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Competitive MS Binding Assays for Dopamine D_2 Receptors Employing Spiperone as a Native Marker

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Dedicated to Professor Fritz Eiden on the occasion of his 80th birthday.

A competitive MS binding assay employing spiperone as a native marker and a porcine striatal membrane fraction as a source for dopamine D_2 receptors in a nonvolatile buffer has been established. Binding of the test compounds to the target was monitored by mass-spectrometric quantification of the nonbound marker, spiperone, in the supernatant of the binding samples obtained by centrifugation. A solid-phase extraction procedure was used for separating spiperone from ESI-MS-incompatible supernatant matrix components. Subsequently, the marker was reliably quantified by LC-ESI-MS-MS by using haloperidol as an internal standard. The affinities of the test compounds, the dopamine receptor antagonists (+)-butaclamol, chlorpromazine and (S)-sulpiride obtained from the competitive MS binding assay were verified by corresponding radioligand binding experiments with [^{3}H]spiperone. The results of this study demonstrate that competitive MS binding assays represent a universally applicable alternative to conventional radioligand binding assays.

Introduction

Competitive-binding assays are a technique commonly used in the biosciences, especially in the drug-discovery process, to determine the affinity of a ligand for a specific binding site.[1] In general, competitive-binding assays are performed with markers, ligands addressing the target of interest, for example, a pharmacological receptor, with high affinity and selectivity, and additionally containing a label to improve their quantification. Most frequently, either a ligand labelled with a radioisotope (e.g. ^{3}H, ^{35}S or ^{125}I) or with a fluorescent moiety is used.[1–4] However, both methods have serious drawbacks. The synthesis of radioligands is, in general, expensive, and special safety precautions have to be observed when radioligand-binding assays are performed. Moreover, the disposal of radioactive waste may result in considerable extra costs. Whereas the latter problems do not apply to assays based on ligands provided with a fluorescent group, the synthesis of such markers is a time-consuming process, as the ligand has to be reoptimised on addition of the fluorescent group[4,5]. There is clearly a need for new and universally applicable methods that allow competitive-binding assays to be performed with unlabelled ligands.

Mass spectrometry (MS) has undergone tremendous technological improvements over the past decade, affecting almost every aspect of MS analysis, including continuous increases in the performance and sensitivity of MS analysis. As a consequence of these improved capabilities, for example, the low limits of quantification, modern MS is now used successfully in diverse binding assays.[6–10] Most of these sophisticated assays are characterised by an affinity selection step of structurally unknown library components followed by elaborate analytical procedures to identify and quantify all the different target-bound library components by employing MS. However, methods that combine the principle of competitive-binding studies with MS detection are still rare,[11–16] even though this approach is especially rewarding, since it offers the opportunity to substitute conventional competitive radioligand-binding assays.

In a recent study, we demonstrated the feasibility of competitive-binding experiments with MS detection at native dopamine D_1 receptors in porcine striatal cell membranes. In contrast to conventional binding assays, these competitive MS binding experiments were performed with concentrations of both the marker and the target in the range of the K_d of the marker for the target. Under these conditions, competitive binding of a test compound to the target can be tracked reliably by quantification of the nonbound marker, instead of the bound marker.[14] An advantage of measuring the nonbound marker is that no additional step for the liberation of the bound maker is required, and the raw samples from the binding experiments can be directly employed in the MS analysis, provided that a suitable buffer system compatible with both steps, the binding experiments and the MS analysis, is used. In the study mentioned above, we found that ammonium for-

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mate was a usable buffer system. It was well tolerated in the binding experiments and was well suited for the MS analysis, which was performed by ESI on a triple-quadrupole mass spectrometer operated in the MRM mode. Several receptors, however, require a specific ionic environment to enable optimal binding that is not suitable for MS analysis. Consequently, we thought it worthwhile to demonstrate that our concept of competitive MS binding experiments is also feasible for receptors requiring this kind of ionic buffer systems, even though the matrix resulting from the binding experiments has to be removed prior to MS analysis. With this goal in mind, we set out to perform MS binding experiments at dopamine D_2 receptors for which a specific buffer system with Tris (50 mM) as the main component, in addition to several inorganic salts, is generally used.

