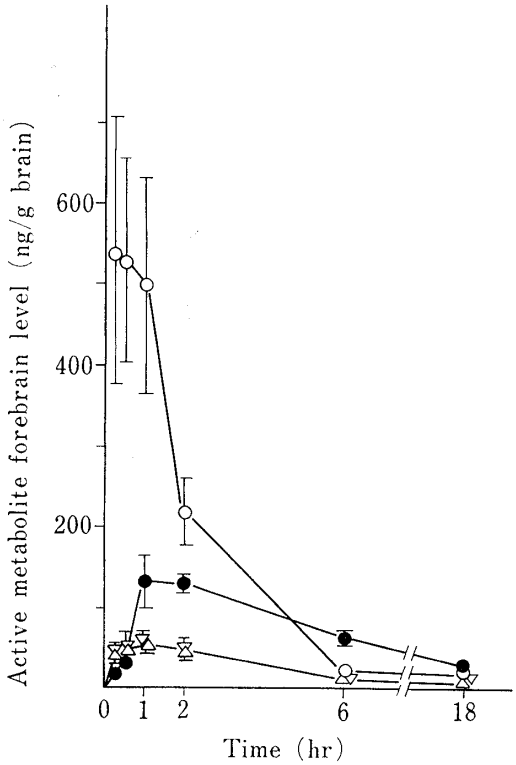

Fig. 6. Forebrain Levels of Pharmacologically Active Benzodiazepines after Administration of **1**

—○—, **2**; —▽—, **3**; —△—, **4**; —●—, **5**. The levels of **3** and **4** were roughly estimated after rechromatography of peak II. Values represent the mean ± S.E. of four rats.

Practically no substance which interfered with the assay was found in samples purified by HPLC. The assay was also fairly insensitive to interfering substances in the ethanol extract. However, the presence of high concentrations of the extract in the assay mixture (20 mg as original tissue wet weight per tube) gave slightly but significantly higher assay values than in their absence. When such samples were to be analyzed, assay blanks had to be set aside with the same amounts of ethanol extract obtained from a rat which had not received the drug (results not shown).

Levels of Pharmacologically Active Benzodiazepines in Rat Brains after Administration of 1

1 was administrated to rats and the levels of benzodiazepines in whole forebrains were determined at designated times by the combination of HPLC and RRA (Fig. 6). A maximum level of **2** was obtained within 15 min after the administration. This level was maintained for approximately 1 hr then decreased rapidly, and after 2 and 6 hr the level had decreased to 40 and 5% of the maximum, respectively. **3** and **4** reached the maximum between 30 and 60 min then decreased gradually. Appearance of a maximum level of **5** was delayed by about 30 min in comparison with those of **3** and **4**. Fairly high levels of **5** (50% of the maximum level, about 0.2 nmol/g brain) were maintained even 6 hr after the administration.

Correlation between the Affinity of Drugs for the Benzodiazepine Receptor and Their Pharmacological Potencies

Table I lists K_i values in ^{3}H-diazepam binding and ED_{50} values in pharmacological tests determined with **2**, **3**, and **5**, and several benzodiazepines which are currently in clinical use. Statistical analysis showed high correlations between K_is and ED_{50}s in antipentylenetetrazol ($r=0.828$, $p<0.01$, Fig. 7a) and taming tests ($r=0.868$, $p<0.01$, Fig. 7d). Cat muscle relaxant, which reportedly has the highest correlation with binding data,[5] showed the

Table I. Affinity for Benzodiazepine Receptors and Pharmacological Potency of Benzodiazepines

	Inhibition of specific ^{3}H-diazepam binding	Anti-pentylenetetrazol induced convulsion in mice	Potentiation of chlorprothixene hypnosis in mice	Taming of mice	Muscle relaxant action in cats
	K_i value (nM)[a]		ED_{50} (mg/kg)[b]		
Benzodiazepine					
Triazolam	1.12	0.04	0.38	0.47	0.08
Diazepam	3.23	1.19	9.1	6.0	6.0
Nitrazepam	4.39	0.23	1.96	2.1	3.73
Flurazepam·HCl	4.60	2.26	17.5	3.6	1.65
Estazolam	4.77	0.72	7.20	2.61	2.53
Bromazepam	13.1	—	—	—	—
Prazepam	76.7	—	—	—	—
Chlordiazepoxide	216	4.90	44.9	27.0	8.0
Medazepam	485	4.76	45.5	10.7	16.0
2	0.88	0.25	0.6	0.73	2.0
3	0.73	0.19	0.57	0.23	2.0
4	1.49	—	—	—	—
Lorazepam (**5**)	1.66	0.25	0.45	0.76	0.5

a) K_i values were determined as described in the preceding paper.[2]
b) The compounds were tested orally in mice or cats for the pharmacological effects according to previously described procedures.[6-9]

