Radioiodinated 2′-Iododiazepam: A Potential Imaging Agent for SPECT Investigations of Benzodiazepine Receptors

Hideo Saji, Yasuhiko Iida, Iwao Nakatsuka, Masaki Kataoka, Kazuhiko Ariyoshi, Yasuhiro Magata, Akira Yoshitake and Akira Yokoyama

Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan and Environmental Health Science Laboratory, Sumitomo Chemical Company Ltd., Osaka, Japan

2′-Iododiazepam (2′-IDZ) is the diazepam analogue iodinated at the 2′-position of C-5 phenyl ring which was synthesized and evaluated as a potential radiopharmaceutical for investigating brain benzodiazepine receptors by SPECT. The ^{125}I-2′-iododiazepam was synthesized by halogen exchange reaction and purified by HPLC. In vitro competitive binding studies with ^{3}H-diazepam, using rat cortical synaptosomal membranes, showed that the affinity of 2′-IDZ for benzodiazepam receptors was higher than that in diazepam and flumazenil (RO15-1788). Biodistribution studies in mice showed that the brain uptake of 2′-iododiazepam was rapid and profound, and in the brain higher accumulation was found in the cortex than in other regions. Furthermore, the cortical uptake was displaced by benzodiazepinergic compounds. In vivo uptake was assessed by autoradiographic studies. Thus, 2′-iododiazepam bound to benzodiazepine receptors in vivo and therefore holds great potential for in vivo benzodiazepine receptor studies.

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Benzodiazepines induce various pharmacological effects which are thought to be mediated via benzodiazepine receptors in the central nervous system (*1,2*). Alterations in the density of benzodiazepine receptors have recently been reported in various disorders, such as epilepsy (*3,4*), Huntington's disease (*5,6*), hepatic encephalopathy (*7*) and Alzheimer's disease (*8*). Visualization of benzodiazepine receptors in human brain has thus been of great interest, and several ^{11}C-, ^{75}Br-, and ^{123}I-labeled benzodiazepines have been developed for this purpose (*9–16*). The successful imaging of benzodiazepine receptors with these radiolabeled compounds and the superior radiation properties of ^{123}I for SPECT prompted us to synthesize a radioiodinated benzodiazepine analogue with high receptor affinity.

In the 1,4-benzodiazepine molecule, structure-activity relationships have shown that substitution with electron-withdrawing groups such as halogens at position 7 and 2′ increases receptor affinity (*2,17,18*). Iodination at position 7 yields 7-iodo-flunitrazepam, and the synthesis and biodistribution of this agent have been recently reported (*13*).

In this study, a diazepam derivative iodinated at the 2′ position, 2′-iododiazepam (2′-IDZ), was synthesized and the in vitro receptor binding affinity and biodistribution in mice, including regional cerebral distribution, were studied. The chemical structures of 2′-IDZ and related compounds are shown in Figure 1.

MATERIALS AND METHODS

Sodium ^{125}I-iodide (specific activity 81.4 TBq/mmole) and ^{3}H-diazepam (3.15 TBq/mmole) were purchased from Amersham International Plc and Du Pont New England Research Products, respectively. Diazepam and flumazenil (RO15-1788) were a generous gift from F. Hoffmann-La Roche (Basel, Switzerland). Fludiazepam was supplied by Sumitomo Chemical Co. Ltd. (Osaka, Japan). Male Wistar rats and ddY mice were supplied by Japan SLC Co. Ltd. (Hamamatsu, Japan).

