Radiosynthesis of a New Radioiodinated Ligand for Serotonin-5HT$_2$-receptors, a Promising Tracer for γ-Emission Tomography.

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SUMMARY

4-Amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide, a compound with high affinity for 5HT$_2$-receptors, was radioiodinated on the 2-position of the phenoxygroup or the 5-position of the methoxybenzamide group. The former (obtained by Cu^{1+}-assisted radioiodo for bromo exchange) showed high lipophilicity and high non-specific binding to rat brain tissue both in vitro and in vivo. The second labelled compound, ^{125}I-4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide, obtained by direct electrophilic substitution, showed high affinity (K_d of 0.11 ± 0.01 nM) and marked selectivity for 5HT$_2$-receptors in vitro. Moreover in vivo studies in rats over the course of 1-3 hrs post i.v. injection of the radioactive ligand, showed steady preferential retention of radioactivity in the frontal cortex, which is enriched in 5HT$_2$ receptors. (Frontal cortex (FC) / Cerebellum ratio of 10 and FC / Blood ratio of 6, amount compound / mg tissue).

Keywords : radioiodination ,^{125}I-4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide, electrophilic substitution, 5HT$_2$ receptor ligand.

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INTRODUCTION.

$5HT_2$-Receptors seem to play an important role in psychiatric disorders. In order to investigate the role of these receptors in pathology there is an increasing interest in obtaining a selective and high affinity radiolabelled ligand suitable for in vitro and in vivo receptor binding studies. For in vitro and rodent studies ^{125}I labelled tracers are preferred as their specific radioactivity is generally at least 50 times higher than that offered by tritiated compounds. Labelled with ^{123}I a suitable ligand offers the opportunity to perform SPE(C)T. At the end of the 1980's 2-radioiodo-ketanserin was proposed as a potential $5HT_2$-receptor tracer for SPECT (1) and this compound was applied for SPECT imaging of $5HT_2$-receptors in depressed patients (2). The iodinated compound showed properties almost comparable to those of ketanserin (poorly selective for $5HT_2$ vis-à-vis H_1 and α_1-receptors) and high binding to serum proteins resulting in rather poor brain receptor to blood ratio.

Recently 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide derivatives have been discovered as a new class of $5HT_2$ antagonists. The parent compound shows very high affinity and selectivity for $5HT_2$-receptors (pIC_{50} for inhibition of [^{3}H]ketanserin binding to rat frontal cortex membranes is 9.2 ; selectivity with regard to other neurotransmitter receptor sites such as $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{2C}$, $5HT_3$, α_1 and α_2 adrenergic, histamine-H_1 and dopamine-D_2 is at least a factor of 50). It was decided to label this compound with radioiodine in order to develop a new radioiodinated ligand with high affinity for $5HT_2$-receptors. In view of the lower increase in lipophilicity (increased lipophilicity causes increased non-specific binding) two sites were suitable for incorporation of the radioiodine, namely: the 2-position in the 4-fluorophenoxy entity in clustered lipophilic compensation position (3,4) and the 5-position, i.e. ortho to the polar amine function which compensates for the increase of lipophilicity by the iodine atom, in the benzamide group.

MATERIALS and METHODS.

Reagents: 4-Amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide.$2H_20$ **1** , 4-amino-N-[1-[3-(2-bromo-4-fluorophenoxy)pro-

pyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide 2 and 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-bromo-2-methoxybenzamide 3 are original products of the Janssen Research Foundation (Beerse, Belgium). The other reagents used were p.a. grade (Merck) or HPLC grade (Lichrosolv quality Merck). Non carrier added sodium [^{125}I] iodide (10^{-2}N NaOH; 74 ± 5.5 TBq/mmol) was purchased from Nordion Europe (Fleurus, Belgium).