Results and Discussion

To establish a competitive MS binding assay for dopamine D_2 receptors (or, to be more precise, D_2-like dopamine receptors), the first step is to select an appropriate marker. [^{3}H]Spiperone is a common marker with a high affinity for D_2 receptors and is often used in radioligand binding assays.[17,18] We therefore examined whether a LC-MS detection method for unlabelled spiperone could be established that would allow reliable monitoring of spiperone with a sensitivity high enough for competitive MS binding experiments. For the LC-MS analysis, a triple quadrupole mass spectrometer with an electrospray interface directly coupled to a HPLC unit equipped with a RP 8 column was used (LC-ESI-MS-MS). When employing a mixture of acetonitrile and 0.1% aqueous formic acid (30:70) as eluent, spiperone could be reliably quantified in the multiple reaction monitoring mode (MRM) at a transition from 396.0 (*m/z*) to 123.0 (*m/z*), even at subnanomolar concentrations (Figure 1).

A porcine striatal membrane fraction was selected to serve as a source for D_2 receptors in the competitive MS binding assay to be developed. The brain tissue is easy to obtain and proved to be well suited for our previous MS binding experiments at dopamine D_1 receptors.[14] Binding assays based on [^{3}H]spiperone as a marker are, however, not fully selective for dopamine D_2 receptors, as spiperone has only limited selectivity for this binding site.[17,18] For example, the 5-HT_2 receptors, which are probably amply present in the porcine striatal brain membrane fraction, are addressed by spiperone as well. Though the selectivity of binding assays based on spiperone can be improved by various methods (e.g. by blocking 5-HT_2 receptors with ketanserin), no attempts were made in this direction, as this was beyond the scope of the present study. For the sake of simplicity, however, the binding sites addressed by spiperone are referred to as D_2 receptors in this paper.

First, we determined the K_d of spiperone (490 ± 50 pM) and the B_{max} of spiperone-labelled binding sites in the porcine striatal membrane fraction (370 ± 70 fmol mg^{-1}), following a classical approach with [^{3}H]spiperone as a radioligand (see Supporting Information). The test assertained that both the affinity and density of D_2 binding sites are high enough to allow us to perform competitive MS binding assays with the concentration

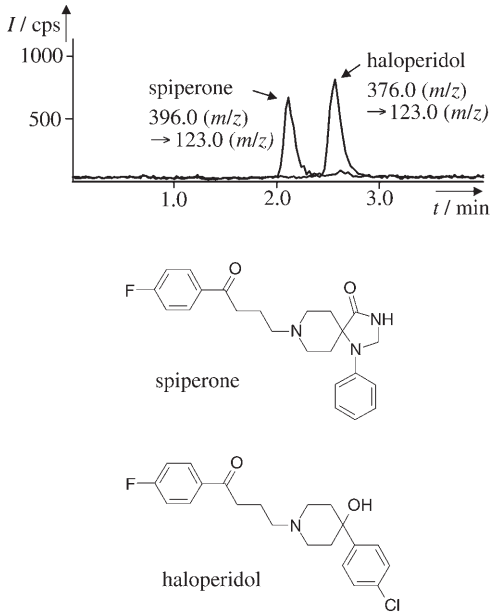

Figure 1. Representative MRM chromatogram of a matrix sample spiked with spiperone (0.875 nM, *m/z* 396.0 to *m/z* 123.0) and haloperidol (0.875 nM, *m/z* 376.0 to *m/z* 123.0) after SPE on an Oasis HLB cartridge followed by LC (RP8 column; solvent: CH_3CN/0.1% HCOOH, 30:70; 150 μL min^{-1}), as described in the Experimental Section.

of both the marker and the binding sites close to the K_d value. This is an important prerequisite if binding experiments are to be tracked by monitoring the amount of nonbound marker.[14] Then, the concentrations of the nonbound marker and the bound marker should be in the same order of magnitude, and the amount of nonbound marker should undergo significant changes when increasing amounts of test compounds are competing with the marker for the binding sites.

