TOTAL VERSUS UNSPECIFIC BINDING: STANDARDIZATION AND OPTIMIZATION OF RECEPTOR-LIGAND BINDING ASSAYS

Christoph A. Seyfried and Peter Tobler

Department of CNS Research, Biological Research, E. Merck, Frankfurter Strasse 250, D-61 Darmstadt, Germany

SUMMARY

Intra- and interassay imprecision was evaluated in 3 *in vitro* ligand binding assays using different types of filtering devices, glass fiber filters and variations in methodology. In the [^{3}H]-spiroperidol test, the already low unspecific binding seemed to be dependent on the type of filters employed, whereas in the [^{3}H]-ketanserin- and [^{3}H]-GR 65630-binding tests, differences were within the range of the normal variabilities. However, in the latter test, which is problematic owing to the high unspecific binding of [^{3}H]-GR 65630, it was found that although the percentage of specific binding was fairly constant on different sheets, large differences in the absolute amounts of total and unspecific binding were observed on consecutive sheets in the same experiment. Thus, it is critically important to filter samples for total and unspecific binding together on the same filter sheet for the calculation of specific binding. Under these precautions, highly reproducible results for IC_{50}-values in the screening of potential $5HT_3$-receptor ligands were obtained in spite of using rat entorhinal cortex, a relatively large area with suboptimal $5HT_3$-receptor density. In contrast, when using rat area postrema, which is optimal with respect to receptor density, more than 10 times the number of rats is necessary for a competition experiment due to the small size of this brain part. Since IC_{50}-values for both areas compare favorably, entorhinal cortex should be used for ethical reasons and to minimize costs.

KEY WORDS

Receptor binding, assay imprecision, D_2-, $5HT_2$-, $5HT_3$-receptors

INTRODUCTION

Although *in vitro* ligand binding assays are very widely used for the screening of new drugs and the characterization of receptor subtypes, relatively few reports exist in the literature on the methodology and the optimal experimental conditions of these tests. This is probably due to the fact that for many receptor types, sufficiently high values for the ratio of total vs unspecific binding are found, i.e., a high signal-to-noise ratio. Typically, these assays are rather robust and insensitive to slight variations in the experimental conditions. On the other hand, if receptor density of a tissue is low, the signal-to-noise ratio may become unfavorable. Thus, under these conditions, the specific binding (the difference between total binding and unspecific binding) may become rather low and highly variable, depending on the variations of the total and the unspecific binding, and IC_{50}-values may differ by several orders of magnitude in different experiments. We therefore investigated several experimental parameters, for instance the influence of the filter blank and the time of pre-soaking, on total and unspecific binding. Furthermore, glass-fiber filters from two different manufacturers and two types of filtering devices were compared.

MATERIALS AND METHODS

Male Wistar rats of 200-250 g body weight were used (Iva: WIWU, Ivanowas, Kisslegg, FRG). Striatum and frontal cortex were dissected out as described /1/ for spiroperidol and ketanserin binding, respectively. These assays were performed according to the literature with slight modifications /2/. Briefly, incubations were performed in 50 mmol/l Tris/HCl-buffer, pH 7.7, final volume 2 ml, containing membranes from about 3 mg or 10 mg of original striatal or cortical tissue, respectively, and final concentrations of about 0.2 nmol/l [^{3}H]-spiroperidol (for D_2-receptors in striatum) and 0.4 nmol/l [^{3}H]-ketanserin (for $5HT_2$-receptors in cortex). Unspecific binding was determined in the presence of 1 μmol/l (+)-butaclamol and 10 μmol/l ritanserin for [^{3}H]-spiroperidol and [^{3}H]-ketanserin binding, respectively. For central $5HT_3$-receptors, [^{3}H]-GR 65630 and membranes from rat entorhinal cortex were used /3/. Entorhinal cortex was dissected according to the stereotactic atlas of König and Klippel at the following coordinates: A: 2.4-1.7;

L: 2.5-6.5; V: 2.0-4.0. Incubations were carried out in HEPES/NaOH-buffer, final volume 2 ml, pH 7.4, containing membranes from about 10 mg original entorhinal cortex tissue and 0.05 or 0.2 nmol/l [^{3}H]-GR 65630. Unspecific binding was obtained in the presence of 1 μmol/l zacopride.