HPLC Equipment :

Analytical 1 : The equipment consisted of a Rheodyne injector (50 μl loop), a Hitachi 655A pump and L-6000 II controller, a 655A variable wavelength UV monitor at 275 nm, a NaI(Tl) detector (Harshaw QS) and appropiate electronics (Canberra), a D2000 Chromato integrator Hitachi and an Ankersmidt R40 one-channel recorder. Quality control was achieved on a Lichrospher 125x4 mm 100 RP 8 (5μ) Merck column with a methanol/acetonitrile/water // trimethylamine/acetic acid (MeOH/ACN/H_2O // TMA/HOAc) : 16/22/62 // 1.5/2 v/v mixture of pH= 4.8 as eluent at a flow rate of 1 ml/min.

Analytical 2 : The equipment consisted of a Rheodyne injector (50 μl loop), a Waters 510 pump, a Waters 486 tunable UV absorbance detector at 275 nm, a Radiomatic A500 (Canberra Packard) with a CaF_2-detector, a Flo-one\ data for Windows software package for instrument control and data analysis and a HP deskjet 500 recorder. Quality control was achieved as described above.

Semi-preparative : Rheodyne injector (2.5 ml loop), a Waters M6000A pump provided with a semi-preparative pumphead, a Waters Lambdamax UV-480 monitor at 275 nm, a NaI(Tl) detector (Harshaw QS) connected to Ortec electronics, a HP 3580 and a Intersmat ICR-18 integrator.

A MeOH/ACN/H_2O//TMA/HOAc : 16/22/62//1,5/2 v/v mixture of pH=4.8 was used as eluent at a flow rate of 6 ml/min on a Lichrocart 250x10 mm Lichrosorb RP Select B (10μ) Merck column.

Synthesis of 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide.2H_2O. 4 .

44.5 μmol of 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide.2H_2O 1 (20 mg) was dissolved in 1 ml of peracetic

acid (glacial acetic acid / 30% H_2O_2 : 2/1) and 50 μmol NaI (7.5 mg) was added to the stirred solution. The reaction proceeded at room temperature. After disappearance of the typical brown iodine color the reaction mixture was cooled in an ice-water bath (0°C) and firstly, 7 ml of H_2O and secondly, 2 ml of 2M Na_2SO_3 were added. After altering the pH to 10.5 by addition of 2N NaOH the iodinated compound was extracted in 3 successive fractions of 10 ml $CHCl_3$. The $CHCl_3$ was then twice washed with H_2O. The organic phase was dried over $MgSO_4$. After evaporation of the chloroform under vacuum, 5 ml of diethyl-ether was added to the oily residue followed by 200 μl H_2O and the solution stored under N_2 and stirred overnight. After evaporation of the diethylether by passage of N_2 at ambient temperature the residue was dried at 30°C under vacuum. A final product (24 mg, white powder) was obtained (yield 91%).
Analytical and semi-preparative HPLC (as indicated in HPLC Equipment using 275 nm (λ_{max} for compound 4)) revealed a purity $\geqslant$ 99%.
Analytical : t_R1 = 6,3 minutes; t_R4 = 21 minutes.
Semi-preparative : t_R1 = 13 minutes; t_R4 = 47 minutes.
^{1}H-NMR (270 MHz,$CDCl_3$) : δ 8.4 (s,1H,piper-NH-CO-Phe) ; δ 7.52 (s,1H,Ar-H_6) ; δ 6.98-6.9 (dd,2H,F-Phe) ; δ 6.82-6.78 (dd,2H,Phe-O-) ; δ 6.25 (s,1H,Ar-H_3) ; δ 4.4 (s,2H,Ar-NH_2) ; δ 3,96-3.92 (d,2H,-O-CH_2-C-) ; δ 3.87 (s,3H,Ar-O-CH_3) ; δ 2.5-2.45 (t,2H,-C-CH_2-piperN) ; δ 2-1.9 (dd,2H,-C-CH_2-C-) ; δ 1.5 (s,3H,CH_3-piperC-NH-CO-).
MS (FAB) (m/e,%RA) : 541 (M+,10), 430 (8), 402 (67), 389 (7), 276 (100), 249 (72).