Radiolabeling

The radioiodination of 2′-IDZ was accomplished by halogen exchange reaction with sodium ^{125}I-iodide. Eighty-five micrograms of 2′-bromodiazepam (BDZ) was dissolved in 107 μl dimethylformamide (DMF). This solution was added to a mixture of sodium ^{125}I-iodide (10 μl, 37 MBq), nonradioactive sodium iodide (0.8 μg in 0.25 μl 0.1 N NaOH), 1-naphthalenesulfonic acid dihydrate (0.26 mg in 5 μl DMF) and copper (II) sulfate pentahydrate (0.26 mg in 5 μl DMF) in a sealed vial. The reaction mixture was heated for 1.5 hr at 100–105°C. After cooling, 400 μl of 10% aqueous sodium carbonate solution was added to the reaction mixture and the product was extracted with chloroform (2 × 0.5 ml). The combined organic layers were evaporated under a stream of nitrogen. The residue was dissolved in 100–150 μl of methanol, applied to a reverse-phase, high-performance liquid chromatography (HPLC) column (Lichrosorb RP-18, 7.5 × 300 mm), and eluted with methanol:water:4-m*M* phosphate buffer (pH 7.4) (11:5:4) at a flow rate of 1.5 ml/min (R_t = 58 min for 2′-BDZ, R_t = 62 min for 2′-IDZ). The fraction corresponding to 2′-IDZ was collected, evaporated to remove the residual organic solvent and sterilized by filtration through a 0.22 μm Milex filter.

The radiochemical purity of the product was determined by

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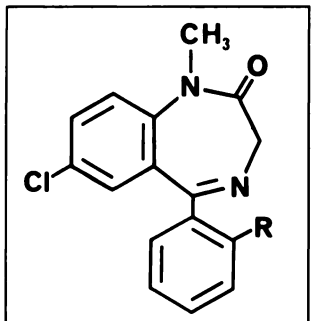
For correspondence or reprints contact: Akira Yokoyama, PhD, Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-01, Japan.



Compound	R
Diazepam	H
Fludiazepam	F
2'-IDZ	I

FIGURE 1. Chemical structures of 2'-IDZ and related compounds.

thin-layer chromatography (TLC) and analytical HPLC. The TLC was performed on a silica gel plate with a chloroform:acetone (4:1) solvent (R_f = 0.62–0.68). HPLC was performed on a 7.5 × 300 mm Lichrosorb RP-18 column eluted with methanol:water:4-m*M* phosphate buffer (pH 7.4) (11:5:4) at a flow rate of 1.5 ml/min (R_t = 62 min).

The nonradioactive 2'-IDZ and 2'-BDZ were synthesized from 2-iodo- or 2-bromobenzoic acid by addition of N-(4-chlorophenyl)-N-methylethylenediamine, followed by cyclization and oxidation, according to the method previously reported for the corresponding fluoro analogue, fludiazepam (*19*). The structures were confirmed by infrared analysis (IR), proton nuclear magnetic resonance (NMR) and mass spectrometric analysis. The details of the synthesis and characterization of these compounds will be published elsewhere.

In Vitro Benzodiazepine Receptor Binding Studies

The affinity of 2'-IDZ for benzodiazepine receptors was measured by displacement of ^{3}H-diazepam from a preparation of synaptosomal membranes, according to the method of Möhler and Okada (*20*). In brief, the cerebral cortex from male Wistar rats (150–200 g) was homogenized in 20 volumes of ice-cold 0.32 *M* sucrose using a Teflon-glass homogeneizer. The homogenate was centrifuged at 1,000x g for 10 min at 4°C. The supernatant was recentrifuged at 20,000x g for 20 min at 4°C. The pellet was resuspended in ice-cold 50 m*M* Krebs-Tris buffer (50 m*M* Tris-HCl buffer (pH 7.4), 118 m*M* NaCl, 4.8 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$) with polytron homogenizer, and diluted with the Krebs-Tris buffer to yield a synaptosomal membrane suspension with a protein concentration of 1.0 mg protein/ml.

The binding assays were performed by incubating 1 ml of the synaptosomal membrane suspension with ^{3}H-diazepam (0.6 n*M*) and different concentrations of competitors in 1 ml of Krebs-Tris buffer. Incubations were performed for 15 min at 4°C, and the samples were rapidly filtered through a Whatman GF/B filter and washed twice with 5 ml of ice-cold assay buffer. The filter was placed in a 20-ml scintillation vial containing 10 ml of ACS II (Amersham). The radioactivity bound to the filter was measured with a liquid scintillation counter (LS 500TA, Beckman). All incubations were performed in triplicate. Nonspecific binding was determined in the presence of 1 μ*M* of diazepam. IC_{50} values were determined from the displacement curves of percent inhibition of ^{3}H-diazepam binding versus inhibitor concentration and were the average of three to five experiments.

