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Research Article

Development of a Radioreceptor Assay for the D_2-Selective Dopamine Agonist N-0437

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N-0437 is a recently developed dopamine (D_2) agonist, theoretically attractive in the therapy of Parkinson's disease and glaucoma. Since its high potency allows small doses of the compound in clinical use and as extensive metabolism occurs in animals, a highly sensitive assay method was required for drug-monitoring purposes. To this end we developed a radioreceptor assay (RRA), a sensitive tool for the assessment of the sample's (dopaminergic) bioactivity. The RRA is based on competition between N-0437 and its tritium-labeled analogue for binding to dopamine receptors. The assay has been optimized for the preparation of the receptor suspension and the incubation conditions. Direct application of the assay for biological samples was impossible because of matrix interferences. Therefore, a solid-phase extraction method was developed in which the combination of a polar Si column and dichloromethane as eluent resulted in an effective elimination of the interferences. Recoveries were better than 90 and 95% for plasma and urine, respectively, even at concentrations at the determination limit of the method (300 pg/ml). Relative standard deviations were less than 15%. Because RRAs are stereoselective, the method discriminates between active and inactive species.

KEY WORDS: radioreceptor assay; dopamine receptors; bioanalysis; N-0437; dopamine agonist.

INTRODUCTION

N-0437 (Fig. 1) is a recently developed, potent dopamine (D_2) agonist of the 2-aminotetraline class, derived from N-0434 by a substitution of the phenyl ring by a thienyl group (1–3). The drug is clinically evaluated for its therapeutic efficacy in Parkinson's disease and glaucoma, among others.

Showing a pharmacological response maximum in rats at doses as low as 10 μg/kg, one may expect that therapeutic plasma levels of N-0437 will be in the range of 100 pg/ml to 10 ng/ml.

Monitoring these low drug levels obliges one to use very sensitive quantitative analytical methods. Besides conventional chromatographic analysis, a radioreceptor assay (RRA) (4), as a highly sensitive tool for the measurement of the sample's (dopaminergic) bioactivity, might be useful. Because of the extensive metabolism of this compound, metabolites with an affinity to the dopamine receptor will be codetermined, and therefore, the outcome should be interpreted as affinity equivalents of N-0437 and not as a true concentration.

The specificity and high-affinity binding of N-0437 to calf caudate D_2-dopamine receptors were thoroughly described by van der Weide *et al.* (3) and were a good starting point for the development of a radioreceptor assay for quantitation of this drug in biofluids. The use of a high-affinity D_2-dopamine antagonist was inappropriate because these compounds label D_2 receptors with both a high and a low affinity for dopamine agonists and are therefore difficult to use for reliable quantitation.

For the development of a RRA for N-0437 we decided to use ^{3}H-labeled N-0437 as the radio ligand, unlabeled N-0437 being the displacer. These compounds compete for binding to D_2-receptors (high-affinity state; D_2-high) from calf striata. Since D_2-high is readily degraded to D_2-low, a receptor state with a low affinity toward agonists, the tissue preparation and the incubation media must be chosen carefully. For example, sodium ions stimulate D_2-high degradation, while magnesium ions are essential to keep D_2-receptors in the high-affinity state (5). Therefore, the RRA has been optimized for the preparation of the receptor suspension and the incubation conditions: tissue (receptor) concentration and concentration of labeled ligand, pH, temperature, and time.

MATERIALS AND METHODS

Chemicals

^{3}H-N-0437 (80.6 Ci/mmol) was supplied by Amersham (Buckinghamshire, England). Unlabeled N-0437 was donated by P. Tepper and A. S. Horn (State University Groningen, Department of Medicinal Chemistry). Plasmasol was used as scintillation liquid and obtained from Packard Instruments (Groningen, The Netherlands). All other chem-

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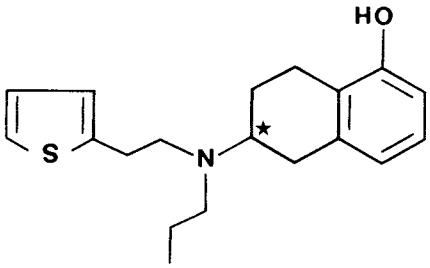

Fig. 1. Structure of N-0437. The star indicates the chiral carbon atom. The ^3H label is present in the propyl side chain.

icals and solvents of analytical grade were obtained from Merck (Amsterdam, The Netherlands).