Radiosynthesis of non carrier added (N.C.A.) ^{125}I-4-amino-N-[1-[3-(4-fluoro-2-iodo-phenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide. 5 .

Stock solution : To 1 mg $SnSO_4$, 25 mg 2,5-dihydroxybenzoic acid, 35 mg citric acid.H_2O and 25 μl glacial acetic acid 1750 μl of water (pro injection quality) was added.

Copper solution : 32.5 mg of $CuSO_4.5H_2O$ was dissolved in 10 ml of H_2O.

Reaction : To 1 mg of 4-amino-N-[1-[3-(2-bromo-4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide 2 respectively 355 μl of the stock

solution and 30 μl of the copper solution were added to a septum closed vial of 1 ml volume. After 10 minutes of sonification the reaction mixture was flushed with N_2 for 5 minutes and the radioiodide solution was added through the septum.The vial, contained in an aluminium safety container, was heated in a thermoblock at 140°C for 60 minutes. The contents of the reaction vial followed by 1.2 ml of rinsing solution (i.e. 0.5 ml MeOH and 0.7 ml semi-preparative HPLC eluent) were sucked through a 0.45 μ filter and injected for semi-preparative HPLC separation.

To the eluent fraction containing the radioactive tracer 1.5 times its volume of H_2O was added and the pH adjusted to 9 with 2N NaOH. The resulting solution was passed through a Baker Bond Octadecyl 100 mg column at a flow rate of 4 ml / minute. The column containing the radioiodinated compound was rinsed with 20 ml of H_2O and blown with air to apparent dryness. The radioactive compound was recovered in 500 μl of ethanol and was stored at -18 °C for further application.

An average labelling yield of 92% was observed. Besides free radioiodine no other radiolabelled compound was present. The overall radiochemical yield amounted to 80%. HPLC inanalytical and semi-preparative conditions (radioactivity detection and mass detection by sensitive UV spectrometry) revealed a radiochemical purity of at least 99% and the amount of starting bromocompound if present, was lower than the detection limit (10^{-13} mol) for the compound (λ_{max} 2 ~ λ_{max} 5 = 275 nm).

Radiosynthesis of N.C.A. ^{125}I-4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide. 6 .

0.7 mg 4-amino-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide.2H_2O 1 was dissolved in 500 μl of glacial acetic acid. The radioiodide was added while stirring followed by the addition of 100 μl of 30% H_2O_2. The reaction was allowed to proceed for 15-20 minutes at room temperature. The reaction vial was transferred to a small ice-bath. To the reaction mixture 2 ml of ice-cold H_2O and 1.8 ml of 1M Na_2SO_3 were added while stirring and the pH was brought to 11 by addition of 2N NaOH. This solution

was passed through a Baker Bond Octadecyl 100 mg column. The column was consecutively rinsed with 10 ml of a NaOH solution of pH 10.5 and 10 ml of H_20.

The non-radioactive compound **1** and radioactive tracer were recovered in 600 μl of MeOH. 300 μl acetonitrile and 500 μl of H_2O were added and the complete mixture was filtered through a 0.45 μ filter. The filtrate was injected for semi-preparative HPLC separation. The pure radioactive tracer was recovered in about 8 ml eluent, approximately 20 ml of H_20 was added and the solution brought to pH 11 with 2N NaOH. Preconcentration was performed on a Baker Bond Octadecyl column as described above. After blowing the column apparently dry, the radioactive tracer was recovered in 500 μl EtOH and stored at -18°C prior to further application.

The labelling yield reached 93 to 98% depending on the amount of radioiodide solution used : 93% for 120 μl and 98% for 20 μl. No radiolabelled side product was observed. Depending on the labelling yield the overall radiochemical yield varied from 75 to 80%. Analytical and semi-preparative HPLC control of the final product revealed a purity of at least 99% and no starting or any other non-radioactive product could be detected (t_R compound **1** : 13 minutes, t_R compound **6** : 47 minutes ($\lambda = \lambda_{max}$ **4**)).