As mentioned above, our intention was to perform the competitive MS binding experiments in the same nonvolatile buffer system (50 mM Tris HCl, 120 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$) commonly employed in [^{3}H]spiperone-binding experiments, in order to demonstrate that this approach is not limited by the buffer system used.

Since the MS dopamine D_2-binding experiments were to be terminated by centrifugation, the resulting samples were assumed to be unsuitable for direct ESI-MS analysis after only a rapid HPLC prepurification. A preliminary experiment following these principles, in which the LC-ESI-MS-MS signal of spiperone was found to be substantially suppressed when the marker was analysed after short retention times, straight out of the supernatant, clearly verified this assumption (data not shown).

A solid-phase extraction procedure (SPE) sample preparation with Oasis HLB cartridges allowed efficient separation of spiperone as well as haloperidol, which was used as internal standard, from the matrix of the supernatant of the binding samples. Following this preparation, spiperone and haloperidol were analysed in a single LC-ESI-MS-MS run (Figure 1) with satisfactory recovery (spiperone: 92 ± 0.4%, haloperidol: 79 ± 3.2%, means ± SD, $n = 6$, for details, see Experimental Section). A control experiment revealed that none of the test com-

Figure 2. Flow chart of competitive MS binding experiments.

pounds used in the subsequent binding assays affected the recovery or the quantification of the marker by the SPE/ESI-LC-MS-MS procedure.

However, the first competitive MS binding experiments with concentrations of the marker and binding sites of about 500 pM failed. This is possibly the result of a high degree of nonspecific binding that leads to a depletion of the marker. Indeed, when the concentration of spiperone was raised to 1.25 nM, while keeping the concentration of binding sites around 400 pM, the competitive-binding assay worked. The concentration of the nonbound marker did now change significantly with varying concentrations of test compound. (The flow chart of the competitive-binding experiment is summarised in Figure 2.)

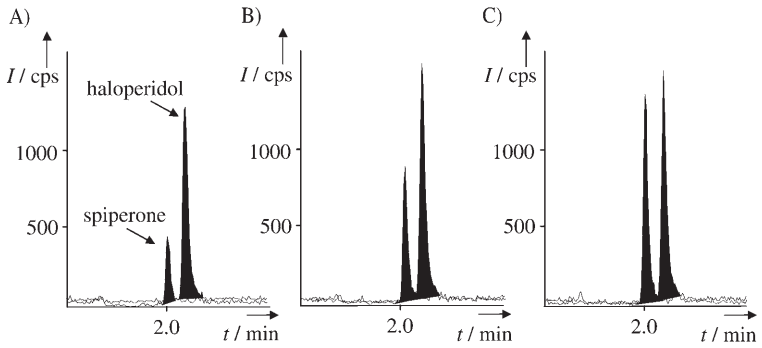

Figure 3. Representative MRM chromatograms of spiperone (*m/z* 396.0 to *m/z* 123.0) from supernatants of binding experiments with spiperone as native marker and a porcine striatal membrane fraction as a source of dopamine D_2 receptors A) in the absence of (+)-butaclamol or in the presence of B) 100 nM or C) 10 μM (+)-butaclamol. ESI-MS-MS was performed after SPE on an Oasis HLB cartridge, followed by LC (RP8 column; solvent: CH_3CN/0.1% HCOOH, 30:70; 150 μL min^{-1}), as described in the Experimental Section. All supernatants were spiked with haloperidol (0.875 nM, *m/z* 376.0 to *m/z* 123.0) as an internal standard. The difference between nonbound spiperone in (C) and (A) represents specific binding.

