J. Pharm. Pharmac., 1972, **24**, 773–780 *Received January* 31, 1972

Localization of [^{3}H]pimozide in the rat brain in relation to its anti-amphetamine potency*

W. SOUDIJN AND I. VAN WIJNGAARDEN

Department of Medicinal Chemistry, Janssen Pharmaceutica, B-2340 Beerse, Belgium

The pharmacological activity of the neuroleptic agent pimozide is well correlated with the concentration of [^{3}H]pimozide in the brain of the Wistar rat. There is an enhanced uptake and retention of [^{3}H]pimozide in the caudate nucleus, an area in which amphetamine and apomorphine act. Its concentration in the caudate nucleus is not correlated with its anti-apomorphine and anti-amphetamine potency. There are, however, significant shifts in the subcellular distribution of [^{3}H]pimozide in the caudate nucleus, from the mitochondrial fraction to the fraction consisting mainly of submicroscopic nerve endings. These shifts correlate well with its pharmacological activity. This points to the small nerve-endings as being the site of action of pimozide. The drug does not influence either the penetration of (+)-[^{14}C]amphetamine into the brain, or its metabolism, indicating that the anti-amphetamine action cannot be due to a decreased penetration of amphetamine into the brain, although a subcellular redistribution of amphetamine induced by pimozide cannot be excluded.

Pimozide, a long-acting potent and orally active neuroleptic drug (Janssen, Niemegeers & others, 1968a), exerts its clinically desirable neuroleptic or anti-psychotic action probably by producing synaptic blockade of the dopaminergic neurons in the midbrain, particularly in the nigrostriatal system.

The anti-dopamine properties of pimozide are clearly demonstrated in the anti-apomorphine test (apomorphine is a fixed-ring dopamine agonist; Ernst, 1967; Ungerstedt, Butcher & others, 1969), by the counteraction of the apomorphine-induced vomiting in dogs, by blocking apomorphine-induced stereotype behaviour in rats, and in the anti-amphetamine test (amphetamine provokes the mobilization of dopamine; Randrup & Munkvad, 1970) by the prevention of amphetamine-induced stereotype behaviour in rats (Janssen & others, 1968a).

The increase of dopamine turnover in mouse and rat brain after pretreatment with pimozide has been explained as a result of the blockade of dopamine receptor sites (Anden, Butcher & others, 1970; Nybäck, Schubert & Sedvall, 1970; Persson, 1970).

From the regional distribution pattern of [^{3}H]pimozide in the dog brain at the moment of peak-effect in the anti-apomorphine test, it appears that the chemoreceptor-trigger zone of Borison and Wang (vomiting centre) and the caudate nucleus, areas rich in dopamine containing neurons, are regions of a selective uptake and retention of pimozide (Janssen, Soudijn & others, 1968b). We set out to find if a similar uptake and retention mechanism is involved in the rat brain using the anti-amphetamine test as a guide for doses of pimozide and time of death.

*Dedicated to Professor Dr. H. Veldstra on the occasion of his retirement from the Chair of Biochemistry of the University of Leiden.

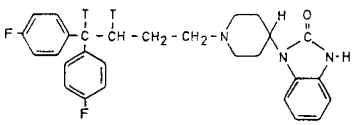
To decide whether the protection of rats against the action of amphetamine by pimozide is due to a decrease of permeability of the brain tissue for amphetamine or to an increase in the metabolism of amphetamine resulting in a decrease of available drug, the distribution of amphetamine in the rat brain was studied in rats pretreated with pimozide and in untreated animals.

The subcellular localization of pimozide and of amphetamine in the caudate nucleus and their possible mutual interference was studied.

MATERIALS AND METHODS

[^3H]*Pimozide*

The synthesis of pimozide specifically labelled with tritium has been described (Soudijn & van Wijngaarden, 1968; Janssen, & others, 1968b). The site of the label (T) is shown in I. The stability was checked and demonstrated by the estimation of unaltered [^{3}H]pimozide by inverse isotope dilution of a boiled solution of [^{3}H]pimozide in 0·1 M tartaric acid (0·31 mg [^{3}H]pimozide/ml, 30 min boiling).



I

Pimozide. Experimental groups of 5 male adult Wistar rats (≈250 g) were kept in metabolic cages at 21° and permitted free access to food and water.

A 0·016, 0·031 or 0·063 mg/ml solution of specifically labelled [^{3}H]pimozide (specific activity 690 mCi/mM) in 0·1 M tartaric acid was injected subcutaneously at doses of 0·16, 0·31 or 0·63 mg/kg.