Radioligand binding in vitro.

Binding of the ligands to membrane preparations of rat frontal cortex and inhibition by ketanserin and ritanserin was essentially carried out as described by Leysen et al. (5,6).

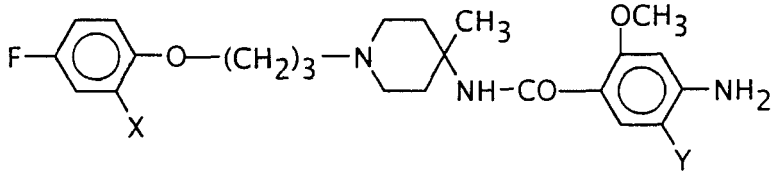
The in vitro stability of the radioiodinated compound **6** was checked for the compound in solution as well as for the compound bound to the membranes. After filtration of the membrane suspension in buffer containing the radioligand an aliquot of the filtrate was injected for HPLC analysis. (conditions as described) The membranes were resuspended in buffer by sonification of the filter and incubation in the presence of 10^{-6}M of ritanserin was performed over the course of two hours. After filtration an aliquot of the filtrate was submitted for analytical HPLC analysis.

Radioactivity detection was carried out with a Radiomatic A500 which allows one to detect very low amounts of ^{125}I radioactivity (2 - 3 Bq can easily be measured above a background of 0,3 Bq, the counting efficiency amounts to 80%). After a total incubation time of six hours no free radioiodide could be detected and only a peak corresponding to the radioligand was present in the chromatogram.

Radioligand binding in vivo.

In vivo experiments were performed in the laboratories of Janssen Research Foundation licenced for this purpose. Radioiodinated ligand 6 (740 kBq) was injected in the tail vein of male Wistar rats. After 0.5, 1.0, 1.5, 2.0 and 3.0 hours the rats were sacrificed, blood was collected and the brain areas were rapidly dissected. Radioactivity in the tissues was counted using a Cobra Packard autogamma counter. Samples of rat serum were treated with an equal amount of a 5% trichloroacetic acid/methanol : 50/50 and after centrifugation an aliquot was injected for analytical HPLC using the high sensitive detector as mentioned before.

RESULTS AND DISCUSSION.



X	Y	Compound
H	H	1
Br	H	2
*I	H	5
H	Br	3
H	I	4
H	*I	6

Radiochemistry.

As mentioned earlier the Cu^{1+} assisted radioiodo for bromo exchange was successful for compound 5 (labelling yield 92%). The purification procedure was shown to be efficient as HPLC analysis shows that for the N.C.A. radioiodinated compound a purity of at least 99% was achieved.

When applying the Cu^{1+} assisted method on the 5-bromo-benzamide group of compound 3 a labelling yield of only 30% was obtained. When using previously described nucleophilic exchange methods (7) virtually no labelling ($\ll$ 5%) was obtained.

This may be due to deactivation of the 5-position of the benzamidegroup by the amino function on the 4-position.

Therefore it was decided to apply the direct electrophilic substitution as described in the experimental session. A mean labelling yield of 95% was obtained. As in theory the 3-position, ortho to both an amino and methoxy group, is more activated for electrophilic substitution than the 5-position a mixture of 3 and 5 iodinated compounds could be expected. Nevertheless the electrophilic substitution occurs nearly entirely at the 5-position. This may be due to steric hindrance by the freely rotating methoxy group on the 2-position.

Less than 0.5% of the compound labelled at the 3-position is observed by HPLC. As the iodine on the 3-position is clustered within two polar functions, i.e. amino and methoxy, the lipophilicity is lower than for the compound substituted on position 5 and due to its shorter retention time the HPLC recovery of the pure required compound 6 is not disturbed (retention time compound 1 : 13 minutes and for compound 6 : 47 minutes).