Measurement of the Octanol/Water Partition Coefficient

The partition coefficient for 2'-IDZ was determined according to a previously reported method (*21*). A 20-μl aliquot of radioiodinated sample was mixed with 3 ml each of 1-octanol and 0.1 *M* phosphate buffer (pH 7.4) in a test tube. The tube was vortexed (3x 1 min), incubated for 1 hr at room temperature, and then centrifuged for 5 min. The 500-μl aliquots of each phase were removed and counted in a well-type NaI scintillation counter.

Biodistribution Studies

Male ddY mice weighing about 30 g were administered ^{125}I-2'-IDZ (18.5 kBq in 0.1 ml ethanolic saline solution) through the tail vein. At the designated times after the injections, the mice were killed by decapitation and their organs were removed. For in vivo brain distribution studies, the brains were dissected on an ice-cold plate according to the method of Glowinski and Iversen (*22*). All samples were weighed, and the radioactivity was counted using a well-type NaI scintillation counter. Results were presented as the %dose/g tissue weight.

The relative binding affinity of ^{125}I-2'-IDZ for the benzodiazepine receptor was determined by using known agonists and antagonists with high affinity, which were injected into mice along with 18.5 kBq of ^{125}I-2'-IDZ. Flumazenil (1.0 mg/kg) was injected simultaneously with the radioligand, but diazepam (10 mg/kg) and flunitrazepam (10 mg/kg) were injected intraperitoneally 30 min before administration of the radioligand. The animals were sacrificed 1, 5, 20 and 60 min after radioligand administration, brain regions were dissected, and tissues were counted as described above. The effect of sodium thiopental on ^{125}I-2'-IDZ uptake was also studied. Mice were injected intraperitoneally with sodium thiopental (100 mg/kg) 10 min before radioligand administration and treated in the same manner as described above.

To demonstrate receptor saturation, 18.5 kBq of ^{125}I-2'-IDZ was coinjected with various doses of cold 2'-IDZ, ranging from 0.01 to 1.0 mg/kg. The animals were killed 5 min after injection and the brains were dissected and counted as described above.

Metabolism

Mice weighing about 30 g were injected intravenously with 55.5 kBq of ^{125}I-2'-IDZ. At designated times afterward, the mice were decapitated, and the brains were removed immediately and homogenized in 1 ml of methanol. After centrifugation, the precipitate was washed twice with 1 ml of methanol, and the washes were combined with the supernatant. The combined methanol extracts were evaporated, and the resulting residue was redissolved in a small volume of methanol and analyzed by HPLC on a Lichrosorb RP-18 column (7.5 × 300 mm) with methanol:water: 4-m*M* phosphate buffer (pH 7.4) (11:5:4) at a flow rate of 1.5 ml/min.

Autoradiographic Studies

Two groups of rats weighing about 250 g were used. One group was injected with 1.11 MBq of ^{125}I-2'-IDZ and the other group received the same dose of ^{125}I-2'-IDZ, but was preinjected with 1.0 mg/kg of flumazenil. At 5 min after injection of the radioligand, the animals were decapitated, and the brains were quickly removed, frozen and cut into 20-μm-thick sections using a cryomicrotome. The sections were placed on x-ray film (MARG-^{3}H type, Sakura, Japan) and exposed for 1 wk.