Polyethylene tubes (10 ml) were obtained from Greiner (Alphen a/d Rijn, The Netherlands). GF/B glass-fiber filters were from Whatman (Maidstone, U.K.). Analytichem extraction columns (Clin Elut & Bond Elut) and a Vac Elut vacuum station were obtained from Betron (Rotterdam, The Netherlands).

The incubation buffer had the following composition: 50 m*M* Tris, 1 m*M* EDTA, 5 m*M* KCl, 1 m*M* $MgCl_2 \cdot 6H_2O$, 2 m*M* $CaCl_2 \cdot 2H_2O$, 0.1 m*M* dithiothreitol (6), and concentrated HCl to pH 7.4

Preparation of the Receptor Suspension

Immediately after killing, calf brains are excised from the skull and the striata are dissected. The striata are immediately deep-frozen on dry ice and kept at −20°C.

The following preparation procedure was finally selected.

(1) Thaw striatal tissue and homogenize 4.5 g in 75 g 0.32 *M* sucrose using a Teflon–glass Potter–Elvejehem homogenizer at 1200 rpm.
(2) Precipitate cell nuclei by centrifugation for 10 min at 600*g*.
(3) Resuspend the pellet obtained in 75 g 0.32 *M* sucrose and centrifuge for 10 min at 600*g*.
(4) Combine the supernatants of steps 2 and 3 and centrifuge for 45 min at 100,000*g*.
(5) Resuspend and homogenize the pellet, containing the cell membranes with dopamine receptors, in 150 ml 50 m*M* Tris/HCl buffer, pH 7.4.
(6) Incubate for 30 min at 30°C in order to eliminate endogenous dopamine.
(7) Precipitate the cell membranes by centrifugation for 20 min at 50,000*g*.
(8) Resuspend and homogenize the pellet in 150 ml 50 m*M* Tris/HCl buffer, pH 7.4.
(9) Centrifuge the homogenate for 20 min at 50,000*g*.
(10) Combine the pellets, divide into 10 portions of ±300 mg, enough for 10 × 40 samples in the RRA (11.25 mg striatal tissue/assay), and store at −20°C.

Before use in the RRA one portion is thawed and resuspended in 50 m*M* Tris/HCl buffer.

Determination of the affinity of N-0437 and the Receptor Density of the Tissue Preparation (Saturation Curve)

To duplicate tubes receptor suspension is added, with increasing concentrations of ^3H-N-0437 over the range 1×10^{-10} to 1×10^{-8} *M*, with or without 1×10^{-6} *M* unlabeled N-0437, according to the procedure described above. The difference in binding (Fig. 2) observed in corresponding tubes with and without unlabeled N-0437 represents receptor-bound radiolabeled ligand (= specific binding). By means of the curve-fitting program LIGAND-MED 58 of Munson and Rodbard (7) (on an Apple IIe), the receptor concentrations and affinity of ^3H-N-0437 for the tissue can be calculated (7).

Final Procedure for Urine and Plasma Using Si Columns

To 1.0 ml urine or plasma, 1 ml Tris/HCl buffer is added and the mixture is vortexed for 1 min. If a sample needs to be spiked, 100 µl of Tris/HCl buffer, containing the required amount N-0437, is added to 1.0 ml of biofluid. The mixture is vortexed for 1 min, and after adding 900 µl of Tris/HCl buffer, the mixture is vortexed again for 1 min.

Bond Elut extraction columns, type Si (straight silica), bed volume 3 ml, manufactured by Analytichem, are used in combination with a Vac Elut vacuum control station. Each column is pretreated by subsequent washings with 3 ml water, 3 ml dichloromethane, and 3 ml Tris/HCl buffer.