The overall yield of N.C.A. compound 6 amounts to 80% and quality control revealed a purity of at least 99%.

Lipophilicity expressed as k'-values.

We have demonstrated before (3,4) that there is an excellent correlation between the k'-values, the solvent accessible area SAA and the log P octanol/buffer values. From the results shown in Table 1 it is clear that substitution of H by a bromine and an iodine atom on the 2-position of the phenoxy entity increases

much more the lipophilicity (almost a factor of 4 and 5 as compared to the original compound 1) than does substitution on the 5-position of the benzamide - group i.e. ortho to the polar amine function.

Compound	k'
1	2.5
2	10.0
5	14.4
3	6.7
4 and 6	8

Table 1 : The k'-values of the different compounds using the HPLC conditions
Column : Lichrocart 125x4 mm, Lichrospher 100 RP8 (5 μ)
HPLC eluent : MeOH/ACN/H_2O//TMA/HOAc : 17/25/58//1.5/2 pH=5
Flow rate : 1 ml/min

In vitro and in vivo binding properties to the rat brain

The affinity binding in vitro of compound 4 for $5HT_2$-receptor sites evaluated by the inhibition of [^{3}H]ketanserin binding to rat frontal cortex membranes showed a pIC_{50} value of 9.45. The selectivity with regard to other $5HT_x$, α_1, α_2, D_2 and D_1 receptors was at least a factor of 50.

The binding of the radioiodinated compound 6 to rat frontal cortex membranes was saturable and is shown in Fig.1. A linear Scatchard plot yields a Kd value of 0.11 ± 0.01 nM and a Bmax value of 38.0 ± 0.7 fmoles/mg. Inhibition of the binding of compound 6 by ketanserin and ritanserin proved that the binding was reversible.

Table 2 presents the frontal cortex (FC) to cerebellum (CER) and the FC to blood activity ratio in the brain of male Wistar rat 30 minutes after i.v. injection of respectively N.C.A. ^{125}I-labelled compound 5 and N.C.A. ^{125}I-labelled compound 6 (the specific radioactivity of both compounds amounts to about 2000 Ci/mmol or 74 x 10^6 MBq/mmol).

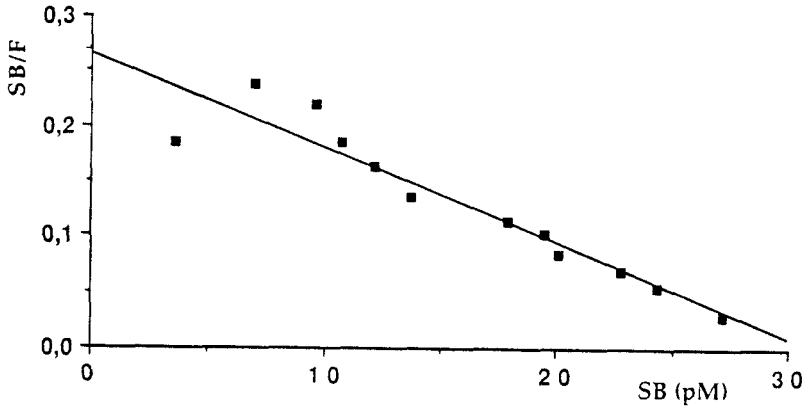


Fig.1: Scatchard analysis of the saturation binding of compound **6** to frontal cortex $5HT_2$ receptors. Incubation was run for 60 minutes at 25 °C.
SB : specific binding definedas the difference between total binding of the radioligand and binding in the presence of 1000 fold excess of BW501.
F : free radioligand, defined as the difference between totally added and totally bound.

It is shown that in vivo the more lipophilic ^{125}I-labelled compound **5** shows a poor FC/CER activity ratio (due to non-specific binding in the cerebellum) and a FC/Blood activity ratio $\langle 1$, making this radioactive compound unsuitable for in vivo and even in vitro application.