The known dopamine receptor antagonists (+)-butaclamol, chlorpromazine and (*S*)-sulpiride were selected as competitors in this new type of binding study. The assays were performed by incubating varying concentrations of the test compounds with porcine striatal membrane fraction (25 °C, 40 min) in the presence of spiperone (1.25 nM). The experiments were stopped by centrifugation, and the amount of nonbound spiperone was then quantified from the supernatants, as described above, by LC-ESI-MS-MS after SPE sample preparation (Figure 3). Based on the data obtained, competition curves describing the concentration of bound spiperone in relation to the concentration of the test compounds could be generated (Figure 4).

The difference between the bottom and the top region of the curve as defined by control samples incubated without any competitor (total binding) or with (+)-butaclamol (10 μM, nonspecific binding), respectively, represents the specific binding of spiperone.

In total, the binding curves disclosed an extraordinarily high amount of nonspecific binding of spiperone. To explain this, diverse factors have to be considered: First, the high amount of nonspecific spiperone binding is clearly the consequence of the high amount of membrane preparation (up to 1 mg total protein in 500 μL) required for the competitive-binding experiment. Secondly, "nonbound" spiperone entrapped in the pellet during centrifugation will substantially contribute to nonspecific binding. Furthermore , the relatively high lipophilicity of spiperone causing adsorption to lipid membranes,[19] as well as additional labelling of binding sites by spiperone with high affinity that are not completely blocked by (+)-butaclamol,[17] could add to the highly nonspecific binding of spiperone, compared to other markers.

Variations in the bottom and top ends of the curves between the different experiments are also striking. This discrepancy is likely to be due to variations between the different membrane preparations employed in the assays. It should be emphasised, however, that the amount of nonspecific binding (binding remaining in the presence of 10 μM (+)-butaclamol) was individually determined for every binding curve, thereby assuring the correct analysis of specific binding in each experiment. In this way, the IC_{50} values (i.e., the concentration of a test compound that reduces the specific binding of the marker to 50%) could be reliably deduced from these binding curves analogously to conventional binding assays and so calculated accordingly (see Table 1).

The mass-spectrometric quantification procedure could, indeed, be verified by a control experiment performed under identical conditions with (*S*)-sulpiride as the competitor and [^{3}H]spiperone as marker, in place of spiperone. The results of this study were in good accordance with the results found in the MS binding experiments (for experimental details and binding curves, refer to the Supporting Information, for results, Table 1).

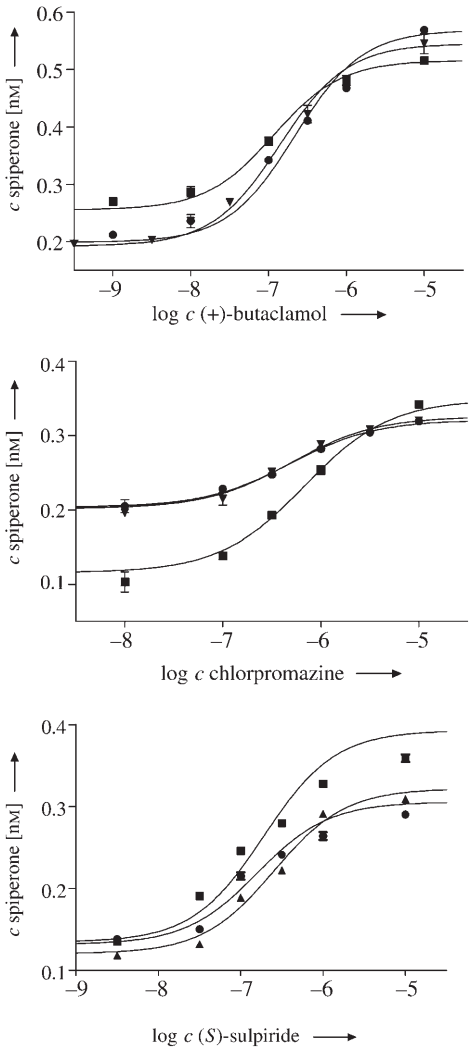

Figure 4. Binding curves for (+)-butaclamol, chlorpromazine and (*S*)-sulpiride as generated by nonlinear regression for competitive MS binding assays. Three binding experiments were carried out for each ligand. The individual points describe nonbound spiperone quantified by LC-ESI-MS-MS from the supernatant of binding samples (performed in triplicate, means $\pm s$).