After removing the final washing by aspiration, the diluted sample is transferred to the washed column and a gentle vacuum (800 mbar) is applied. The columns are rinsed with 1 ml Tris/HCl buffer under gentle vacuum. Elution of adsorbed N-0437 is achieved by passing 5 ml dichloromethane through the column under a moderate vacuum. The eluate is collected in a 10-ml polyethylene tube, and the eluate is evaporated to dryness under a gentle stream of nitrogen.

To the residue is added 50 µl of a solution containing 1×10^{-8} *M* ^3H-N-0437 and 450 µl of receptor suspension in Tris/HCl buffer, so that 11.25 mg of striatal tissue is present

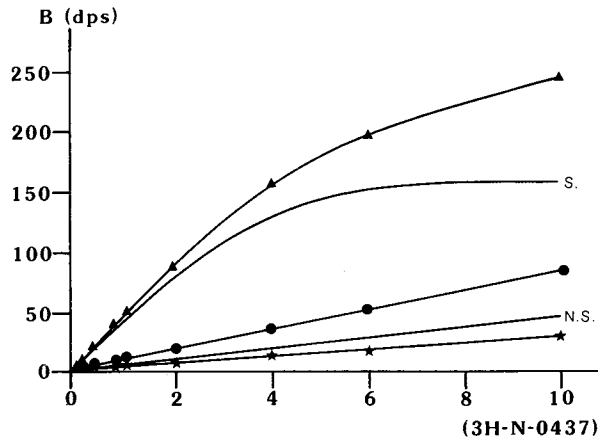

Fig. 2. Saturation curve, binding at several concentrations of ^3H-N-0437 with or without unlabeled N-0437 (1×10^{-6} *M*). Specific binding (S.) is the difference between total and nonspecific binding. Nonspecific binding consists of ^3H-N-0437 filter binding (★) and binding to nonreceptor proteins (N.S.). (▲) Total binding; (●) total nonspecific binding.

in the assay. The mixture is vortexed for 15 sec and incubated for 45 min at 30°C in a water bath. Thereafter, 4 ml of ice-cold Tris/HCl buffer is added and the tubes are vortexed for 5 sec. Then the mixture is immediately filtered through Whatman GF/B glass-fiber filters under vacuum using a filtration apparatus (Multividor 40 S, Janssen Scientific Instruments, Beerse, Belgium). The tubes are rinsed with 4 ml ice-cold buffer which was also filtered. The filters are washed with 4 ml ice-cold buffer and transferred to polyethylene counting vials, 3.5 ml Plasmasol is added, and the vials are shaken for 120 min. The total filtration, rinsing, and washing process, taking place in approximately 15 sec, is carried out on each tube in turn. The vials are counted for 10 min or 40,000 counts in a liquid scintillation counter (Minaxi, Packard, Groningen, The Netherlands). The amount of bound labeled ligand is given as dps (disintegrations per second) values, which are obtained by converting the cps values; the counting efficiency of the samples is always between 47 and 50%. Quench correction is made automatically, based on the spectral index of the sample.

RESULTS

Assessment of the Optimal Incubation Conditions

In Fig. 2, a representative saturation curve is presented for the dopamine D_2-receptors labeled with ^{3}H-N-0437 indicating a reasonable ratio of specific/nonspecific binding. The calculated receptor density (25 fmol/mg striatal tissue) and equilibrium binding constant K_a (2×10^8 to 4×10^8 M^{-1}) are in good agreement with the data of van der Weide *et al.* (3).

The optimum incubation pH turned out to be 7.4. Higher or lower pH values were accompanied by increases in nonspecific or decreases in specific binding, respectively.

As was expected on basis of the results in Fig. 2, tracer concentrations below 2 n*M* offered the best ratio of specific/nonspecific binding and an acceptable sensitivity. An assay concentration of 1 n*M* ^{3}H-N-0437 in combination with 11.25 mg striatal tissue per assay met our demand, to count at least 40,000 counts/10 min, at the maximum binding level and assuring reliable quantitation (4).

Incubation at 25 (3) or 30°C during 45 min gave the highest ratios of specific/nonspecific binding (ca. 4.2). For practical reasons an incubation temperature of 30°C, which is easier to maintain, is preferable. Attempts to reduce nonspecific filter binding were without success.