Compound	FC / CER	FC / BLOOD
5	1.7	0.6
6	3.1	2.6

Table 2 : Ratio of the amount of radioactivity in brain tissue (FC : frontal cortex, CER : cerebellum) and blood (fg/mg), 30 min. p. injection in tail vein of rat.

As shown in Fig.2 for the less lipophilic radioiodinated compound **6** the FC to CER and FC to Blood ratio increases from about 3 at 30 minutes p. injection

to a steady value of about 10 for FC / CER and 6 for FC / Blood up from 60 minutes to 3 hours. The striatum (ST) / CER ratio and the ST / blood ratio amounted to about halve that value. Binding in the frontal cortex and in the striatum was shown to be saturable.

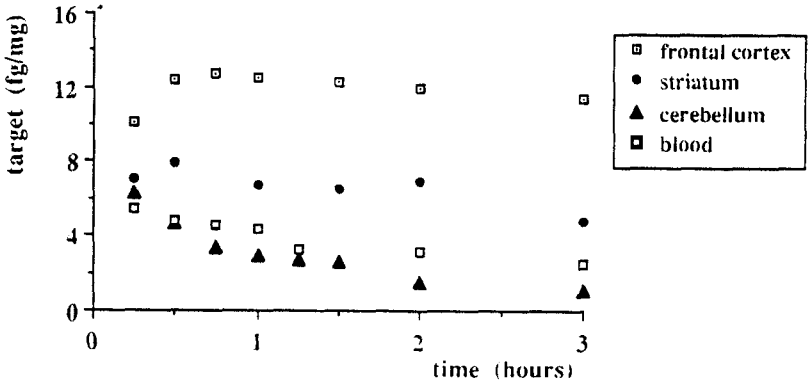


Fig.2 : Distribution of compound **6** in the rat brain (fg / mg tissue) after i.v. injection(5 ng or 740 kBq /rat) as a function of time (hours).

Displacement by ketanserin, a ligand showing high affinity for $5HT_2$ receptors, which was i.v. injected 60 minutes after the administration of the radioactive compound, showed that in the in vivo brain the binding of compound **6** was reversible and occured at $5HT_2$ receptor sites.

As after three hours the unchanged radioiodinated ligand represented more than 95% of the radioactivity present in the serum fraction of the rat blood and as ketanserin displaced the radioactivity in the brain, it could be assumed that the net bound radioactivity in the frontal cortex and in the striatum represented the unchanged radioligand. Two hours post injection the %ID/g in FC was 0.24 and the ratio of radioactivity in the FC to the rest of the brain was at least 4.

CONCLUSION

N.C.A. Radioiodination on the 5-position of the methoxybenzamide group of 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxy-benzamide yields a radioiodinated ligand that shows high affinity and selectivity for $5HT_2$-receptors sites in vitro as well as in vivo and if labelled with ^{123}I it can be a promising tracer for SPE(C)T.

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REFERENCES.

1. Mertens J., Gysemans M., Piron C., Thomas M., J. Labelled Comp. Radiopharm 28,731-739 (1990).
2. D'Haenen H., Bossuyt A., Mertens J., Piron C., Gysemans M., Kaufman L., Psychiatry Research, Neuroimaging, 45, 227-237 (1992).
3. Mertens J., J. Labelled Comp. Radiopharm. 30, 363 (1991).
4. Mertens J., Moereels H., Yu J., Zolle I., J. Labelled Comp. Radiopharm. 32, 245 (1993).
5. Leysen J.E., Niemegeers C.J.E., Van Nueten J.M., Laduron P.M., Mol. Pharmacol., 21, 301-314 (1982).
6. Leysen J.E., Gommeren W., Mertens J., Luyten W.H.M.L. et al., Psychofarmacology, 110, 27-36 (1993).
7. Mertens J., Vanryckeghem W., Gysemans M., Eersels J., Finda E., Carlsen L., Eur. J. Nucl. Med. 13, 380-381 (1987).