Animals were decapitated exactly 1, 2, 4, 8, 16 and 32 h after the injection, the brain rapidly removed and the caudate nucleus dissected. Brain parts were pooled and kept at −20° until analysed.

Total radioactivity. The brain parts were weighed after thawing and extracted in the presence of 50 μg of carrier pimozide with 70% ethanol by homogenizing the tissue with an Ultra-Turrax TP 18/2 disintegrator at 20 000 rev/min. The homogenate was centrifuged at 3500 rev/min. The supernatant was decanted and the extraction procedure was twice repeated (no more carrier-pimozide was added). A 1 ml aliquot of the combined supernatants was pipetted into a counting vial containing 0·5 ml of carrier pimozide solution in methanol, made alkaline with NH_4OH (10 mg pimozide per ml). The contents of the counting vials were dried at 75° and the residue was dissolved in 10 ml of scintillator solution (4 g BBOT/litre *p*-xylene-*m*-xylene-abs. ethanol 6:3:1). Quenching correction was applied by internal standardization.

Residual radioactivity of the sediments was estimated by using the solubilization technique of Mahin & Lofberg (1966).

Estimation of pimozide. After the estimation of the total radioactivity, the remainder of the extracts of the caudate nucleus (about 15 ml) and 10 ml of the extract of the remainder of the brain (about 170 ml) were transferred to 50 ml Erlenmeyer flasks containing 50 mg of unlabelled pimozide. By adding methanol or absolute ethanol and chloroform an opalescent solution was obtained. The solutions were evaporated to dryness in a current of nitrogen by warming.

The residues were boiled with 5 ml of isopropanol and filtered while hot through a small cotton plug. Erlenmeyer flasks and filters were rinsed with 2 ml of boiling isopropanol.

The filtrates were concentrated to 2 ml under a current of nitrogen and boiled again to clear the solution from which on cooling in ice and scratching, pimozide crystals were isolated and washed with cold isopropanol. The specific activity in d/min per mg was estimated from a small dried portion and the remaining pimozide was recrystallized until the specific activity was constant.

The pimozide content is calculated from $x = (s\ m/b) \times 100$ in which x = % pimozide, s = specific activity of the isolated pimozide in d/min per mg, m = weight of the added pimozide in mg, b = total radioactivity of the used extract in d/min.

(+)-*Amphetamine.* A 2·5 mg/ml solution of (+)-[7-^{14}C]amphetamine sulphate (specific activity 0·8 mCi/mM) was injected intravenously at 5 mg/kg (=3·67 mg amphetamine base/kg) into adult male Wistar rats of ≈250 g.

The animals were kept separately in observation boxes at 21° (5 animals per experiment) and their behaviour observed. Exactly 1, 2 or 4 h after the injection the animals were decapitated and the brain dissected and analysed for total radioactivity and amphetamine content as for pimozide.

Radioactivity estimations. Aliquots of 200 μl of the brain extracts were counted in 10 ml scintillator solution (4 g BBOT per litre of toluene–isopropanol 7:3).

Estimation of amphetamine. 100 mg of unlabelled (±)-amphetamine sulphate was dissolved by warming in e.g. 10 ml of brain extract. The solution was acidified with 2–3 drops of dilute H_2SO_4 and evaporated to a small volume under a current of nitrogen.

Water was added and the suspension was boiled, cooled, filtered through a cotton plug, made alkaline with solid K_2CO_3 and extracted twice with chloroform. The chloroform extracts were dried on $MgSO_4$, filtered, acidified with solid cyclohexylsulphamic acid and evaporated to dryness under nitrogen. The residue was dissolved on warming in 3 ml of acetone. On cooling, the amphetamine-cyclohexylsulphamate crystallized in leaflets which were isolated by filtration through a coarse sintered glass filter (G. 2) and washed with cold acetone.

After two recrystallizations from acetone:isopropanol(≈4:1), a constant specific radioactivity which did not change by further recrystallization from isopropanol, was obtained.

Gradient-centrifugation. The caudate nuclei of 5 male Wistar rats were dissected 1, 4 or 16 h after pretreatment with [^{3}H]pimozide (0·31 mg/kg, s.c.) and 2 h after (+)-[^{14}C]amphetamine sulphate (5 mg/kg, i.v.).

The caudate nuclei (total weight approximately 175 mg) were pooled in a Potter tube containing 2 ml of 0·25 M sucrose (Mann Research Lab. N.Y. ultra pure) cooled in ice.