Generally, filter sheets were pre-soaked in the corresponding assay buffers for 30 min, although shorter or longer time periods (15 min to 3 h) did not influence the results.

In two experiments (indicated by an asterisk in Table 4), filter sheets were also pre-soaked in 0.1% polyethyleneimine for 3 h. Filter blanks were prepared by filtering the incubation mixture lacking the membrane preparations. Glass-fiber filter sheets Whatman® GF/B (lot # 7584 or 7823) were purchased from Bender and Hobein, 8 München, F.R.G. and GF/C (without lot #) from Brandel, Gaithersburg, MD, U.S.A. Alternatively, glass-fiber filter sheets Ederol® T60 (lot # 1539) and T61 (lot # 1239) from J.C. Binzer GmbH, 3559 Hatzfeld/Eder, F.R.G., were used. Radioactive ligands, i.e. [^{3}H]-spiroperidol, 888 GBq/mmol (24 Ci/mmol, NET-565), [^{3}Hl-ketanserin, 2.83 TBq/mmol (76.5 Ci/mmol, NET-791) and [^{3}H]-GR 65630, 3.22 TBq/mmol (87.0 Ci/mmol, NET-1011) came from DuPont, Biotechnology Systems Division, 6072 Dreieich, F.R.G. All other chemicals were of at least analytical-grade purity. For filtration, either a 24-sample Brandel harvester (M-24R) or a 30-sample Brandel harvester (M-30R) in combination with a 30-sample Brandel deposit and dispensing system (PD-030) were used (Brandel, Gaithersburg, MD, U.S.A.). The diameter of the deposed filters was 18 mm. Radioactivity was counted in a Tricarb 2500TR (Canberra Packard) liquid scintillation counter at an efficiency of 40-50%.

RESULTS

As can be seen from Table 1, virtually no differences were observed in the results obtained using either the M-24R harvester and transferring the filters manually to the scintillation vials or the M-30R harvester in combination with the automatic deposit system, the latter method being much less time consuming. Differences between the two systems were in most cases within the range of the standard deviations of parallel assays for [^{3}H]-spiroperidol or [^{3}H]-GR 65630. Similar results were obtained for the [^{3}H]-ketanserin binding test (data not shown). Furthermore,

Table 1

Comparison of different filtering devices and intra-assay imprecision

Device	Assay	Filter type	Total binding dpm ± SD	CV	Unspecific binding dpm ± SD	CV	Filter blank dpm ± SD	CV
M-24R	[^{3}H]-Spiroperidol	GF/B	4446 ± 90 (6)	2	391 ± 56 (3)	14	282 ± 17 (3)	6
M-30R/ PD-030	[^{3}H]-Spiroperidol	GF/B	4350 ± 123 (6)	3	247 ± 31 (3)	13	175 ± 38 (3)	22
M-24R	[^{3}H]-Spiroperidol	T 61	4192 ± 210 (6)	5	140 ± 6 (3)	4	67 ± 3 (3)	4
M-30R/ PD-030	[^{3}H]-Spiroperidol	T 61	4241 ± 102 (6)	2	136 ± 3 (3)	2	61 ± 11 (3)	18
M-24R	[^{3}H]-GR 65630	GF/B	3575 ± 505 (12)	14	2289 ± 437 (6)	19	701 ± 29 (3)	4
M-30R/ PD-030	[^{3}H]-GR 65630	GF/B	3780 ± 331 (9)	9	2012 ± 279 (6)	14	894 ± 71 (3)	8
M-24R	[^{3}H]-GR 65630	T 61	3697 ± 59 (6)	2	2463 ± 66 (3)	3		
M-30R/ PD-030	[^{3}H]-GR 65630	T 61	3678 ± 82 (6)	2	2550 ± 155 (3)	6	431 ± 21 (3)	5