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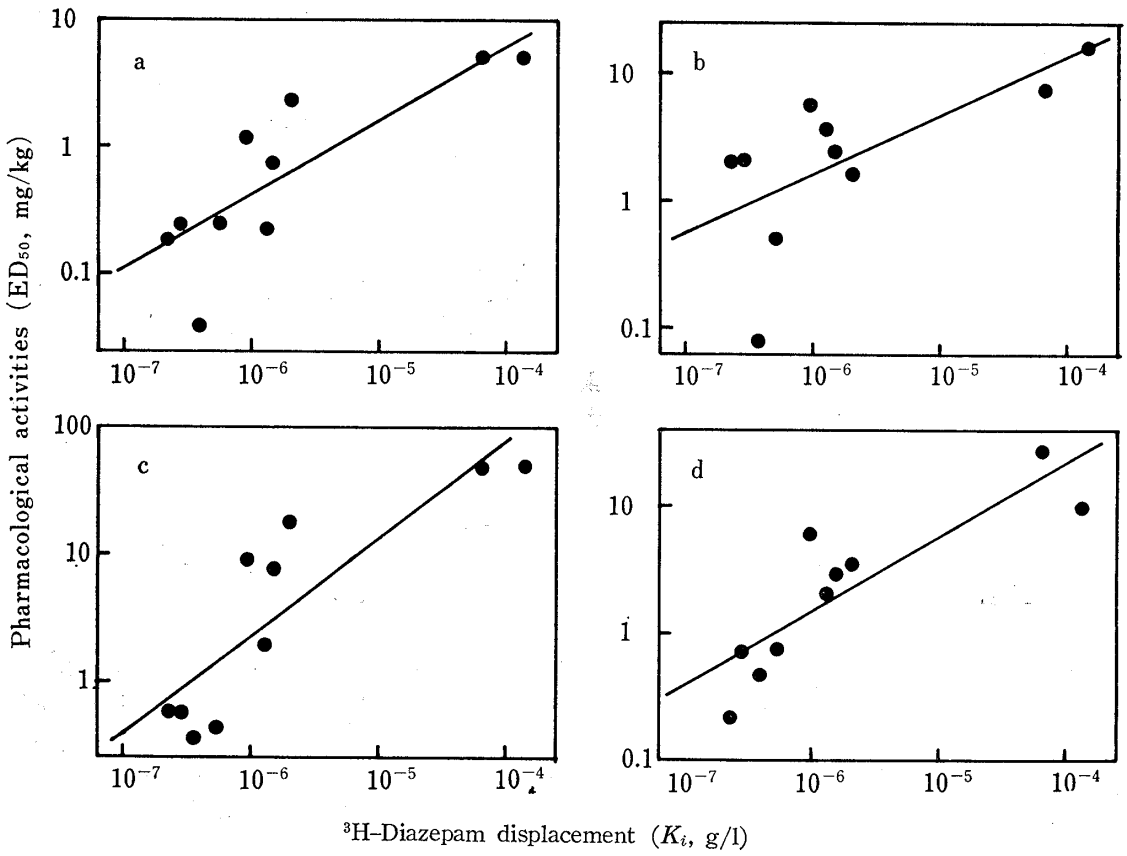

Fig. 7. Correlation between K_i Values of Drugs in Benzodiazepine Receptor Binding and Their Pharmacological Potencies

The plots show the correlation between K_is in ^{3}H-diazepam binding and *a*) anti-pentyleneterazol convulsions in mice (ED_{50}, mg/kg *p.o.*); *b*) cat muscle relaxant effect (ED_{50}, mg/kg *p.o.*); *c*) the intensifying effect on chlorprothixene hypnosis (ED_{50}, mg/kg *p.o.*); and *d*) taming of mice (ED_{50}, mg/kg *p.o.*). The correlation coefficients are given in the text.

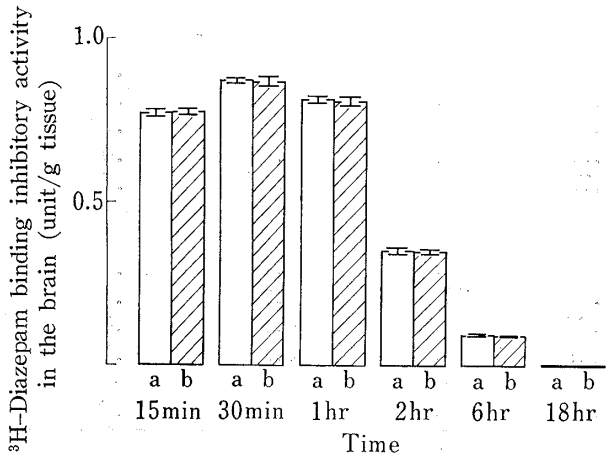

Fig. 8. Effect of Preincubation of Brain Extracts of **1**-Treated Rats with Crude Synaptosomes on ^{3}H-Diazepam Binding