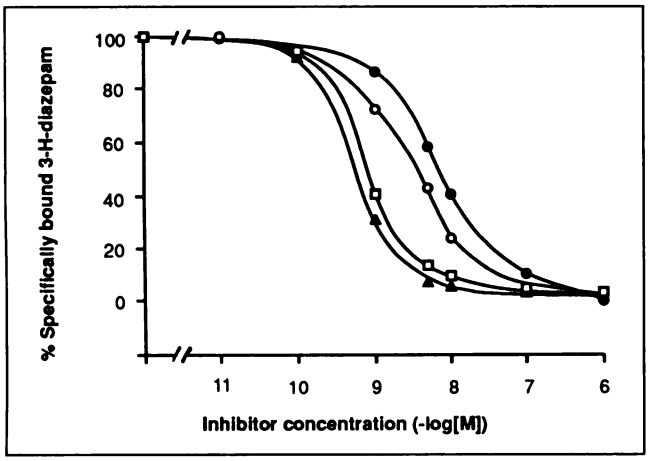


FIGURE 2. Inhibition of ^{3}H-diazepam binding to rat brain synaptosomal membranes by diazepam (●), fludiazepam (○), flumazenil (□) and 2′-IDZ (▲). Values shown are the mean of three to five independent experiments.

TABLE 1
IC_{50} Values of Benzodiazepines in Inhibiting ^{3}H-Diazepam Binding to Benzodiazepine Receptors on Synaptosomal Membranes from Rat Cortex*

Compound	IC_{50} (n*M*)
Diazepam	6.70
Fludiazepam	3.36
2′-IDZ	0.74
Flumazenil	1.05

*Each value represents the mean of three to five independent experiments.

RESULTS

In Vitro Binding

Using the reference compounds, diazepam, fludiazepam and flumazenil, the affinity of 2′-IDZ for brain benzodiazepine receptors was measured by competition for ^{3}H-diazepam binding sites in rat cortical membranes. Figure 2 illustrates competitive binding curves representative of these compounds. The IC_{50} values were determined from the curves and are summarized in Table 1. All compounds tested competed with ^{3}H-diazepam for binding in the synaptosomal membranes, and the binding affinity order from highest to lowest was 2′-IDZ, flumazenil, fludiazepam and diazepam. Thus, among the compounds evaluated, 2′-IDZ showed the highest affinity, which was approximately nine times that of diazepam, five times that of fludiazepam and slightly higher than flumazenil.

Radiolabeling

Iodine-125-2′-IDZ was prepared by a iodide-bromide exchange reaction. The desired labeled product, ^{125}I-2′-IDZ, was effectively separated from the 2′-BDZ starting material, by reverse-phase HPLC. The total radiochemical yield, after HPLC purification, was approximately 50%. The radiochemical purity of the product was greater than 98%, and the specific activity was 6.66 – 8.51 TBq/mmole, estimated by the uv absorbance at 284 nm.

Lipophilicity

The lipophilicity of 2′-IDZ was assessed by octanol-phosphate buffer (pH 7.4) extraction. The partition coefficient of 2′-IDZ was higher than that of diazepam (log P; 2′-IDZ: 3.2, diazepam: 2.1).

Biodistribution in Mice

Iodine-125-2′-IDZ rapidly entered the brain and a high uptake of radioactivity was observed during the early phase, but declined with time (Table 2). The radioactivity in blood was cleared rapidly, and the highest brain-to-blood ratio of 3.7 was obtained at 5 min after injection. High initial uptake was also observed in the kidneys, lungs and heart, but the radioactivity in these organs cleared rapidly. The liver showed a rapid increase in radioactivity, reaching a maximum at 5 min postinjection, which gradually decreased with time. Uptake in the stomach was low.

The regional distribution of the radioactivity in the mouse brain is shown in Table 3. Differences in the regional distribution of radioactivity were observed: i.e., the

TABLE 2
Biodistribution of ^{125}I-2′-IDZ in Mice

Organ	Time after injection (min)			
	1	5	20	60
Blood	2.27 ± 0.27*	1.50 ± 0.02	1.64 ± 0.03	1.89 ± 0.10
Intestine	2.67 ± 0.30	3.02 ± 0.21	3.98 ± 0.13	6.67 ± 0.72
Liver	4.20 ± 0.75	11.00 ± 0.29	8.82 ± 0.40	8.96 ± 0.43
Kidney	11.98 ± 1.27	7.46 ± 0.45	6.15 ± 0.33	5.81 ± 0.36
Stomach	1.53 ± 0.27	1.66 ± 0.21	2.02 ± 0.34	6.66 ± 2.14
Lung	8.88 ± 2.12	4.84 ± 0.83	3.06 ± 0.24	2.68 ± 0.22
Heart	10.50 ± 0.37	4.95 ± 0.37	4.51 ± 0.31	3.68 ± 0.45
Brain	6.21 ± 1.06	5.53 ± 0.63	3.47 ± 0.29	2.78 ± 0.49
Br/Bl	2.74 ± 0.57	3.69 ± 0.42	2.11 ± 0.18	1.47 ± 0.27

*Each value is the mean ± s.d. for four animals (%dose/g organ).
Br/Bl = brain-to-blood ratio.