Values are means ± SD of the number of parallel assays in parentheses. Final concentration of [^{3}H]-spiroperidol or [^{3}H]-GR 65630 was about 0.2 nmol/l. In each experiment, all parallel assays were obtained from one single filter sheet.

pre-soaking of the filter sheets for periods from 15 min to 3 h in the corresponding assay buffers had no discernible effect on the results (data not shown). It is also clear from the data in Table 1 that with [^{3}H]-spiroperidol, in contrast to [^{3}H]-GR 65630, the unspecific binding poses a minor problem, the ratio total/unspecific binding being >10, and most of the unspecific binding seems to be due to binding to the filter material. In the [^{3}H]-GR 65630 test, filter binding seems to contribute much less to the unspecific binding.

In Table 2, the inter-assay imprecision in the [^{3}H]-spiroperidol test is summarized. It is noteworthy that the variability of the unspecific binding and the filter blank is rather high in spite of the relatively low variability of the total binding. An explanation might be that the precision of the radioactivity counting decreases at low radioactivity levels at the constant counting periods used. The rather high variability of the unspecific binding is reflected in the high variability of R. Nevertheless, with $R \geq 10$, any determinations of IC_{50}-values of competing ligands will be very precise and differ, according to our experience, only by a factor of 2 to 3, which is sufficient for the conventional *in vitro* screening of drugs, by which differences of up to six orders of magnitude are assessed. With respect to the different filter types, T 60 or T 61 filters give even lower unspecific binding with R-values $\geq$ 20, at least with the batches of filter sheets employed in this investigation. This trend is also evident in the data given in Table 1.

In the [^{3}H]-ketanserin test (Table 3), the results were similar to the [^{3}H]-spiroperidol test, with a trend for higher R-values with T 60 or T 61 as compared to GF/B filters, although the ranges for the R-values overlap for all three types of filters, all giving favorable ratios. As in the [^{3}H]-spiroperidol test, most of the unspecific binding seems to be due to the filter blank.

Owing to the high unspecific binding in the [^{3}H]-GR 65630 test, variabilities at two ligand concentrations, i.e. 0.2 and 0.05 nmol/l, were determined. As mentioned above, most of the unspecific binding in this test is apparently not related to the filter blank but stems from binding to membrane proteins different from the $5HT_2$-receptor-sites that is not displaced even at the high zacopride concentration of 1 μmol/l used. As can be seen (Table 4), the R-values at 0.2 nmol/l [^{3}H]-GR 65630 ($\sim$ 80000 dpm/2 ml) cluster around 1.60, whereas at 0.05 nmol/l ($\sim$ 20000 dpm/2 ml), values even above 2.00 are reached, though the difference is probably not statistically significant. Compared to the [^{3}H]-spiroperidol and [^{3}H]-ketanserin binding assays, these R-values are rather

Table 2

Inter-assay imprecision in the [^{3}H]-spiroperidol binding assay

Filter type	n	Total binding dpm ± SD	CV	Unspecific binding dpm ± SD	CV	R ± SD	CV	Filter blank dpm ± SD	CV	Ligand conc. dpm/2ml ± SD	CV
GF/B	5	4250 ± 158	4	354 ± 84	24	12.6 ± 3.2	25	263 ± 65	25	22233 ± 1462	7
T 60	6	4183 ± 233	6	186 ± 27	15	23.0 ± 4.0	17	145 ± 32	22	22433 ± 2355	10.
T 61	4	4255 ± 70	2	161 ± 40	25	27.5 ± 5.5	20	105 ± 68	65	21481 ± 544	3

n = number of independent assays carried out within one year. Values are means ± SD of n. Each assay consists of the means of at least 6 or 3 parallel determinations of total binding or unspecific binding and filter blank, respectively, similar to the experiments shown in Table 1. Final concentration of [^{3}H]-spiroperidol was about 0.2 nmol/l. R is the ratio of total binding/unspecific binding for each individual assay.