Under the conditions presented here, the radioreceptor assay for N-0437 is not sensitive to minor variations in the incubation conditions and is similar in principle to the RRA employed for the characterization of dopamine D_2-receptors. Nevertheless, the modifications and obtained reproducibility of binding described here are essential for application of the RRA as a bioanalytical tool.

Application of the RRA to Biological Samples

A critical factor in the development of a bioassay is the interference caused by the biological matrix in which the drug of interest is present. Depending on the degree of interference by the biological matrix in the RRA, two alternatives exist.

1. *Direct assay:* A certain volume of a biofluid, after proper dilution and/or pH adaptation, is added to the incubation medium and assayed. This is the method of choice when there is little or no matrix interference. A direct assay has several advantages in that it is simple and relatively inexpensive and there is no risk of drug losses.
2. *Indirect assay:* The biofluid is pretreated in such a way that the drug is separated from its endogenous matrix or at least, from the interfering components in the matrix. This method has to be used when a direct assay is not possible and/or if a preconcentration step is required in order to obtain detectable drug concentrations.

When blank plasma was added to the RRA, the ^{3}H-N-0437 binding was inhibited. The addition of 200 µl blank plasma caused over 90% inhibition. This may be explained by nonspecific binding of the label to plasma proteins. Thus, a direct assay for N-0437 in plasma was not possible.

Also, when blank urine was used in the RRA, the ^{3}H-N-0437 binding was inhibited. The addition of 25–250 µl urine caused an inhibition of about 43–85% of the maximum binding (Fig. 3). In contrast to plasma, the decrease in specific binding appeared to be proportional to the amount of urine added. Therefore, a direct assay may be possible when using only small volumes of urine. Yet a prerequisite is the availability of a representative sample of blank urine. On the other hand, with samples containing relatively high drug concentrations, dilution of the samples may be possible to the extent that matrix effects become negligible, with drug concentration remaining at levels at which they can still be assayed.

Isolation of N-0437 from Urine and Plasma

Initial experiments concentrated on the isolation of N-0437 by liquid/liquid extraction. As urine usually does not contain appreciable amounts of protein, it was anticipated that a relatively simple liquid/liquid extraction procedure would suffice.

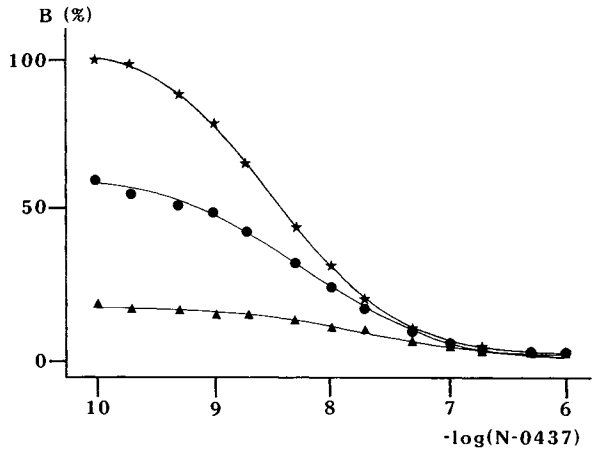

Fig. 3. Inhibition of ^{3}H-N-0437 binding by urine, containing increasing concentrations of unlabeled N-0437. (★) Inhibition curve in Tris/HCl buffer; (●) with 25 µl urine; (▲) with 250 µl urine.

To this end, various organic solvents (diethyl ether, dichloromethane, dichloroethane, and chloroform) were tested in combination with different extraction pH levels, ranging from 2 to 10. Dichloromethane at pH values of 4–6 appeared to give the best results, with recoveries roughly between 85 and 105%. However, it was then established that blank female urine caused inhibition of binding by more than 50% compared to buffer solutions and to blank male urine. Moreover, we discovered that male urine, after previous alcohol intake, also gave inhibition of binding of at least 50%.

These findings prompted us to abandon the liquid/liquid extraction experiments and to switch to column extraction procedures. With these, extraction of a sample can be seen as a three-step process:

(1) retention step, in which the polar matrix component passes the column more or less unhindered and the drug is retained as completely as possible;
(2) washing step, removing additional interfering material while the drug is kept on the column; and
(3) elution step, in which the drug is eluted from the column.