All further manipulations were made at ⩽4°. With a Teflon pestle (l = 50 mm, d = 12·5 mm, clearance =0·25 mm) the tissue was homogenized at 950 rev/min for 15 s and centrifuged in a Sorvall centrifuge, type SS-1 at 1000 *g* for 5 min. 0·7 ml of the supernatant was layered on a discontinuous sucrose gradient. The gradient was prepared in a 5 ml polyallomer tube (Beckmann no. 326819 size 1/2″ dia × 2″) by carefully layering of sucrose solutions (M); 1·0 ml 1·25, 0·5 ml 1·20, 0·5 ml 1·15, 0·5 ml 1·10, 1·0 ml 1·00 and 0·5 ml 0·75 and equilibration of the gradient for 1 h at 4° before use.

After centrifugation for 1 h at 3° using a swing-out rotor (SW 65 L) at 120 000 *g* (Beckmann L2-65B class G), the identical layers of 2 tubes were combined after

isolation by aspiration and 0·2 ml was assayed for radioactivity in 0·8 ml water +10 ml Instagel (Packard).

The amount of [^{3}H]pimozide or of [^{14}C]amphetamine present in the combined layers was estimated by inverse isotope dilution.

The subcellular distribution of the labelled drugs added to cooled isolated caudate nuclei of untreated rats just before homogenization, served as a control.

The drug concentration in the control caudate nuclei was about the same as that in the caudate nuclei of treated rats.

The subcellular fractions of the gradient were identified by electron-microscopy. The layers were isolated and transferred to 5 ml polyallomer centrifugation tubes. After addition of 3·5 ml of a solution of 250 g glutaraldehyde, 21·4 g sodium cacodylate and 70 g sucrose in 750 ml distilled water, the tubes were stored for 4 h at +4°. After centrifugation at 100 000 *g* and +3° for 1 h, the pellets were fixated in osmium tetraoxide. Ultrathin sections were obtained with an L.K.B. ultratome and stained on the grids with uranylacetate and lead citrate.

RESULTS AND DISCUSSION

The concentration of unaltered pimozide in the *total brain* reflects the activity of pimozide in the amphetamine test (Table 1, Fig. 1).

Between 2 and 8 h after administration of [^{3}H]pimozide (0·31 mg/kg), at the period when all the rats are protected against the action of amphetamine, a nearly constant level of about 15 pg of pimozide per mg wet brain tissue is maintained. One and 16 h after injection, when pimozide has little protective action, the concentration in the brain is below 15 pg/mg tissue.

At other time intervals and doses, at the moment that pimozide fails to protect the animals (e.g. 0·16 mg/kg at 1 h; 0·63 mg/kg at 32 h), the concentration in the brain is well below that at the moment of the lowest ED 100 (Table 1).

Up to 4 h after administration, about 80–90% of the total radioactivity in the brain is due to pimozide (Table 1). After 4 h the percentage of penetrating metabolites increases: at 8 h there is 63% of unaltered drug, at 16 h 45% and after 32 h 19%.

So the total radioactivity (pimozide plus metabolites) in *the total brain* does not correlate with the anti-amphetamine activity, whereas the amount of unaltered pimozide does so.

The caudate nucleus of the Wistar rat is an area of enhanced uptake and retention of [^{3}H]pimozide (Table 1). This is in agreement with results from dogs (Janssen, & others, 1968b).

The concentration of pimozide in the caudate nucleus is higher than that in the remainder of the brain and is not correlated with its pharmacological action (Table 1, Fig. 1); e.g. 16 h after administration of 0·31 mg/kg and 32 h after 0·63 mg/kg, although the pimozide concentration in the caudate nucleus is comparable to that at the moment of peak effect (2 h), its action is virtually nil. That this may be due to a subcellular redistribution of [^{3}H]pimozide from sites of action to sites of loss was confirmed by subcellular distribution experiments.

Fig. 2A shows that 1 h after subcutaneous injection of [^{3}H]pimozide (0·31 mg/kg), when the animals are relatively unprotected against the action of amphetamine, the distribution pattern of [^{3}H]pimozide is similar to the distribution pattern of [^{3}H]-pimozide added to the isolated caudate nuclei of untreated rats just before homogenization.