Table 3

Inter-assay imprecision in the [^{3}H]-ketanserin binding test

Filter type	n	Total binding dpm ± SD	CV	Unspecific binding dpm ± SD	CV	R ± SD	CV	Filter blank dpm ± SD	CV	Ligand conc. dpm/2ml ± SD	CV
GF/B	3	19205 ± 3116	16	1585 ± 65	4	12.1 ± 2.1	17	1314 ± 77	6	129911 ± 10155	8
T 60	5	16946 ± 2944	17	1138 ± 338	30	16.0 ± 6.2	39	1090 ± 228	21	130714 ± 6527	5
T 61	3	15669 ± 1290	8	994 ± 259	26	16.2 ± 2.8	17	577 ± 118	20	128528 ± 10460	8

Final concentration of [^{3}H]-ketanserin was about 0.4 nmol/l. For other details, see Table 2.

unfavorable. However, sufficiently precise estimations of IC_{50}-values can be obtained if assay conditions are carefully controlled and kept constant. The effect of pre-soaking the filter sheets in 0.1% polyethyleneimine was also checked, one representative example being given in Table 4. As expected, filter blanks are reduced dramatically by this procedure; however, the unspecific binding is not reduced in the same proportion; in fact, R-values are about the same with and without the polyethyleneimine treatment. The data in Table 4 suggest that at the ligand concentration of 0.05 nmol/l, more favorable R-values, i.e. 1.82-2.15, are obtained than at 0.2 nmol/l, where the range of R-values was 1.43-1.77, irrespective of the types of filter material used.

One important result in the present investigation with the GR 65630-test is the finding that relatively constant R-values can only be calculated if the total binding and the unspecific binding are obtained together on the same filter sheet. Otherwise, gross discrepancies arise, as illustrated in several examples in Table 4. For instance, in the experiment with the T61 filters, when the identical samples were filtered together on the same sheet, R-values of 1.76 and 1.51 were obtained for two consecutive sheets. However, both total and unspecific binding were considerably higher on the second sheet. Thus, if one were to filter the total binding on the first and the unspecific binding on the second sheet (or vice versa) in this experiment, R-values of 1.06 (i.e. 2303/2163) and 2.50 (i.e. 3267/1307) would be obtained, leading to unacceptable variation of IC_{50}-values.

DISCUSSION

The results of the present investigation, obtained with three representative ligands under carefully controlled conditions, underscore several points that might be important to consider when setting up new ligand binding assays in the laboratory. Highly reproducible results were obtained by either manual or automatic (and batchwise) transfer of the filter discs into counting vials. As for the different types of filters, considerable differences in the amount of unspecific binding may be encountered for some, but not all, types of ligands. Thus, T61 filters seem especially well suited for the [^{3}H]-spiroperidol assay. Recently, also Bylund and Yamamura /4/ noted that "in certain assays, the choice of the filter material may be very important"; however, no examples are given. Conceivably,