TABLE 3
Regional Cerebral Distribution of ^{125}I-2′-IDZ in Mice*

Region	Time after injection (min)			
	1	5	20	60
Cortex	6.95 ± 1.31	6.27 ± 0.66	3.72 ± 0.49	2.92 ± 0.57
Striatum	5.73 ± 1.03	4.75 ± 0.47	2.97 ± 0.43	2.53 ± 0.46
Hippocampus	5.01 ± 0.88	4.97 ± 0.84	3.45 ± 0.47	2.63 ± 0.65
Cerebellum	5.83 ± 0.93	5.09 ± 0.59	3.21 ± 0.24	2.63 ± 0.48

*Each value is the mean ± s.d. for 6–8 animals (%dose/g tissue).

cortex showed higher uptake than other regions. The regional distribution parallels the distribution of benzodiazepine receptors from an in vitro study (*23*) and from PET (*16,24–27*). Furthermore, the effects of various drugs on the cortical uptake of ^{125}I-2′-IDZ was studied. As shown in Figure 3, the administration of diazepam, fludiazepam and flumazenil reduced uptake of radioactivity in the cortex. However, the administration of sodium thiopental did not influence cortical uptake, but caused a delay in the clearance of the radioligand. All tested compounds showed no effect on the radioactivity in the blood.

Autoradiographic studies in the rat brain were in agreement with the results of the regional brain distribution in mice described above. Figure 4A shows the regional cerebral distribution of ^{125}I-2′-IDZ in rat brain sections at 5 min postinjection. The high accumulation of radioactivity was observed in the cerebral cortex. Figure 4B shows the effect of flumazenil on radioligand brain distribution. Treatment with flumazenil reduced the accumulation of radioactivity in regions of high uptake in the untreated experiment and resulted in nearly identical levels of radioactivity in all brain regions.

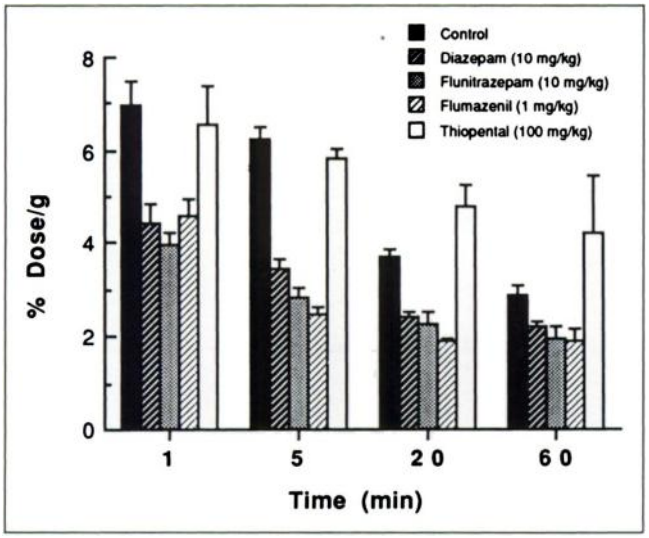


FIGURE 3. Effect of various drugs on the cortical uptake of ^{125}I-2′-IDZ in mice. Iodine-125-2′-IDZ was intravenously injected 30 min after the administration of diazepam and flunitrazepam 10 min after administration of sodium thiopental or simultaneously with flumazenil. Results are expressed in %dose/g of tissue ± s.e.m. for four to eight animals.