The following Bond Elut columns (1-ml bed capacity) were tested for urine samples:

CN, functional group = cyanopropyl;
2OH, functional group = diol;
Si, functional group = silica (no bonded phase); and
NH_2, functional group = aminopropyl.

The columns were washed with 3 ml water, 3 ml eluent, and 2 ml Tris/HCl buffer, pH 7.4, respectively, before use.

These columns were evaluated with a mixture of 50 μl urine and 950 μl Tris/HCl buffer, pH 7.4, to which N-0437 was added to yield a final concentration of 5×10^{-9} *M*. Elution was studied for a variety of organic solvents, i.e., acetone, dichloromethane, chloroform, ether, and hexane. In each case 3 ml of organic solvent was used. After elution, the organic phase was evaporated under nitrogen, the residue was redissolved in 300 μl Tris/HCl buffer, and the samples were analyzed by RRA. All columns were able to retain N-0437 from aqueous solutions essentially complete as well as the main part of the interfering compounds.

All ether extracts completely inhibited ^{3}H-N-0437 binding in the RRA, probably caused by the stabilizer of the solvent. Hexane did not elute a reasonable amount of drug from the columns. Acetone eluted the interfering compounds from the CN, 20H, and Si columns, whereas with the NH_2 columns a low recovery for N-0437 was seen. Dichloromethane and chloroform were more selective in the elution of N-0437, without eluting the interfering compounds. The combination of Si columns and dichloromethane was slightly better than the seven other combinations, so that Si columns were selected for the final procedure. For practical reasons, 3-ml columns were employed.

The inhibition of ^{3}H-N-0437 binding in the RRA by plasma constituents (proteins) has a detrimental effect on the sensitivity of the assay. Attempts were made to precipitate the plasma proteins by the addition of an organic solvent. From the solvents tested, methanol offered the best opportunities for satisfactory precipitation and a reasonable solubility for N-0437, however, low recoveries (<10%) and inadequate removal of interfering endogenous compounds forced us to apply an extraction step.

After developing the above procedure with Si columns for urine, we also tested it with plasma and found that it can be used for plasma as well.

In parallel experiments we tested five nonpolar bonded silicas for the extraction of N-0437 from plasma (Bond Elut, cyclohexyl, phenyl, C_{18}, C_8, and C_2) in combination with water–methanol mixtures for washing and elution. The C_2 columns with 100% methanol as eluent turned out to be the best combination, with a recovery between 91 and 95% for radiolabeled N-0437. However, in order to avoid losses of N-0437 during the evaporation of the methanol extract, a small quantity of acetic acid should be added. The use of Si columns for the extraction of N-0437 from plasma allows a much faster evaporation of the (DCM) extract and obliviates the addition of acetic acid.

The recovery of this procedure for urine was determined with ^{3}H-N-0437 for concentrations ranging from 5×10^{-10} to 2.5×10^{-6} *M* and was found to be between 94 and 101%, independent of the concentration.

The above procedure with Si columns was also used for plasma. The recovery from plasma ranged from 89 to 94% between 5×10^{-10} and 2.5×10^{-6} *M* and was independent of the concentration.

Validation of the Method with Si Columns

Validation experiments with 1-ml plasma and 1-ml urine samples and with N-0437 concentrations ranging from 5×10^{-10} to 5×10^{-8} *M* (158 pg/ml to 15.8 ng/ml) were carried out in duplicate series on 3 different days.

The data are presented in Tables I and II. For both plasma and urine, the detection limit (the level at which 10% inhibition occurs; IC_{10}) was 150 pg, using 1-ml samples. When using larger samples a lower detection limit can be achieved. It should be noted, however, that in actuality only the active enantiomer is determined in the RRA, which accounts for 50% in spiked samples.

It can be seen that the accuracy for plasma and urine is satisfactory and that no systematic errors appear to occur. The precision is also satisfactory and, especially for plasma, it may be considered quite good for this type of assay. As expected with an S-shaped response curve, the accuracy and precision are better in the steep part of the curve. If one transforms this response curve in a logit-type curve, a straight line can be obtained, but this will not improve the accuracy and precision.