Table 4

Inter- and intra-assay imprecision in the [^{3}H]-GR 65630 binding test

Filter type	n	Total binding dpm ± SD	CV	Unspecific binding, cpm±SD	CV	R ± SD	CV	Filter blank dpm ± SD	CV	Ligand conc. dpm/2ml ± SD	CV
GF/B	4	3827 ± 333	9	2163 ± 174	8	1.77 ± 0.11	6	891 ± 42	5	77479 ± 1625	2
T 60	4	4113 ± 465	11	2431 ± 131	5	1.69 ± 0.18	11	730 ± 246	34	78307 ± 7157	9
T 60	4	870 ± 200	23	410 ± 108	26	2.15 ± 0.30	14	201 ± 113	56	19809 ± 2697	14
T 60, Sheet 1	1	597 ± 27 (6)	5	310 ± 21 (3)	7	1.93		91 ± 10 (3)	11	17091 ± 299 (3)	2
T 60, Sheet 2		713 ± 26 (6)	4	380 ± 25 (3)	7	1.88		111 ± 14 (3)	13		
T 60, Sheet 1	1	455 ± 20 (6)	4	224 ± 22 (3)	10	2.03		71 ± 7 (3)	10	19069 ± 462 (3)	2
T 60, Sheet 2		584 ± 25 (6)	4	292 ± 3 (3)	1	2.00		70 ± 7 (3)	10		
T 60, Sheet 1	1	538 ± 26 (6)	5	295 ± 17 (3)	6	1.82		81 ± 10 (3)	12	18340 ± 345 (3)	2
T 60, Sheet 2		619 ± 16 (6)	3	341 ± 42 (3)	12	1.82		106 ± 9 (3)	8		
T 61, Sheet 1	1	2302 ± 171 (6)	7	1307 ± 29 (6)	2	1.76		347 ± 48 (6)	14	81931 ± 426 (3)	1
T 61, Sheet 2		3267 ± 102 (6)	3	2163 ± 153 (6)	7	1.51		436 ± 59 (6)	14		
GF/C, Sheet 1	1	2271 ± 236 (6)	10	1529 ± 57 (6)	4	1.49		816 ± 90 (6)	10	≈ 80000	
GF/C, Sheet 2		3151 ± 112 (6)	4	2020 ± 192 (6)	10	1.56		928 ± 102 (6)	11		
GF/C, Sheet 1*	1	3537 ± 54 (6)	2	2226 ± 76 (6)	3	1.59		286 ± 16 (6)	6	75330 ± 828 (3)	1
GF/C, Sheet 2*		4152 ± 145 (6)	3	2895 ± 140 (6)	5	1.43		332 ± 23 (6)	7		

* Filter sheets pre-soaked in 0.1 % polyethyeneimine for 3 hrs. Final concentration of [^{3}H]-GR 65630 was about 0.2 nmol/l (≈ 80000 dpm/2ml) or 0.05 nmol/l (≈ 20000 dpm/2ml). The coefficient of variation (CV) is calculated either from the number (n) of independent assays (inter-assay variation) or from the number of parallel samples (in parentheses) of the individual assays. For other details, see Table 2.

this question may become critical when peptidergic ligands are used or, with non-peptidergic ligands, in other cases where receptor density is low, as with the $5HT_3$-receptor in the [^{3}H]-GR 65630 assay. However, for the latter case, only marginal differences were found with respect to different filter types, whereas, unexpectedly, the sequence of the filtrations was highly critical for obtaining constant, although rather low, levels of specific binding. As seen in Table 4, the difference between total and unspecific binding is fairly constant if both filtrations are performed together on the same sheet, although the absolute values on different sheets may vary considerably. It is also noteworthy that, in many experiments with replicates, the values for total and unspecific binding were found to be lower on the first sheet than on the following sheet (see Table 4). The reason for this phenomenon is unclear: it might be connected with differences in the vacuum immediately after the onset of filtration, leading to different depths of penetration of the membrane preparations into the glass fibers of the filter. It is clear that care must be taken to avoid calculating the specific binding using the total binding on one sheet while using the unspecific binding from a different one, which can lead to enormous variations (see Results). However, when these precautions are taken, the reproducibility of IC_{50}-values is adequate. For example, for the potent $5HT_3$-antagonist zacopride, we obtained an IC_{50} of 0.29 ± 0.12 nmol/l (mean ± SD for 4 independent determinations). This value is in accordance with literature data for zacopride obtained also with other $5HT_3$-receptor ligands: [^{3}H]-BRL 43694, K_i = 0.10 ± 0.01 nmol/l /5/; [^{3}H]-quipazine, K_i = 0.42 ± 0.2 nmol/l /6/; [^{3}H]-GR 67330, IC_{50} = 0.99 ± 0.02 nmol/l /7/ and [^{3}H]-zacopride, K_i = 1.98 ± 0.56 nmol/l /8/. The differences observed may be accounted for by the different radioactive ligands and different types of brain tissue used. However, it should be mentioned that even with assays with low unspecific binding, interassay differences of more than one order of magnitude may be observed (for review see /9/). Thus, in 43 distinct experiments accumulated over several years, the K_D-values for [^{3}H]-rauwolscine at rat cortical α_2-receptors had a range of 1.0-14.1 nmol/l. Importantly, the frequency distribution of results in this, as well as in many other *in vitro* tests, is of the log-normal type /10/.