In order to prove the validity of the results obtained in our competitive MS binding experiments, we also performed conventional [^{3}H]spiperone binding assays (Figure 5).

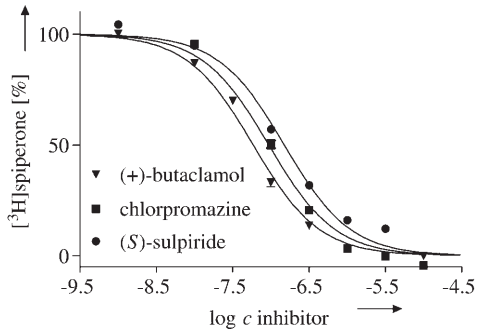

Figure 5. Representative binding curves for (+)-butaclamol, chlorpromazine and (*S*)-sulpiride from conventional radioligand-binding assays monitoring bound [^{3}H]spiperone after filtration of the binding samples (each performed in triplicate, means $\pm s$) on GF/C filters by means of a cell harvester.

These experiments were conducted with a much smaller amount of the porcine striatal membrane fraction; about a tenth of that used in the MS binding experiments. It should be noted that the MS binding experiments described in this paper required a much higher concentration of binding sites than conventional radioligand-binding assays based on the analysis of the bound marker by liquid scintillation. In addition, in the case of the radioligand assay, the incubation was stopped by filtration on glass fibre filters, which were finally measured in a liquid scintillation counter.

To compare the affinities determined in the competitive MS binding assay with conventional radioligand binding assays, all IC_{50} values were converted to K_i values. Since marker depletion in the competitive MS binding assays (as well as the identically performed control experiments with [^{3}H]spiperone) had surpassed the critical limit (>10%) for the calculation, an equation was used to take this into account.[20,21] The K_i values of the conventional radioligand-binding assays were calculated according to Cheng and Prusoff as, in this case, depletion of the marker was considerably below 10%.[20,22] The K_i values obtained in the conventional [^{3}H]spiperone-binding experiments are roughly in accord with the results from our competitive MS binding assays (Table 1). The observed discrepancy is probably a result of the very high amount of membrane material employed in the competitive MS binding assays, which, in turn, would cause an extraordinarily high amount of nonspecific binding of the test compounds. Consequently, such a substantial amount of nonspecific binding would lead to a significant

Table 1. Affinities for dopamine antagonists obtained by competitive MS binding experiments and radioligand binding assays, respectively.

	Spiperone (nonbound)[a]		[^{3}H]Spiperone (nonbound)[b]		[^{3}H]Spiperone (bound)[c]	
	IC_{50}[d]	K_i [nM][d]	IC_{50}[d]	K_i [nM][d]	IC_{50}[d]	K_i [nM][d]
(+)-butaclamol	140 ± 50	43 ± 10		n.d.[e]	44 ± 8	8.7 ± 1.8
chlorpromazine	560 ± 90	220 ± 20		n.d.[e]	120 ± 10	23 ± 3
(*S*)-sulpiride	210 ± 30	65 ± 8	110 ± 25	45 ± 9	120 ± 20	25 ± 4

[a] Nonbound spiperone determined by LC-ESI-MS-MS from the supernatant of the binding samples obtained by centrifugation; [b] Nonbound [^{3}H]spiperone determined by liquid scintillation counting from the supernatant of the binding samples obtained by centrifugation; [c] Bound [^{3}H]spiperone measured by liquid scintillation counting bound marker after filtration on GF/C filters by means of a cell harvester; [d] Means ± SEM from three experiments each performed in triplicate; [e] Not determined.

depletion of the test compound and an enhancement of the K_i values. This assumption is supported by the observation that the deviations between the MS K_i values and those from the conventional [^{3}H]spiperone-binding experiments are markedly higher for the lipophilic compounds (+)-butaclamol (log D at pH 7 = 4.25)[23] and chlorpromazine (log D at pH 7 = 3.01)[23] than for sulpiride (log D at pH 7 = −1.49).[23] However, further investigations are necessary to better understand this problem.