Furthermore, the effects of the carrier on brain uptake of ^{125}I-2′-IDZ was investigated using various doses of cold ligand (0.01 to 1 mg/kg). As shown in Figure 5, the uptake of radioactivity in the cerebral cortex decreased in a dose-dependent manner. The radioactivity level in the blood was not significantly different at any dose level.

Metabolism

Analysis of brain homogenates was carried out at various intervals after injection of ^{125}I-2′-IDZ. Approximately 95% of the radioactivity in the homogenate could be extracted by methanol at each time of analysis. Analysis of the methanol extracted fraction was performed by HPLC. The results indicated that there were four metabolites which eluted faster from the reverse-phase column (fraction A: 8 min, B: 33 min, C: 42 min, D: 44 min, 2′-IDZ: 62 min). The time-course of relative distribution of each metabolite is summarized in Table 4. The results are expressed relative to the total radioactivity of the methanol-extracted fraction at each time of analysis. A high percentage (94%–98%) remained as 2′-IDZ by 5 min, but afterward its relative contribution to total brain radioactivity declined with time. The radioactive contents in fraction C and D increased steeply after 20 min, and fraction B was observed at 60 min after injection. Fraction A was a hy-

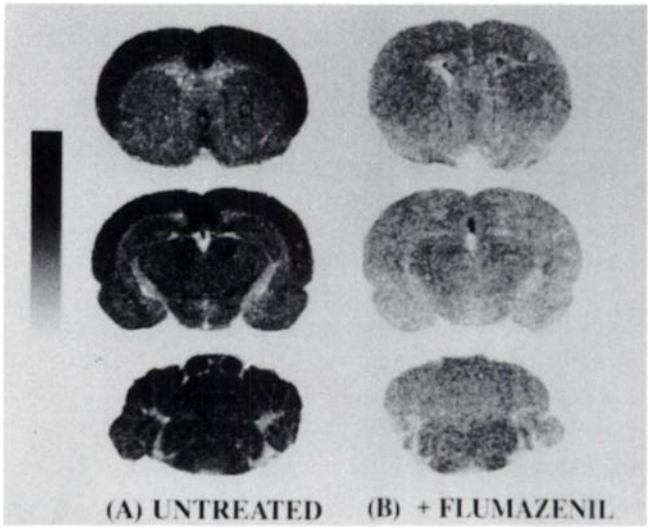


FIGURE 4. In vivo autoradiography of rat coronal brain sections at 5 min after injection of ^{125}I-2′-IDZ. (A) ^{125}I-2′-IDZ alone and (B) flumazenil (1 mg/kg) administered simultaneously with ^{125}I-2′-IDZ.

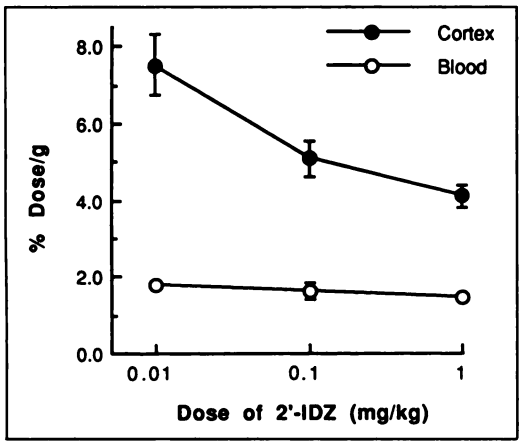


FIGURE 5. Effect of doses of unlabeled 2'-IDZ on cortical and blood uptake of ^{125}I-2'-IDZ in mice. Mice were coinjected with ^{125}I-2'-IDZ and various doses of unlabeled 2'-IDZ and were killed 5 min after injection. ● = cortex; ○ = blood. Each point is the mean ± s.d. of four animals.

drophilic metabolite including free iodide, which accounted for less than 3% throughout the experiment. The retention time of fraction D corresponded with N-desmethyl 2'-IDZ (2'-iodonordiazepam).

DISCUSSION

The basic requirements for the effective use of radioligands for in vivo studies of brain benzodiazepine receptors include high affinity to the receptors and rapid and quantitatively significant brain uptake following peripheral administration (*28–30*).