Table 1. *Distribution of [³H]pimozide in the brain of the Wistar rat after administration of [³H]pimozide; 0·16, 0·31, or 0·63 mg/kg s.c.*

Time (h)		Amphetamine* Apomorphine test 0·16 mg	0·31 mg	0·63 mg	Distribution of pimozide in Caudate nucleus (0·31 mg dose) a	b	c	Brain (0·31 mg dose) a	b	c	Caudate nucleus (0·16 mg dose) a	b	c	Brain (0·16 mg dose) a	b	c
1	Am.	0	3	9	11	10	90	9	8	87	6	5	89	4	4	85
	Ap.	0	0	6	11	7	69	7	6	82	10	5	47	4	3	85
								9	7	81				4	4	89
2	Am.	4	10	10	22	19	88	15	13	90						
	Ap.	1	9	10	28	23	82	18	15	81						
					26	22	86	18	17	94						
4	Am.	7	10	10	35	32	90	20	16	82						
	Ap.	3	10	10	36	28	78	19	14	77						
					36	33	91									
											(0·63 mg dose)			(0·63 mg dose)		
8	Am.	5	9	10	45	29	64	18	11	61	66	51	77	44	30	68
	Ap.	0	7	10	49	35	72	23	15	64	71	56	79	44	31	70
											76	57	75	47	31	65
											66	50	76	47	27	57
16	Am.	1	2	5	37	22	60	21	10	45						
	Ap.	0	0	0	43	26	60	20	9	46						
32	Am.	0	0	0	24	11	44	16	3	19	50	19	37	35	6	17
	Ap.	0	0	0												

a = pg radioactive material per mg of wet tissue.
b = pg [³H]pimozide per mg of wet tissue.
c = % [³H]pimozide in a.
* Amphetamine test, apomorphine test: number of protected rats in a group of 10 rats treated with a standard dose of (+)-amphetamine (5 mg, i.v.) or apomorphine (1·25 mg, i.v.). Detailed description of the test: Janssen & others, 1967, 1968b.
Each figure represents a group of five animals (pooled organs) in 1–4 experiments e.g. 0·63 mg/kg 8 h: 4 groups of 5 rats, 32 h: 1 group of 5 rats etc.

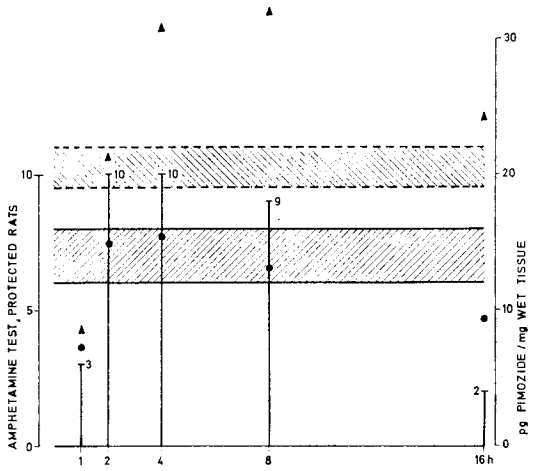


FIG. 1. Concentration of [³H]pimozide in brain and caudate nucleus of the Wistar rat in relation to its anti-amphetamine activity. Vertical lines show number of rats in a group of 10 protected against amphetamine-induced stereotype behaviour.
The limits of the minimal concentration of [³H]pimozide (0·31 mg/kg s.c.) in the caudate nucleus (▲) or in the brain (●) necessary to evoke maximal effect are shown by cross hatching between broken and solid lines respectively.

The highest percentage of the total radioactivity was found in fraction 9, which was identified by electron-microscopy to consist mainly of mitochondria.

Four h after administration, when pimozide is fully effective in the anti-amphetamine test, there is a shift in the distribution profile and now the highest percentage of the total radioactivity is found in fraction 3, a fraction rich in small nerve-endings filled with vesicles.

After 4 h the ratio $\frac{\%\text{ radioactivity in fraction 3}}{\%\text{ radioactivity in fraction 9}} = 1{\cdot}82$, is the inverse of the ratio after 1 h (0·55).

Inverse isotope dilution shows that in both fractions after 4 h about 85% of the radioactivity is due to [^{3}H]pimozide and 15% probably to the radioactive metabolites 4-bis(*p*-fluorophenyl)butyric acid and bis(*p*-fluorophenyl)acetic acid (Soudijn & van Wjingaarden, 1969).

Sixteen h after the injection, when the action of pimozide is weak, there is another shift in the distribution profile from the small nerve-ending fraction 3 to the fractions 2 (myelin) and 1 (supernatant).

In the other subcellular fractions e.g. fraction 5, consisting mainly of post synaptic membranes and fraction 7, which is similar to fraction 5 but is slightly contaminated with mitochondria, no significant distribution shifts were observed.

Thus we may conclude, that although the pimozide concentration in the caudate nucleus does not correlate with its anti-amphetamine activity, the pimozide concentration in the small nerve-endings does so.