Conclusion

The principle of competitive MS binding assays characterised by the mass-spectrometric quantification of a native marker has been applied to dopamine D_2 receptors. Competitive-binding experiments were performed in a nonvolatile incubation buffer by employing spiperone as a native marker for dopamine D_2 receptor binding sites in a porcine striatal membrane fraction (additional labelling of other binding sites, for example, 5-HT_2 receptors cannot be excluded). Quantification of nonbound spiperone from the supernatant of such binding samples could be realised by LC-ESI-MS-MS after SPE sample preparation; this allowed the determination of the affinities of several dopamine receptor antagonists for D_2 receptors. The results produced by this novel approach agree well with control experiments performed identically by monitoring nonbound [^{3}H]spiperone and are roughly in accord with conventional radioligand-binding assays of bound [^{3}H]spiperone. The explanation for the differences between the K_i values from conventional radioligand binding assays and MS binding assays remains speculative. It is possible, however, that the depletion of the test compounds in the MS-based assay, which is due to the high amounts of membrane material required in the competitive-binding experiments, adversely affects affinity determination. As this is obviously the result of an insufficient density of target sites in the porcine striatal membrane preparation, it should be easily overcome by using a more appropriate source for the target sites, such as heterologously expressed D_2 receptors. Alternatively, the competitive MS binding assays might be performed with quantities of membrane material that represent receptor concentrations markedly below the K_d value of the marker. This approach would have exceeded the sensitivity of the mass spectrometer employed in this study. However, with the new generation of triple-quadrupole mass spectrometers on the market, which display a sensitivity up to 100 times higher than that of the instrument we used, such binding experiments should be feasible.

Nevertheless, the approach presented is rather straightforward and offers an attractive alternative to conventional binding assays based on markers labelled with radioisotopes or fluorescent groups, since competitive MS binding assays are simple to perform and work well with demanding targets, such as native membrane-bound receptors, as demonstrated in this study.

Clearly, further investigation is necessary in order to improve the throughput of this procedure; for example, by scaling up the assay format to 96-well plates, simplifying sample preparation, miniaturising HPLC, utilising column-switching systems or employing less time-consuming MS techniques. Additionally, the possibility should be examined of whether monitoring the bound marker in competitive MS binding experiments yields a more favourable ratio of specific versus nonspecific binding. Regarding mass-spectrometric quantification of the bound marker, it should be noted that such a procedure would parallel conventional competitive radioligand-binding assays more closely and might thereby gain wider attention. Bearing in mind the continuously increasing sensitivity of mass spectrometers, the realisation of this idea appears to be feasible.

To sum up, this novel approach has two major advantages. First, a ligand for a binding site does not have to be labelled and, secondly, it is universally applicable. This will ensure that this new method will gain increasing importance as an attractive tool in primary drug screening.

Experimental Section

Chemicals: The compounds employed were purchased from the following sources: spiperone, pimozide and (*S*)-sulpiride from RBI/Biotrend, Köln, Germany; chlorpromazine, (+)-butaclamol and haloperidol from RBI/Sigma, Taufkirchen, Germany; [^{3}H]spiperone (814 GBq mmol^{-1}) from Amersham Bioscience, Freiburg, Germany.

Membrane preparation: Striatum from pig brains (from the local slaughterhouse) was homogenised in 10 volumes of sucrose (0.32 M) with a potter (PotterS, Braun, Melsungen, Germany, 1200 rpm, 10 up-and-down strokes), and centrifuged (1000 *g*, 10 min, 4 °C). The supernatant was centrifuged again (20000 *g*, 10 min, 4 °C). The resulting pellets (P_2) were resuspended in Tris-HCl buffer (50 mM, pH 7.4) and centrifuged (30000 *g*, 20 min, 4 °C). The last centrifugation was repeated. The final pellet was resuspended in Tris-HCl buffer, and the protein was determined according to Bradford, with BSA as standard, after treatment with an equal volume of NaOH (1 M) for 1 h.[24] Portions of the porcine striatal membrane fraction were frozen at −80 °C.