Based on structure-activity data for 1,4-benzodiazepines, the 2' position of C-5 phenyl ring appeared to be the most attractive position for iodination (*2,17,18*). The in vitro receptor binding studies showed that 2'-IDZ, a diazepam derivative with iodine at the 2' position, did indeed show higher receptor affinity than diazepam, the parent compound. Furthermore, 2'-IDZ had about 1.5 times higher affinity than flumazenil, which was reported to be a useful ligand for imaging studies of benzodiazepine receptors (*3,7,11,16,24–27*). Thus, iodination at the 2' position provided marked increase in affinity for the benzodiazepine receptor, and the results suggest that the 2' position is a good site for iodination of 1,4-benzodiazepines.

In vivo biodistribution studies with ^{125}I-2'-IDZ showed rapid and high accumulation in the brain. The uptake was in accordance with the high octanol-to-phosphate buffer partition coefficient.

The studies on regional brain distribution showed that 2'-IDZ had higher uptake in the cortex than any other brain region evaluated. This regional distribution confirms the known distribution of benzodiazepine receptors (*16,23–27*). In addition, the administration of benzodiazepinergic compounds reduced the cortical uptake of ^{125}I-2'-IDZ. The cortical uptake of ^{125}I-2'-IDZ was also found to decrease with increasing doses of cold ligand. These results indicate that 2'-IDZ binds to benzodiazepine receptors in the brain following intravenous injection. Autoradiographic studies were consistent with the results.

In the displacement studies, however, a significant amount of the radioactivity in the cortex remained when compared to radiolabeled flumazenil (*24,25,31,32*). A similar phenomenon was noted with flunitrazepam, methylclonazepam, and RO16-0154 binding in benzodiazepine receptors (*15,31,32*). Although the cause of this finding is not certain, it may be due to the presence of high capacity binding sites, nonspecific binding or reoccupation, as mentioned for RO16-0154 (*15*). The treatment with sodium thiopental slowed the clearance of cortical radioactivity. Slow clearance of brain radioactivity by treatment with barbiturates was reported for ^{123}I-RO16-0154 (*33*). Barbiturates have been shown to act allosterically to enhance the affinity of agonists for benzodiazepine receptors (*23,34*). Barbiturates also tend to lower body temperature (*33*), and these actions may be related to the effect of sodium thiopental.

The HPLC analysis of brain homogenates after injection of ^{125}I-2'-IDZ showed that there were three main metabolites. The 1,4-benzodiazepines are known to be extensively metabolized to a number of pharmacologically active metabolites (*35–38*). If 2'-IDZ is metabolized in a manner similar to diazepam (*35–37*), the three possible metabolites of 2'-IDZ would include the N-desmethyl analogue, the 3-hydroxy analogue and the 3-hydroxylated N-desmethyl analogue, which correspond to fractions D, C and B, respectively, as judged by the retention times on HPLC.

TABLE 4
Relative Distribution of Radioactivity in the Methanol Extractable Fraction of the Brain After Intravenous Injection of ^{125}I-2'-IDZ in Mice*

	Time (min)			
	1	5	20	60
Fraction A	0.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 0.5
Fraction B	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.6	15.8 ± 1.8
Fraction C	0.0 ± 0.0	2.5 ± 1.0	18.0 ± 3.0	25.5 ± 5.7
Fraction D	0.8 ± 1.3	3.2 ± 1.2	26.6 ± 3.0	31.4 ± 5.6
2'-IDZ	97.6 ± 8.2	94.4 ± 1.4	54.6 ± 5.4	24.7 ± 6.6

*Each value represents the percentage of the total counts in the methanol extractable fraction as the mean ± s.d. for four animals.

Further studies are required to confirm the metabolism of 2′-IDZ.

In conclusion, the results obtained in this study indicate that 2′-IDZ, iodinated at 2′ position of C-5 phenyl ring of diazepam, shows high affinity for benzodiazepine receptors. The ^{123}I-2′-IDZ analogue is thus a potential radioligand for use in SPECT investigations of brain benzodiazepine receptors in humans.

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