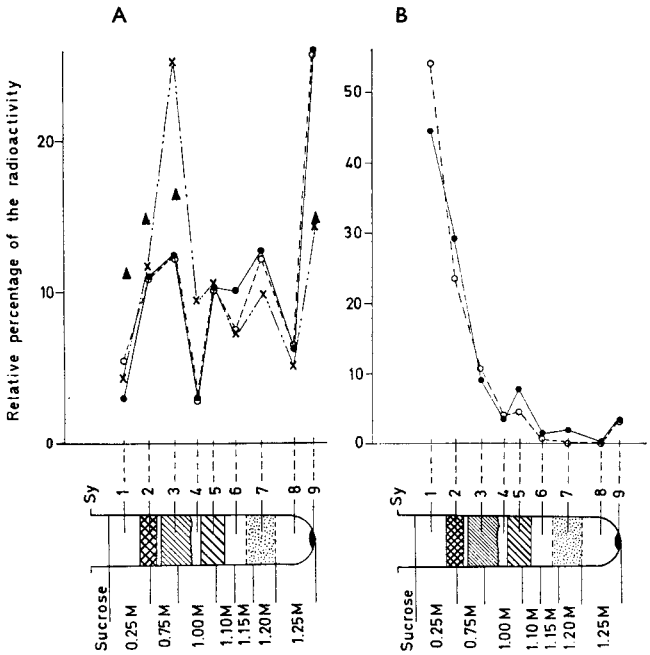


FIG. 2A. Subcellular distribution of labelled material in the caudate nucleus of the Wistar rat, after administration of [^{3}H]pimozide (0·31 mg/kg, s.c.). ○ - - ○ 1 h. × - - × 4 h. ▲ 16 h. ●—● added to caudate nuclei of untreated rats.

B. Subcellular distribution of (+)[7-^{14}C]amphetamine in the caudate nucleus of the Wistar rat, 2 h after administration of (+)-[^{14}C]amphetamine sulphate (5 mg/kg, i.v.). ○ - - ○ *in vivo*. ●—● added to caudate nuclei of untreated rats.

The caudate nucleus is rich in small dopaminergic nerve-endings so it is tempting to assume that pimozide acts by masking the dopamine receptor sites.

The protective action of pimozide against the amphetamine-induced stereotype behaviour and agitation cannot be a result of a decrease in permeability or an increase in amphetamine metabolism, for the amphetamine concentration in brain or caudate nucleus at the moment of peak effect of both drugs (1 h after amphetamine and 4 h after pimozide) is the same whether the rats are pretreated with pimozide or not (Table 2). However the possibility of subcellular displacements of amphetamine cannot be excluded.

Gradient centrifugation of homogenized caudate nucleus of rats pretreated with (+)-[^{14}C]amphetamine (2 h, 5 mg/kg, i.v.) showed that 75% of the amphetamine present in the organ was found in supernatant and myelin fraction (Fig. 2B). The subcellular distribution of (+)-[^{14}C]amphetamine added to the isolated caudate nuclei just before homogenization was identical to the distribution in the *in vivo* experiment, indicating that amphetamine is not, or very weakly, particle bound, and homogenization may result in redistribution, thus masking the real distribution in the organ *in vivo*.

Table 2. *Concentration of radioactive material in the brain of the Wistar rat, 1 h after (+)-[7-^{14}C]amphetamine sulphate (5 mg per kg, i.v.) after pretreatment with unlabelled pimozide (0·31 mg/kg, s.c.) or with saline 3 h before the amphetamine administration.*

Tissue	Pretreatment	
	Saline	Pimozide
Caudate nucleus	a. 4436	4554
	b. 100	96
	c. 4454	4386
Brain	a. 4021	3997
	b. 98	100
	c. 3928	4001

a. pg radioactive material per mg of wet tissue.
b. % [^{14}C]amphetamine in a.
c. pg [^{14}C]amphetamine per mg of wet tissue.
Each figure represents a group of 5 animals (pooled organs).

Amphetamine treatment of the animals had no influence on the pimozide concentration in rat brain and caudate nucleus, nor on the subcellular distribution of [^{3}H]pimozide in the caudate nucleus, 4 h after [^{3}H]pimozide administration and 1 h after administration of amphetamine (van Wijngaarden, unpublished results).

It seems very likely that the anti-amphetamine activity of the neuroleptic agent pimozide is a result of masking the dopamine receptor sites in the caudate nucleus, an area of enhanced uptake and retention of pimozide in rats as well as in dogs.

Acknowledgements

This investigation was supported by the Netherlands Organization for the Advancement of Pure Research.

We thank Dr. J. Schaper for the preparation and interpretation of the electron microscopic data and Mr. F. M. Lenaerts for his skilled help with the animal experiments.

We wish to thank Smith Kline and French Laboratories USA for the gift of (+)-[7-^{14}C]amphetamine.

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