Competitive spiperone-binding assays: A portion of the membrane fraction was thawed, centrifuged (48000 *g*, 30 min, 4 °C) and resuspended in Tris-salt buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl and 1 mM EDTA pH 7.4). Aliquots representing about 200 fmol specific spiperone-binding sites of this membrane suspension (estimated from the B_{max} for each preparation determined in [^{3}H]spiperone saturation assays, as described in the Supporting Information, and from the total amount of protein determined according to Bradford) were incubated in the presence of spiperone (1.25 nM), test compounds in (at least 6) varying concentrations and Tris-salt buffer in a total volume of 500 μL in polypropylene tubes (1.5 mL) in a shaking water bath (40 min, 25 °C). The samples were repeatedly vortexed to avoid sedimentation of the membrane particles. The incubation was stopped by centrifugation (50000 *g*, 20 min, 4 °C). The supernatant (400 μL) was transferred to polypropylene tubes (1.5 mL) and frozen at −18 °C for further use. Control samples without test compounds were used to define total binding. Nonspecific binding was determined in the presence of (+)-butaclamol (10 μM). Samples without spiperone and without test compounds were treated in the same manner as described above to obtain matrix samples.

SPE and sample preparation: The frozen samples from the competitive spiperone-binding assays (see above) were thawed at room temperature (1 h). Haloperidol (30 μL, 5 nM) in acetonitrile and 0.5% formic acid (25:75; all ratios for solutions are expressed

as *v/v*) was added to each sample as an internal standard, whereas additional matrix samples were spiked with spiperone and haloperidol (30 μL, 5 nM each), each in acetonitrile/0.5% formic acid (25:75). Subsequently, the samples were treated with 0.5% aqueous ammonia (370 μL) and briefly vortexed. Oasis HLB extraction cartridges (30 μm, 1 cm^3, 10 mg, Waters, Eschborn, Germany) were preconditioned with acetontrile/methanol (90:10, 1 mL) followed by 0.5% aqueous ammonia. From the final samples (800 μL, binding samples as well as matrix samples), aliquots (700 μL) were loaded onto the SPE cartridges. The loaded cartridges were aspirated (flow rate $\leq 2\ mL\ min^{-1}$) by using a LiChrolut vacuum manifold (Merck, Darmstadt, Germany). Subsequently, the cartridges were washed with 0.5% aqueous ammonia (1.5 mL). After the cartridges had been dried at maximal vacuum (20 s), the analyte was eluted four times (0.5 mL) with acetonitrile/methanol (90:10), into polypropylene tubes. The eluent collected was evaporated at 40 °C to complete dryness by using a Christ RVC 2–18 vacuum centrifuge (Christ, Osterode, Germany). Finally, the residues were reconstituted in acetonitrile/0.1% formic acid (25:75, 150 μL) and filtered through 0.45 μm GHP Acrodisc Syringe Filters (Waters, Eschborn, Germany) into autosampler vials.

To determine the recoveries of spiperone and haloperidol, matrix samples spiked with spiperone (30 μL, 5 nM) or haloperidol (30 μL, 5 nM) as well as void matrix samples were processed, as described above. The evaporated spiked matrix samples were reconstituted in acetonitrile/0.1% formic acid (25:75, 150 μL). These samples were compared with the void matrix samples, which were reconstituted in acetonitrile/0.1% formic acid (25:75, 150 μL) containing spiperone (0.875 nM) or haloperidol (0.875 nM) by LC-ESI-MS-MS, as described below.