Table I. Validation of N-0437 in Plasma by RRA (1-ml Samples)

Conc. N-0437 added		Mean Conc. N-0437 found (N = 6)		CV
M	ng/ml	*M*	ng/ml	(%)
5×10^{-10}	0.158	6.16×10^{-10}	0.194	22.4
1×10^{-9}	0.315	1.10×10^{-9}	0.347	4.71
2×10^{-9}	0.630	1.86×10^{-9}	0.586	11.68
5×10^{-9}	1.575	4.92×10^{-9}	1.550	4.52
1×10^{-8}	3.15	1.05×10^{-8}	3.31	2.76
2×10^{-8}	6.30	2.10×10^{-8}	6.62	9.33
5×10^{-8}	15.75	5.00×10^{-8}	15.75	16.13

Table II. Validation of N-0437 in Urine by RRA (1-ml Samples)

Conc. N-0437 added		Mean conc. N-0437 found ($N = 6$)		CV
M	ng/ml	*M*	ng/ml	(%)
5×10^{-10}	0.158	4.70×10^{-10}	0.149	51.89
1×10^{-9}	0.315	1.20×10^{-9}	0.378	19.27
2×10^{-9}	0.630	2.23×10^{-9}	0.702	9.28
5×10^{-9}	1.575	4.86×10^{-9}	1.531	6.01
1×10^{-8}	3.15	0.96×10^{-8}	3.02	8.05
2×10^{-8}	6.30	1.92×10^{-8}	6.05	4.49
5×10^{-8}	15.75	6.08×10^{-8}	19.15	7.24

If we accept a coefficient of variation (CV) of 20%, quantitation in plasma and urine can be done at concentrations of ≥300 pg/ml. It should be realized, though, that concentrations higher than 15 ng/ml ($>5 \times 10^{-8}$ *M*) will require dilution.

CONCLUSIONS

Because of its high affinity to D_2-receptors a sensitive radioreceptor assay for N-0437 could be developed. Calf striatum proved to be a good source for dopamine receptors. Nonspecific binding could be kept low and interference of striatal dopaminergic compounds could be circumvented. However, adaptation of the RRA to the analysis of N-0437 in plasma and urine showed that a direct assay was impossible. Therefore, an extraction procedure, utilizing silica columns, was developed, based on the combination of adequate removal of matrix interferences in the RRA and a high and reproducible recovery of N-0437.

At this time we are not aware of the presence of active metabolites or whether we are able to isolate these with the validated procedure. Therefore, the availability of an alternative method for plasma with C_2 columns can be advantageous for a selective extraction of the parent compound and/or the active metabolite. This aspect should be investigated further after sufficient insight into the metabolism of N-0437 has been obtained.

The validated procedures have a detection limit of 5×10^{-10} *M* (150 pg/ml) for both plasma and urine, whereas reliable quantitations (CV <20%) can be done at concentrations of 1×10^{-9} *M* (300 pg/ml) and up. Blank samples of plasma and urine are required to prepare adequate calibration curves.

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REFERENCES

1. M. Beaulieu, Y. Itoh, P. G. Tepper, A. S. Horn, and J. W. Kebabian. *Eur. J. Pharmacol.* 105:15–21 (1984).
2. J. van der Weide, J. B. de Vries, P. G. Tepper, and A. S. Horn. *Eur. J. Pharmacol.* 125:273–282 (1986).
3. J. van der Weide, J. B. de Vries, P. G. Tepper, and A. S. Horn. *Eur. J. Pharmacol.* 134:211–219 (1987).
4. K. Ensing and R. A. de Zeeuw. *Trends Anal. Chem.* 3:102–106 (1984).
5. M. Watanabe, S. R. George, and P. Seeman. *J. Neurochem.* 45:1842–1849 (1985).
6. E. T. Suen, E. Stefanini, and Y. C. Clement-Cormier. *Biochem. Biophys. Res. Comm.* 96:953–960 (1980).
7. P. J. Munson, and D. Rodbard. *Anal. Biochem.* 107:220–239 (1980).