The method for screening of $5HT_3$-receptor ligands described in the present paper, using rat entorhinal cortex, represents a reasonable compromise between costs for screening and precision of obtained data. Although some investigators use larger areas of

rat cortex, to increase the yield of membranes per rat, this procedure has frequently resulted in a less favorable percentage of specific binding than with entorhinal cortex. On the other hand, with brain tissue where $5HT_3$-receptor density is still higher, i.e., rat area postrema and cervical vagus nerve preparations /3/, tissue from about 50 rats is required for one competition experiment due to the small size of these preparations. In contrast, with entorhinal cortex, less than 5 rats are necessary for a comparable study. In the present method, we obtained the following IC_{50}-values (nmol/l) for various $5HT_3$-ligands, which agree well with the data of Kilpatrick *et al.* /3/ (given in parentheses) who employed [^{3}H]-GR 65630 and rat area postrema: Quipazine: 1.5 (0.87); ICS 205-930: 1.2 (1.1); GR 38032F: 1.8 (2.8); MDL 72222: 13 (27.9); Metoclopramide: ~ 900 (816).

ACKNOWLEDGEMENTS

We would like to thank Karlheinz Heider for the preparation of tissue samples and Silvia Krichbaum for expert typing of the manuscript.

REFERENCES

1. Glowinski J, Iversen LL. Regional studies of catecholamines in the rat brain-I. The disposition of ^{3}H-norepinephrine, ^{3}H-dopamine and ^{3}H-DOPA in various regions of the brain. J Neurochem 1966; 13: 655-669.
2. Klockow M, Greiner HE, Haase AF, Schmitges C-J, Seyfried CA. Studies on the receptor profile of bisoprolol. Arzneim-Forsch/Drug Res 1986; 36: 197-200.
3. Kilpatrick GJ, Jones BJ, Tyers MB. Binding of the $5HT_3$-ligand, [^{3}H]-GR 65630, to rat area postrema, vagus nerve and the brains of several species. Eur J Pharmacol 1989; 159: 157-164.
4. Bylund DB, Yamamura HI. Methods for receptor binding. In: Yamamura HI, Enna SJ, Kuhar MJ, eds., Methods in Neurotransmitter Receptor Analysis. New York: Raven Press, 1990; 1-35.
5. Nelson DR, Thomas DR. [^{3}H]-BRL 43694 (Granisetron), a specific ligand for $5HT_3$ binding sites in rat brain cortical membranes. Biochem Pharmacol 1989; 38: 1693-1695.
6. Milburn CM, Peroutka SJ. Characterization of [^{3}H]-quipazine binding to 5-hydroxytryptamine$_3$ receptors in rat brain membranes. J Neurochem 1989; 52: 1787-1792.

7. Kilpatrick GJ, Butler A, Hagan RM, Jones BJ, Tyers MB. [^{3}H]-GR 67330, a very high affinity ligand of $5HT_3$ receptors. Naunyn-Schmiedeberg's Arch Pharmacol 1990; 342: 22-30.
8. Barnes NM, Costall B, Naylor RJ. [^{3}H]-zacopride: Ligand for the identification of $5HT_3$ recognition sites. J Pharm Pharmacol 1988; 40: 548-551.
9. Hancock AA. Factors influencing apparent radioligand affinity. Biotech Update, Du Pont 1990; 5: 12-14.
10. Hancock AA, Bush EN, Stanisic D, Kyncl JJ, Lin T. Data normalization before statistical analysis: keeping the horse before the cart. Trends Pharmacol Sci 1988; 9: 29-32.