LC-ESI-MS-MS analysis: LC-MS Analysis was carried out on an Agilent 1100 HPLC instrument (vacuum degasser, quaternary pump, autosampler and oven, Agilent, Waldbronn, Germany) coupled to an API 2000 triple-quadrupole mass spectrometer with an electrospray ionisation source (Applied Biosystems, Darmstadt, Germany). A Phenomenex Luna C8 (Phenomenex, Aschaffenburg, Germany) column (50×2.0 mm, 3 μm particle size, 100 Å pore size) with a Phenomenex C8 security guard column (4.0×2.0 mm) was employed for chromatographic separation under the following conditions: column temperature 25 °C, mobile phase: acetonitrile/0.1% formic acid (30:70), flow-rate: $150\ \mu L\ min^{-1}$; injection volume: 25 μL (followed by a washing step with methanol). The operating parameters of the MS detector in the MRM mode were set as follows: source temperature 480 °C, ion-spray voltage +3000 V, collision energy 61 V, nitrogen was used as the curtain (96 kPa), as the nebulising (483 kPa), as the auxiliary (207 kPa) and as the collision gas (82.7 kPa). The entrance potential, the declustering potential, the focusing potential and the collision cell exit potential were set to 10.5 V, 36 V, 240 V and 2 V, respectively. The transitions from 396.0 (*m/z*) to 123.0 (*m/z*) for spiperone and from 376.0 (*m/z*) to 123.0 (*m/z*) for haloperidol were monitored by operating Q1 and Q3 under low mass-resolution conditions and dwell times of 500 ms for a total acquisition time of 5 min. For routine quantification, the effluent up to 1.5 min and effluent from 3.5 min to 5.0 min was diverted to waste by a Valco valve in order to protect the mass spectrometer. Data were collected and quantified (by means of the internal standard without further manipulation of the data like smoothing etc.) by using Analyst 1.2 (Applied Biosystems, Darmstadt, Germany).

Radioligand-binding assays: Radioligand binding experiments were performed according to described [^{3}H]spiperone assay methods.[25–27] A portion of the porcine striatal membrane fraction was thawed, centrifuged (48000 *g*, 20 min 4 °C) and resuspended in 3 volumes of Tris-salt buffer. In competition experiments, aliquots (about 100 μg protein) were incubated in the presence of [^{3}H]spiperone (about 2 nM), test compounds in varying concentrations and Tris-salt buffer (in total 500 μL) in polystyrene tubes in a shaking water bath (40 min, 25 °C). Incubation was terminated by filtration through Whatman GF/C filters presoaked (1 h) in 0.5% polyethylenimine by using a Brandel M-24R harvester (Gaithersburg, MD, USA). The filters were rapidly rinsed with cold buffer (4× 2 mL), and [^{3}H]spiperone bound on the filters was counted in Rotiszint Eco Plus (3 mL, Roth, Karlsruhe, Germany) by using a Packard TriCarb 1600 (Perkin–Elmer Life Sciences, Freiburg, Germany) liquid scintillation counter. Total binding and nonspecific binding were defined as described for competitive spiperone binding.

Analysis of binding experiments: In all experiments, specific binding—defined as the difference between total and nonspecific binding—was analysed. The concentration of a competing drug that inhibits 50% of specific binding (IC_{50}) was calculated with Prism 2.01 (GraphPad Software, San Diego, CA, USA) for sigmoidal dose-response curves ($n_H = 1$ or -1) by fixing A (bottom) and B (top) to the values obtained for the controls without competitor (total binding) or with (+)-butaclamol (10 mM), respectively. K_i values were calculated according to Cheng and Prusoff[22] when bound [^{3}H]spiperone was analysed. K_i values were calculated with Equation (1) when nonbound spiperone was analysed.[21]

$$K_i = \frac{IC_{50}}{2^{\frac{(L^* - L_0^*)}{L_0^*}} + 1 + \frac{L^*}{K_d}} \quad (1)$$

L^*: concentration of spiperone at the IC_{50}; L_0^*: concentration of free spiperone in the absence of a competing ligand.

Unless otherwise stated, all data are expressed as mean ± standard error of the mean of three separate experiments, each performed in triplicates.

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Keywords: binding assays · dopamine receptors · drug screening · mass spectrometry · medicinal chemistry

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