In the experiment in Fig. 6, the brain of a rat was set aside at each time point. The brains were extracted with ethanol and the inhibition of ^{3}H-diazepam binding was determined before (a) and after (b) preincubation with brain crude synaptosomes at 37° as described in an accompanying paper.[2] The inhibition potency was calculated using the standrd curve for **2** (see *Fig. 5*). One unit in the ordinate is equivalent to 1 μg of **2**. Values given are means±S.E. of 5 replicates.

lowest correlation in the present experiment ($r=0.658$, $p<0.05$, Fig. 7b). In contrast, ED_{50} values in the chlorprothixene test in mice, which have not previously been examined for correlation with binding data, showed the highest correlation with K_i values ($r=0.861$, $p<0.01$, Fig. 7c).

Failure to Detect 1 in Rat Brain

As was reported in the preceding paper,[2] **1** has a low affinity for benzodiazepine receptors itself, but the incubation of **1** with crude synaptosomes from rat brain at 37° results in the formation of compounds having high affinity for the receptors. Therefore, if the brain contained **1**, the value determined after incubation of brain extract with the synaptosomes should be larger than that determined without incubation.

In the experiment indicated in Fig. 6, the brains of some rats were extracted with ethanol and the extracts were prein-

cubated with the synaptosomes at 37° for 30 min. No significant difference was found between the values determined before and after the incubation throughout the experimental period (Fig. 8).

Discussion

Several analytical methods for benzodiazepines have been reported,[10–13] and among them the electron-capture gas chromatographic (EC–GC) method and radioimmunoassay (RIA) can measure nanogram quantities of the drugs.[11,12]

Practically no detailed description exists on the receptor binding of ^{3}H-diazepam as a tool for quantitative assay of benzodiazepines, although Chang and Snyder[14] determined benzodiazepines in rat brains by RRA. The present results indicate that RRA is a convenient and reliable assay method having high sensitivity and reproducibility. The method is superior to EC–GC and RIA in several respects: 1) RRA is simpler in procedure than EC–GC, 2) RRA is more sensitive than EC–GC and existing RIA, 3) the combination of RRA and HPLC allows separate determination of several pharmacologically active benzodiazepines in the brain, which is probably impossible with RIA, where the cross-reactivity of various benzodiazepines with the antibodies is not correlated with the CNS activities of the drugs.

From the data presented in Fig. 6, **1** is likely to be converted *in vivo via* the sequences depicted in Fig. 1. As suggested in the preceding paper,[2] enzymatic cleavage of a peptide bond of **1** and subsequent nonenzymatic ring closure may result in the formation of **2**. **2** might be further converted into **3** and **4** by either N_1-demethylation or oxidation at the C_3 position. As the route which leads to the formation of **5** is not clear at the moment, we tentatively show both **3** and **4** as being converted into **5**. The postulated metabolic pathways from **2** to **5** are analogous to those from diazepam to oxazepam.[15]

1 could not be detected in brains throughout the experiments. In experiments similar to those presented in Fig. 8, we tried to detect **1** in the ethanol extracts of jejunum, liver, lung, kidney, and blood. **1** could not be detected from 5 to 60 min after the administration of **1**, except in the jejunum, where small but significant amounts of **1** could be detected at 5 and 8 min, but not after 15 min (results not shown). This result suggests that the *in vivo* conversion of **1** to compounds having a benzodiazepine ring is a rapid process, and it is likely that **1** is converted into **2**, **3**, **4**, and **5** before reaching the brain. In any case, **1** seems to act as a prodrug of benzodiazepines.

The close correlations between receptor-binding data and pharmacological data (Table I and Fig. 7) suggest that the benzodiazepine levels in brains determined by the present method at a certain moment may provide a direct indication of the pharmacological activity of the administered drug at that moment. Since the pharmacological activity of **1** lasts for about 6 hr,[16] the sum of active benzodiazepines at 6 hr after administration (about 0.4 nmol/g brain, Fig. 6) may be the level required for CNS activity. This level is one or two orders of magnitude higher than *in vitro* K_i values of benzodiazepines determined at 37°, at which the K_i values are 2 to 20 times higher than those determined at 0°.[17] However, this level coincides fairly

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well with the *in vivo* K_i values of benzodiazepines determined by Chang and Snyder,[14] and supports their view that a large percentage of benzodiazepines in the brain is unavailable for binding.

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